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DISSERTATION

Feasibility Studies for Development of Specific Re-loadable Binding Sites for Drug Delivery Systems Immobilized onto Implant Surfaces

Verfasserin

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All art is autobiographical, the pearl is the oyster's autobiography

Federico Fellini

I dedicate this work to people who through their presence gave me strength and hope during the ups and downs of this work

Kamal, Roua, Raniem

Gisi & Fritz

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Abbreviations

Ab	Antibody
ABS	Absorbance
Ag	Antigen
AP	Alkaline phosphatase
APS	Ammonium peroxodisulphate
APTS	3-**A**mino**p**ropyl**t**riethoxy**s**ilane
AU	Absorbance units
BBB	Blood brain barrier
ß-LG	Beta-lactoglobulin
ßME	2-Mercaptoethanol
ß-MEA	2-Mercaptaethanol amine
BMS	Bare metal stent
BODIPY 493/503	(4,4-Difluoro-1,3,5,7,8-pentamethyl-4-boro-3a,4a-diaza-s-indacene)
BrdU	5-bromo-2-desoxyuridine
BSA	Bovine serum albumin
CaCo2	Human epithelial colorectal adeno carcinoma
CBB-R250	Coomassine Billant-Blue-R250
CH_2Cl_2	Dichloromethane
CLISA	Cluster-Linked Immunosorbent Assay
c-m-PEG	Carboxy-methoxy-polyethylene glycol
CS	Chitosan
CS-Ab	Mouse monoclonal Anti-ß-O-linked N-Acetylglucosamine (O-GlcNAc) IgM isotype
CS-NPs	Chitosan-nanoparticles
CV	Coefficient of variation
dd. H_2O	Double distilled water

DDDs	Drug delivery systems
DESs	Drug eluting stents
DIC	N,N`-Diisopropylcarbodiimide
DIC	Different interference contrast
DMF	Dimethyformamide
DMSO	Dimethyl sulfoxide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany
DTNB	5,5´-Dithio-bis-(2-nitrobenzoic acid)
ECP	Eosinophil cationic protein
EDC	(1-**E**thyl-3-(3-**d**imethylaminopropyl) **c**arbodiimide hydrochloride
ELISA	Enzyme-linked immunosorbent assay
EtAc	Ethyl acetate
FDA	Food and drug administration
$FeCl_3$	Ferric chloride
GA	Glutaraldehyde
GCCs	Gold colloidal clusters
GlcN	4-linked D-glucosamine
GlcNAC	1,4-linked N-acetyl-D-glucosamine
H_2SO_4	Sulfuric acid
HAc	Acetic acid
$HAUCl_4$	Tetrachloric acid
HCl	Hydrochloric acid
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNO_3	Nitric acid
HRP	Horseradish peroxidase
HSA	Human serum albumin

Ig	Immunoglobulin
IaIp	Inter-alpha inhibitor protein
KCN	Potassium cyanide
LDH	Lactat dehydrogenase
Lipoic acid	6, 8-dithiooctanoic acid
MACE	Major adverse cardiac events
mc-Ab	Monoclonal antibody
$MgCl_2$	Magnesium chloride
m-PEG	Methoxy-PEG
MPS	Mononuclear phagocytic system
MRI	Magnetic resonance imaging
MS	Massenspectrometry
NaN_3	Sodium azid
$Na_2S_2O_4$	Sodium hydrosulfite
Na_3PO_4	Sodium phosphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogencarbonate
NaOAc	Sodium acetate
Na_2SO_4	Sodium sulfate
NBT	Nitro blue tetrazolium chloride
NC	Nitrocellulose
NH_2 -m-PEG	Amino-methoxy- polyethylene glycol
NH_2-PEG-NH_2	Bis-amino- polyethylene glycol
NHS	N-hydroxysuccimide ester
NP(s)	Nanoparticle(s)
O-GlcNAc	O-linked N-Acetylglucosamine
OPA	o-Phthaldialdehyde
PAGE	Polyacrylamide gel electrophoresis

PES	Paclitaxel-eluting stent
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
pc-Ab	polyclonal antibody
PEG	Polyethylene glycol
PET	Positron emission tomography
PGI_2	Prostacyclin
pI	Isoelectric point
PLGA	Poly (D,L-lactide-co-glycolide)
PLI	Polydispersity index
PLL-g-PEG	Poly (L-lysine)-g-poly(ethylene glycol)
p-NPP	para- Nitro Phenyl Phosphate
RG	Resomer
SAM	Silica-amino-microspheres
SDS	Sodium dodecyl sulphate
SES	Sirolimus-eluting stent
SH-PEG	(O-[2-(3-Mercaptopropionylamino)ethyl]-O´-methyl-polyethyleneglycol
$SOCl_2$	Thionylchloride
Sulfo-NHS	Sulfo-N-hydroxysulfosuccinimide
Sulfo-SDTB	Sulfosuccinimidyl-4-o-(4,4-dimethoxytrityl) butyrate
Sulfo-SMCC	Sulfosuccinimidyl 4-(N-maleimido-methyl) -cyclohexane-1-carboxylate
SVG	Saphenous veingraft
TBS	TRIS buffer saline
TE	Tris/EDTA buffer
TEMED	N,N,N´,N´-Tetramethylenediamine

THF	Tetrahydrofurane
TMB	Tetramethylbenzidine
TNB	5-thio-2-nitrobenzoic acid
TNBS	2,4,6-Trinitrobenzenesulfonic acid solution
TNP	Trinitrophenol
TPP	Sodium tripolyphosphate
Tris	Tris (hydroxymethyl)aminomethane
Tween 20	Polyethylene glycol sorbitan monolaurate
ZrO_2	Zirconia (ceramics)

Abstract

The overall objective of this thesis is to develop novel, bio-compatible coatings for medical implant materials with binding sites for bio-degradable nanoparticles that may serve as drug carriers. The drugs are released gradually during decomposition and after particle degradation the binding sites can be reloaded with new drug delivering particles.

To achieve this goal, we used stainless-steel and zirconia as test implant materials. To improve biocompatibility of these materials a so called “passive coating” procedure is used where biocompatible polymers such as polyethyleneglycol (PEG) act as a barrier. Furthermore a specific receptor system was introduced for binding drug containing biodegradable nano- or micro-particles to mediate local pharmacological activity, the so called active coating. For this purpose specific antibodies or antibody-fragments were generated and coupled to the implant material surface as capture molecules for the drug releasing particles.

Poly (D,L-lactide-co-glycolide) (PLGA) and chitosan were used to produce these biodegradable nanoparticles. The particles are then degraded either by hydrolysis or by means of enzymes circulating in the bloodstream (mere hydrolysis for PLGA and lysozyme for chitosan) while releasing the drugs locally. After complete biodegradation the receptor system is then free again for reloading with new particles containing the respective drugs, making this a completely new mean of drug administration on medical implants.

Such coatings can be the basis for a number of applications that require controlled, local drug delivery at the interface between an implant and healthy tissue. Upon the need for medication, drug-loaded nanoparticles can be injected into the bloodstream, bind to the particle-specific antibodies on the target implant and gradually release their drug content during degradation. As nanoparticles can be loaded with different drugs, this technology allows easy adjustment to patient-specific medication needs as well as the administration of compounds that are easily degradable or difficult to dose.

Analytical proofs of principle on these materials have proved to be difficult, as the currently available optical methods for testing the successful chemical setup could not be applied because of quenching effects on metal surfaces and the rough surface of zirconia (ceramics). Therefore specific binding of the nanoparticles onto the

implant surface was tested on glass substrates, coated similarly as the implant materials, by means of fluorescence microscopy. Furthermore for proof of the successful chemical setup and biorecognitive binding several new analytical test methods had to be developed and evaluated.

One of the major challenges we had to overcome was to find suitable biorecognitive systems for binding of the drug containing particles, as these have to be biocompatible too. Special efforts were made to identify and generate specific antibodies against biocompatible materials. In case of chitosan, antibodies were commercially available, whereas for PLGA a very stringent immunisation protocol had to be developed for antibody production in rabbits, in addition to optimization of the purification and titer determination protocols.

Using these tools, we were able to perform specific experiments that demonstrate a proof of concept for the possibility to generate a reloadable targeting device for local drug release and potential of our approach in targeted drug delivery and release. This new system provides the basis for a wide array of applications in regenerative medicine such as stents, implants of artificial bones and other artificial tissues, requiring local drug action at the interface between an implant and healthy tissue in a controlled manner. The delivery system protects the incorporated drugs from degradation prior to action e.g. growth factors and immunosuppressives, and facilitates the transport of poorly absorbable or insoluble drug candidates to the intended site of action. Due to the high local drug concentration, the absorption rate of poorly soluble drug molecules will be improved, which would significantly reduce undesired, adverse systemic effects. Furthermore after implantation also a change of drugs is possible, as with each reloading procedure appropriate drugs according to patients needs may be chosen.

Zusammenfassung

Ziel dieser Dissertation war die Entwicklung eines neuartigen biokompatiblen Beschichtungssystems für medizinische Implantatmaterialien, die Bindungsstellen für bioabbaubare, mit Wirkstoffen beladene Nanopartikel (Drug Carriers) tragen. Diese Wirkstoffe werden während des Abbaus der Drug Carriers dort kontrolliert freigesetzt. Nach dem Abbau der Partikel sind diese Bindungsstellen wieder frei um mit neuen Drug-Carriers beladen zu werden.

Zum Erreichen dieses Ziels wurden als Testsystem die Implantatmaterialien Edelstahl und Zirkon-Keramik verwendet. Zur Verbesserung der Biokompatibilität wurde ein sogenanntes Passivcoating mit Polymeren wie z.B. dem medizinisch zugelassenen Polyethylenglykol (PEG) aufgebracht, um eine Schutzschicht zu bilden. Außerdem wurde ein geeignetes Rezeptorsystem zu verankert, das in der Lage ist, biodegradable mit Wirkstoffen beladene Mikro- oder Nanopartikel (Drug Carriers) zu binden, um eine lokale pharmakologische Wirkung zu erzielen (Aktives Coating). Zu diesem Zweck wurden spezielle Antikörper bzw. auch Antikörper-Fragmente als Capture-Moleküle an die Oberfläche des Implantatmaterials gebunden.

Zur Herstellung der Drug Carriers wurden sowohl ein Copolymer aus Milch- und Glycolsäure (PLGA) als auch Chitosan verwendet. Die Drug Carriers werden dann entweder durch Hydrolyse oder mit Hilfe von Enzymen im Blut (Hydrolyse für PLGA, Lysozym für Chitosan) abgebaut und setzten die in ihnen enthaltenen Wirkstoffe lokal frei. Nach dem vollständigen Bioabbau liegen die Capture-Moleküle wieder frei vor und können mit neuen Drug Carriers beladen werden, womit ein vollkommen neuartiges System für Wirkstoffabgabe von medizinischen Implantaten zur Verfügung steht.

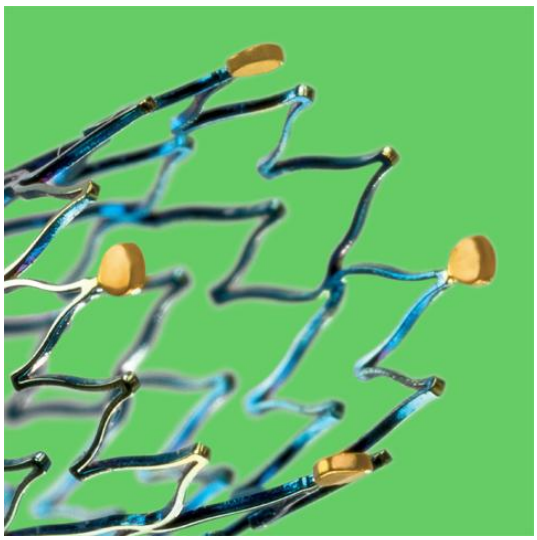
Solche Beschichtungen können die Basis für eine Vielzahl von Anwendungen sein, die eine gezielte Wirkstoffabgabe an der Grenzfläche zwischen Implantat und gesundem Gewebe erfordern. Bei Bedarf können die Drug Carriers in die Blutbahn injiziert werden, binden an das Rezeptorsystem auf der Implantatoberfläche und setzen im Zuge ihres Abbaus die Wirkstoffe frei. Da sie mit unterschiedlichen Wirkstoffen beladen werden können, ermöglichen sie eine patientenspezifische Anpassung der Medikation, aber auch die Gabe von Wirkstoffen, die sonst zu rasch abbaubar oder schwierig zu dosieren wären.

Der analytische Nachweis des erfolgreichen chemischen Setups mit Hilfe optischer Methoden erwies sich als schwierig auf Grund von Quenching Effekten im Fall von Metall bzw. wegen der rauen Oberfläche bei Zirkon (Keramik). Daher wurde die spezifische Bindung der Nanopartikel an der Implantatoberfläche an Glasoberflächen mit gleicher chemischer Beschichtung mit Hilfe von Fluoreszenzmikroskopie getestet. Außerdem mussten für den Nachweis des erfolgreichen chemischen Aufbaus und der biorecognitiven Bindung verschiedene neue analytische Tests entwickelt und evaluiert werden.

Ein schwieriges Problem, das in dieser Arbeit gelöst werden musste, war das Finden eines brauchbaren Biorekognitionssystems für das Binden der biokompatiblen Drug Carrier Nanopartikel. Es braucht spezielle Lösungsansätze, um Antikörperbildung gegen biokompatible Materialien zu induzieren. Im Fall von Chitosan, waren Antikörper kommerziell erhältlich, während für PLGA ein sehr stringentes Immunisierungsprotokoll in Kaninchen entwickelt werden und auch Probleme hinsichtlich Reinigung und Titerbestimmung gelöst werden mussten.

Durch die durchgeführten Experimente war es möglich ein „Proof of concept“ für ein wiederbeladbares Targeting-system zur lokalen Medikamentenfreisetzung an Oberflächen zu geben. Dieses neuartige System ist eine Basis für ein breites Anwendungsgebiet in der regenerativen Medizin wie z.B. Stents, artifizielle Knochenimplantate und andere künstliche Gewebe, die eine lokale und kontrollierte Medikamentenfreisetzung an der Grenzfläche zwischen Implantat und gesundem Gewebe benötigen. Das Drug-Delivery System schützt die eingeschlossenen Wirkstoffe vor vorzeitigem Abbau – wichtig z.B. bei Wachstumsfaktoren und Immunsuppressiva und erleichtert auch den Transport von schwer absorbierbaren oder schwer löslichen Wirkstoffen zum erwünschten Wirkort. Dank der hohen lokalen Wirkstoffkonzentration wird die Absorption schwerlöslicher Substanzen verbessert, wodurch unerwünschte systemische Nebenwirkungen vermindert werden können.

Introduction



Introduction

1 Background

Advances in modern material sciences have brought many life saving products to the clinic, in particular to the operation theatre in the form of various implants. Optimal biocompatibility is of utmost importance when developing novel implant materials and a number of challenges still need to be addressed in this area. In addition, the implanted material needs to have specific mechanical properties (e.g. durability and resistance to the chemical stresses) within environment at the implanted site. Single compound materials rarely possess all the above-mentioned qualities. During the last decade, surface modification with biocompatible materials has emerged as one possible method to improve the biocompatibility of medical implants. Another goal that has become feasible recently is to design implant materials able to stimulate healing, and improve restoration of diverse tissue functions. For this purpose drug eluting implants have been developed. However the following problems are still the drawback of available drug eluting implants.

1.1 Re-stenosis after coronary stent implantation

Despite advances of modern intervention in coronary artery heart infarction, re-stenosis continues to be the main problem after balloon angioplasty treatment (Liu M. et al. 1989). Compared to angioplasty alone, coronary stent implantation has been proven effective in the reduction of re-stenosis in native coronary vessels. Nevertheless, re-stenosis rates after bare-metal stent implantation are still as high as 20% to 40% at 6 months post intervention (Scheller B. et al. 2004). The physiopathology of re-stenosis after balloon angioplasty and stent implantation suggests 5 major mechanisms: i) elastic recoil; ii) thrombus formation at the injury site; iii) inflammation; iv) proliferation of smooth muscle cells; v) neointimal hyperplasia. Thrombogenicity of the stents is associated predominantly with activated thrombocytes, which may be induced by shear forces and through contact with biomaterials (Courtney JM. et al. 1994). Furthermore, it has been suggested that metal ions can induce activation of blood platelets as well as other cell types (Ruygrok P. et al. 1996). Stented blood vessels show reactive inflammatory

infiltrates, composed of lymphocytes, histiocytes and eosinophiles, surrounding the stent struts (Karas SP. et al. 1992). It is assumed that this inflammatory reaction is a mixed response to both, vessel injury, and non-specific activation mediated through metal ions released from the alloy of the stent. More over eosinophils have been identified in post-mortem studies as important players of both restenosis and thrombosis after drug-eluting stent (DES) implantation. Further studies are warranted to establish whether ECP (eosinophil cationic protein, a marker of eosinophil activation) is a risk marker or plays a contributory pathogenetic role (Niccoli G. et al, 2009).

1.1.1 Neointimal hyperplasia

The innermost cell layer, or intima, of a blood vessel is composed of a single layer of cells that are adapted to respond to mechanical stress in a highly specific manner, as they are constantly exposed to the shear forces generated by blood flow. Intimal hyperplasia after balloon injury is a complex process involving platelets, growth factors, endothelial cells, smooth muscle cells, mechanical injury, wall shear stress, and probably other unknown factors (Liu M. et al. 1989). Stent implantation often leads to intimal hyperplasia, perturbed tissue growth with characteristic inflammatory cells, proliferating smooth muscle cells and extracellular matrix, consisting of collagen, elastin and several types of glycoproteins (Strauss B. et al. 1994). However it has been assumed for a long time that excessive cell proliferation is the major mechanism of intimal hyperplasia (Schwartz RS. et al. 1992). Three major stent-related factors influence the extent of intima proliferation: i) stent material; ii) stent design; and iii) extent of vascular injury.

In order to reduce re-stenosis and stent-associated thrombosis, significant efforts have been devoted to improve the biocompatibility of the stent material (Ruygrok P. et al. 1996). Ideally the stent material should combine both, excellent mechanical properties and good biocompatibility. New stent designs, changes in the composition of the materials, and surface modifications such as coatings have been applied (Ruygrok P. et al. 1996). Recently, the use of drug-eluting stents, coated with anti-proliferative substances has become a milestone in interventional cardiology.

1.1.2 Drug eluting stents (DES)

Recently, drug-eluting stents have been introduced as a new and promising technique to reduce re-stenosis after stent implantation. There are three different approaches for binding pharmacologically active compounds onto coronary stents:

- The drug is bound by a polymer onto the stent surface (Suzuki T. et al. 2001).
- The drug is bound by an inorganic stent coating (Kataoka T. et al. 2001).
- The drug is directly applied onto the stent surface without any coating (Kaluza GL. et al. 2002).

Several substances with immunosuppressive, antiproliferative and cytostatic properties such as Sirolimus, Eveolimus, and Paclitaxel have been tested as active stent coatings and were shown to reduce re-stenosis in humans (Sousa J. et al. 2001, Grube E. et al. 2004, Chieffo A. et al. 2002). Despite these advances, re-stenosis rates still remain substantial in patients with high-risk for re-stenosis, such as patients with long coronary lesions, complex lesion morphologies or lesions at bifurcations of blood vessels (Park S. J. et al. 2008). Moreover, results from small selected patient groups showed potential risks when delivering anti-proliferative drugs from coronary stents. As these substances do not only inhibit smooth muscle proliferation, but also disturb re-endothelialization of stent struts, the risk of acute and sub-acute stent thrombosis might be increased. Another alarming observation was a significant increase of incomplete stent strut apposition with Sirolimus eluting stents (Moreno R. 2005, Anderson R. et al. 2005). Roiron C. et al. study shown that a trend to an increased risk of stent thrombosis after Paclitaxel eluting stent implantation was found, whereas the risk of stent thrombosis was similar with Sirolimus eluting stent and bare-metal stents (Roiron C. et al. 2006). Paclitaxel is cytotoxic, whereas sirolimus is cytostatic. In general drug-eluting stents may be associated with an increased rate of late stent thrombosis, myocardial infarction and death (David E. et al. 2009).

1.1.3 Long coronary lesions

A study by Faraasen S. et al. compared the efficacy of the Sirolimus-eluting stent (SES), the Paclitaxel-eluting stent (PES) and the bare metal stent (BMS) in patients with elongated coronary lesions. The two DES groups had longer stented segments

than the BMS group. After six months, angiographic follow-up showed lower in-segment re-stenosis rates in the SES (9.3%) and PES (21%) groups than in that of the BMS group (42.5%). The rate of major adverse cardiac events (MACE) including death, myocardial infarction, and target lesion revascularization at 9 months was higher in the BMS group (26.6%) than in the SES (13.0%) and PES (15.7%) groups (Faraasen S. et al. 2003). In general SES showed significantly greater suppression of neointimal hyperplasia in complex coronary lesions compared to PES (Yun-Kyeong C. 2009).

1.1.4 Saphenous vein graft stenting

Saphenous vein graft (SVG) stenting is still a challenge for modern engineering, cardiology and basic research. In this approach, a part of the saphenous vein of the patient is implanted at the site of coronary lesion to fulfil stent-like function. However, SVGs tend to degenerate rather quickly and up to 50% of SVGs show significant re-stenosis and nearly 40% are completely occluded after 10 years (Kim Y. et al. 2006, Firzgibbon G. et al. 1991). Furthermore SVG stenting is associated with re-stenosis rates as high as 50% at 6 months, and does not significantly improve the survival rate (Firzgibbon G. et al. 1996, Keeley E. et al. 2001). De Jaehere et al. DES implantation in SVG lesions appears safe (De Jaehere P. et al. 1996). However, patients were followed only for 6 months. Longer follow-up in SVG interventions is especially important because it is known that vein graft related complications continue to develop after 6 months. DES is effective in reducing target vessel revascularization at 9 months, although most of this advantage is lost at 2 years (Bruce R. et al. 2009). A study by Ge et al. compared the efficacy of SES with bare metal stents in patients who underwent SVG intervention within one year. SES is clinically safe and feasible, but is not associated with a decrease of clinical events up to one year compared with BMS. The most likely explanation is the small size of SESs due to the unavailability of larger stents. The small size of SESs allows only limited contact of the drug with the blood vessel wall in relation to the dimensions of the lesion (Ge L. et al. 2005).

1.1.5 Current limitations of drug loading

Currently, drug-eluting implants, including stents, are prepared by incubating the implant in the pharmaceutical drug followed by drying. Drug release is then achieved either by passive diffusion or by degradation of the implant matrix (Wu P. et al. 2006). However, this approach allows only limited control of such important parameters as drug dosing, release kinetics, and release duration. Another study showed that increasing Rapamycin concentrations (0.5, 2,2%) can lead to reduced re-stenosis via a polymer-free on-site stent coating compared to BMS. Rapamycin acts analogous to sirolimus. In-segment restenosis was significantly reduced from 25.9% with BMS to 18.9%, 17.2 and 14.7% with 0.5, 1.0 and 2.0% respectively (Chu W. et al. 2006). The main drawback of small drug eluting implants is their limited drug loading capacity. Furthermore, currently used methods allow only single drug loading onto the implant, and after implantation the drug cannot be changed according to varying patient needs throughout different stages of the healing process.

Another approach is the use of nanoparticulate drug carriers specifically transfecting arterial walls. Here the problem arises that only very small particles of about 110 nm are suitable, whereas larger particles (> 300nm) show only very short residence time on the arterial wall surface and accumulate in liver and spleen and lung. (Brito L., Amici M. 2007).

2 Our approach: Design of reloadable implant materials in this study

The overall objective of my thesis is to investigate and develop possible solutions to these limitations for coronary stent implant materials with the hope of extending these solutions to other types of implants.

The general design approach developed in this thesis is outlined in Figure 1.

Implant materials were coated with a biocompatible layer that was covalently bound to the implant backbone. This biocompatible layer forms a passive protection layer to prevent rejection of the implant material. Additional binding sites were then anchored in this protective layer and were subsequently used to load biodegradable, drug containing nanoparticles onto the implant via specific bio-recognition sites. Use of these specific binding sites overcomes problems as outlined by Brito L. and Amici M.

2007 as the particles will act site directed on the implant material and unspecific accumulation in liver, lungs and spleen will be minimized. After implantation into the patient, such a structure will serve as a re-loadable drug delivery system. While the particles are degraded, either by hydrolysis or by enzymes in the surrounding body fluids, the drugs will be released gradually. At the end of this process the bio-recognitive sites are regenerated and become free to bind newly injected drug containing particles again. The possibility of re-loading allows not only patient-adequate dosage of drugs but also change of medication after implantation, i.e. using different drugs at different stages of the healing process.

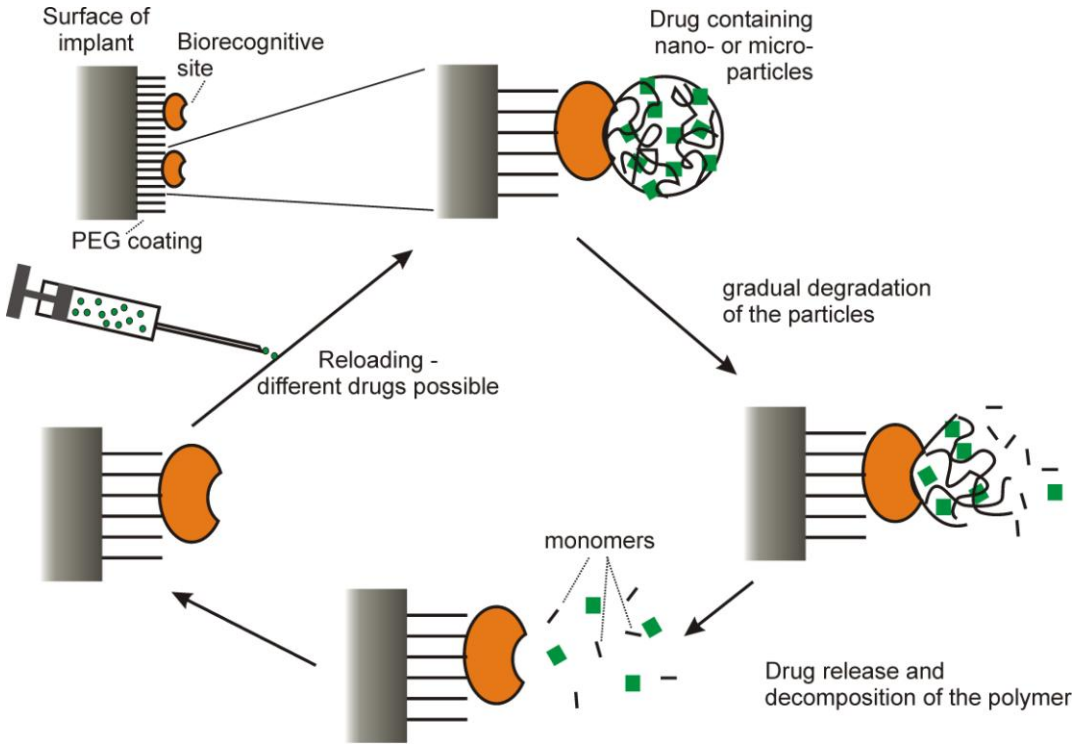


Figure 1. Principle of nanoparticle binding, drug release and reloading.

A similar solution to the one proposed in this thesis was already described in a published Patent *WO 02/067849,* however the patented concept has many drawbacks and limitations. Patent *WO 02/067849* demonstrates in principle a novel biomedical device that potentially can overcome several of the abovementioned prior-art limitations by allowing targeted delivery and activation of therapeutic agents at a specific tissue site. To achieve this goal several general engineering methods were described to produce such a device. However, the practical realisation of such

a proposed device is still missing. The principal design concept of the biomedical implant described in this patent is similar to that developed in this thesis. However, the bio-recognitive principles illustrated in the patent were described in rather general terms as a listing of known biorecognitive interactions that consist of binding of one of the biorecognitive partners to the implant material and the other to a drug containing particle. The only recognitive principle mentioned explicitly in this patent was based on avidin-biotin interaction. The avidin-biotin system represents one of the strongest biorecognition reactions with respect to binding, and therefore cannot be the reaction of choice for a reloadable drug delivery implant system, as was claimed in the patent. Binding reaction systems for reloadable systems need to be strong enough to keep the drug releasing nanoparticles tightly bound for a certain period of time, but on the other hand weak enough to allow reloading after the gradual decomposition of the beads. Furthermore, avidin is immunogenic in humans, leading to inflammatory response, and this immunogenicity is increased after binding to a carrier-particle as was suggested in the patent. Moreover, the iso-electric point of avidin is 10. At physiological conditions in the human body avidin will be positively charged and adheres, non-specifically, to cell surfaces and other negatively charged structures. Furthermore, biotin (vitamin H) is endogenous in the human body, and therefore biotinlyated particles as described in this patent will compete with vitamin H for avidin-binding sites, thus not allowing an implant site specific application.

3 Specific aims

In order to realise the development of the drug delivery system for implant materials the following specific aims were pursued.

Specific aim 1: To produce a covalently bound biocompatible coating for implant materials

Specific aim 2: To introduce binding sites for degradable, drug loaded nanoparticles into the biocompatible coating

Specific aim 3: To search and develop a biorecognitive reloadable binding system for drug delivery system e.g. nanoparticles

Specific aim 4: To provide a proof of principle testing of the interaction between the nanoparticles and their in biocompatible coating embedded binding site

3.1 Specific aim 1

To produce a covalently bound biocompatible coating for implant materials (Chapter 1)

Implant Material Coating

The implant materials selected for use in this study were stainless-steel L316, or ceramics (zirconium dioxide ZrO_2), which were chemically activated for binding the passive coating layer and the bio-recognitive system. The passive coating of the implant materials with the partially functionalized polymer polyethyleneglycol (PEG) was intended to enhance the biocompatibility of stainless-steel L316 or ceramics. The biorecognitive system – in our approach receptor molecules capable of binding nanoparticles – was embedded into the PEG coating. This biorecognitive system had to be chosen very carefully to avoid any interactions with other components circulating in the blood. Furthermore, the binding between receptor molecules and nanoparticles had to be strong enough to keep the drug releasing nanoparticles tightly bound for a certain period of time, but on the other hand weak enough to allow reloading after the gradual decomposition of the particles. In our approach we used antibodies as receptors for the drug loaded nanoparticles. The nanoparticles used in this study were either made of PLGA (Poly (D,L-lactide-co-glycolide)) that is degradable by hydrolysis, or of chitosan that is degraded by the lysozyme circulating in human blood.

3.1.1 Passive coating

In general, passive coating with hydrophilic, protein-repellent polymers provides good biocompatibility as it acts as a barrier between the implant-material and the host defence mechanisms that initially rely on opsonisation by endogenous marker molecules. In this study the well-described polymer polyethyleneglycol (PEG) was used for passive implant-coating. PEG is water-soluble and non-ionic and approved by the FDA (food and drug administration) for many pharmaceutical and biomedical

applications. It can be chemically modified for attachment to other molecules and surfaces (Li J. et al. 2003). PEG is widely used in the food additives and cosmetics industries and has many biochemical, industrial, and pharmaceutical applications as a potent fusogenic agent (Bailey F. et at. 1976). In clinical practice, PEG is often employed to study the permeability of organs such as the gut (Parlesak A. et al. 1994) and bile duct (Roma M. et at. 1991) under normal and pathological conditions. Several enzymes and proteins with therapeutic potential have been covalently modified with varying sizes of PEG in order to enhance their biological efficacy (Katre N. 1993). PEG conjugates of several clinically important proteins have been shown to exhibit increased circulatory lifetime as well as reduced antigenicity and immunogenicity (Katre N. 1993, Lang G. et al. 1992, Zimmerman R. et al. 1989, Meyers F. et al. 1991, Wada H. et al. 1990, Chaffee S. et al. 1992). PEGylated drugs such as PEG-interferon and PEG-asparaginase are already available on the market. In addition, PEG confers tolerogenic properties to a number of allergens (Holford-Strevens V. et at. 1982). These observations have emphasized the potential usefulness of PEG in enhancing the therapeutic index of many biologically important molecules.

The generation of protein resistant surfaces is a basic requirement in the design of many medical devices and implants that come in contact with blood, such as catheters or vascular stents. Protein resistant surfaces are necessary to avoid or reduce non-specific protein adsorption, platelet adhesion, and thrombus formation and thus prevent undesired reactions of the living system to the device or implant. Since implant materials, such as steel or titanium and its alloys, covered by their respective surface oxides (passive film), are often applied in blood-contacting devices, a reliable method for reducing protein adsorption on oxide surfaces *in vivo* would be highly desirable. Moreover, protein resistant surfaces are increasingly applied in bioaffinity sensing where the selectivity and sensitivity of the antigen/antibody assay is directly proportional to the suppression of non-specific adsorption. In other words, protein resistant surfaces are needed to reduce non-specific adsorption in order to allow implant site-specific ligand recognition.

Non-specific adsorption can be avoided by pre-coating the implant surface with a material resistant to protein adsorption. Such materials are typically non-ionic and very hydrophilic. For example, polyvinyl alcohol (Amanda A. et al. 2001),

polyacrylamide as e.g. poly HEMA (Park S. et al. 2000), hyaluronic acid (Matsuda T. et al. 1992), dextrane (Holland NB. et al. 1998), and PEG (Scott M. et al. 1998, Zalipsky S. et al. 1992, Kenausis G. et al. 2000, Huang NP. et al. 2001, Van de Vondele S. et al. 2003) have been used as coating materials for such purpose. PEG has also been used to sterically stabilize red blood cells from lectin-induced haemagglutination and fibroblasts from adhesion to fibronectin-coated surfaces (Elbert D. et al. 1998).

***In this thesis we set out to develop a biocompatible coating for the implant materials stainless-steel 316L and ceramics (zirconia, ZrO_2-TZP-A BiO-HIP). The covalently bound polymer layer conveys biocompatibility by reducing protein adsorption. On the other hand, the polymer layer also contains functional groups that provide anchorage for a bio-recognition system that makes this layer a pharmacologically active coating*.**

3.2 Specific aim 2

To introduce binding sites for degradable, drug loaded nanoparticles into the biocompatible coating (Chapters 2, 7)

3.2.1 Bio-recognitive system for drug delivery system

A difficult issue to solve in this thesis was to find suitable biorecognitive systems for binding of the drug containing particles. The design of the receptor is of great importance as any bio- or chemorecognitive interactions with other components circulating in the blood should be avoided. Furthermore, the binding between the receptor and the nanoparticles has to be strong enough to keep them tightly bound during their lifetime, but on the other hand allow reloading after final degradation. Antibodies against the biocompatible nanoparticles were bound to the implant surface as biorecognitive site for reloading of the particles.

In this thesis we chose an antibody which serves as a binding site for the bio-recognitive system for drug containing nanoparticles. The immobilization of Ab molecule or its Fab´ fragment onto the biocompatible coated carriers is shown

using fluorescence microscopy. More over a newly optimized method to create two Fab` fragments using dihydrolipoamide was developed.

3.3 Specific aim 3

To search and develop a biorecognitive reloadable binding system for a drug delivery system e.g. nanoparticles (Chapters 3, 4, 5, 6).

3.3.1 Drug-delivery and targeting-systems

The method of drug delivery can have a significant effect on the efficacy of the drug. Most drugs have an optimum concentration range with a maximum benefit, and concentrations below this range produce minimal or no therapeutic benefit, whereas concentrations above this range may be toxic. On the other hand, the very slow progress in improving efficacy of treatments in severe diseases has suggested a growing need for specific delivery of therapeutics to targets tissues. The “magic bullet” concept, (Ehrlich P. 1913), brought drug targeting systems to the fore front of pharmaceutical research, however the practical implementation of these “magic bullets” continues to be a challenge. Since then new concepts for controlling the pharmacodynamics, pharmacokinetics, immunogenicity, biorecognition, non-specific toxicity, and efficacy of drugs have been developed. These drug delivery systems (DDSs) are based on interdisciplinary approaches combining pharmaceutical research, polymer sciences, molecular biology and bio-conjugate chemistry. Other approaches to drug delivery are focused on crossing particular physical barriers, such as e.g. the blood brain barrier (BBB), in order to improve targeting of the drug and also improve its effectiveness. This approach is an alternative and acceptable way for delivery of protein drugs as opposed to delivery via the gastro-intestinal tract, where degradation can occur very rapidly. Other challenges for DDS are related to the properties of the carrier that needs to maintain the drug in a stable and active form while it is travelling through the body to its specific target site. Non-specific interactions need to be avoided during this time. Various DDSs and drug targeting systems are currently under development to minimize drug loss and degradation, to prevent harmful side-effects, and to increase drug bioavailability at the target site.

Among such drug carriers are micro- or nano-particles made of biodegradable natural and synthetic polymers, lipoproteins, liposomes and micelles. According to a number of studies, nanoparticles show better performance than other drug carriers (Kaparissides C. et al. 2006). Nanoparticles have a solid structure with good physical stability and can be designed using a wide range of materials to control release of the encapsulated drug. Drugs can be bound to nanoparticles in various forms such as a solid solution, dispersed or adsorbed onto the surface, or chemically bound. To prolong the circulation of nanoparticles in the blood their surface can be modified, coated or attached with targeting ligands to achieve site-specific drug delivery (Saeed Arayne M. et al. 2007).

In biological systems nanoparticles show different properties depending on their surfaces (Figure 2). The bio-distribution of nanoparticles is largely determined by their physical and biochemical properties, such as the nature of the polymer and drug, particle size and biochemical surface properties (Cheng J. et al. 2007). The polymeric matrices of "conventional structures" (Figure 2) tend to be hydrophobic and are slightly coated by serum proteins that act as opsonins. However, opsonisation of the particles can be reduced by coating nanoparticles with flexible hydrophilic polymers, particularly polymers containing polyethylene glycol (PEG), which allows them to remain in the circulation for extended periods of time (Garnett M. et al. 2006). PEG as a polymer for "nonfouling" surfaces can resist the adsorption of proteins and/or cells. The matrix of polymeric nanoparticles can be modified with cell-specific ligands (e.g. antibodies) for "target-orientation". Such target-oriented nanoparticles are commonly used in chemotherapy. However, phagocytic cells, such as macrophages, may recognize injected particulate drug carriers as foreign materials and remove them via nonspecific phagocytosis via the mononuclear phagocytic system (MPS) (Thomasin C. et al. 1996). In this case the MPS-clearance might be decreased by reducing the particle size to < 100 nm. These nanoparticles were also reported to resist opsonisation.

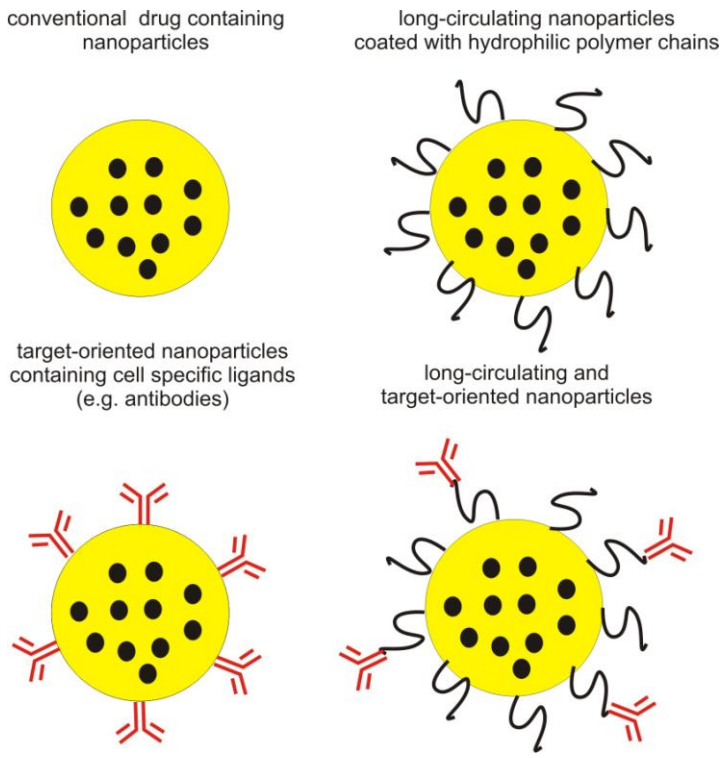


Figure 2. Surface variations of nanoparticle drug carriers.

3.3.2 Controlled drug release

The mode of drug delivery can make the difference between a drug's success and failure. Moreover the choice of drug is often influenced by the way it is administered. Drugs can be released at a controlled rate (continuous release), by diffusion, or by degradation of the polymer over time. Pulsed release, which closely mimics the way of natural hormone release, such as insulin, is often the preferred method of drug delivery. It is achieved by using drug-carrying polymers that respond to specific stimuli such as changes in pH, temperature or ultrasonication (Chouhan R. and Bajpai AK. 2009; Husseini G. and Pitt W. 2009).

Controlled drug release and the subsequent bio-degradation of the nanoparticle polymer are important aspects in the development of successful formulations. The rate of bio-degradation of nanoparticles can be controlled via the hydrophobic/hydrophilic ratio. Release rates depend on:

- Diffusion through the NP-matrix
- Diffusion (in case of nanocapsules) through the polymer wall

- Desorption of the surface-bound/adsorbed drug
- Erosion of nanoparticle-matrix
- A combination of erosion and diffusion

3.3.2.1 Biodegradable PLGA-based nanoparticles

PLGA {poly (D, L-lactide-co-glycolide)] is a random copolymer of glycolic acid and lactic acid, it is insoluble in water and soluble in organic solvents, such as ethyl acetate, chloroform, dichloromethane, tetrahydrofurane, DMSO, dioxane, toluene, acetone, ethanol or petroleum ether. The ratio of glycolic acid to lactic acid determines the rate of degradation, generally considered as hydrolysis. The hydrophilicity of PLGA increases with decreasing percentage of glycolic acid. Degradation of PLGA yields lactic acid and glycolic acid, both also products of the endogenous metabolism. A large number of *in vivo* and *in vitro* studies have shown the full biocompatibility and biodegradability of PLGA nanoparticles and their degradation product (Athanasiou K. et al. 1996, Anderson J. 1997, Tracy M. 1999).

In clinical applications, PLGA nanoparticles can be administered parenterally, vascularly or orally for site-specific delivery. However, the nonspecific adsorption of plasma proteins on PLGA micro- and nanospheres remains one of the main limitations of drug targeting (Müller M. et al. 2003). Müller et al. (2003) demonstrated a strong decrease of protein adsorption on poly (L-lysine)-g-poly(ethylene glycol) (PLL-g-PEG)-coated PLGA microspheres for the tested proteins HSA (human serum albumin), Fibronectin, Fibrinogen, and IgG as representatives for abundant human blood proteins. The choice of PLGA as a polymer for nanoparticles originated from their use in the medical device industry to make bioabsorbable sutures. However, their unique properties, including versatile degradation kinetics, established safety and biocompatibility, made them appear ideal for drug delivery applications. Moreover, PLGA nanoparticles not only modify the pharmacokinetics of encapsulated drugs, but they also protect the occluded drugs from enzymatic attack.
Currently, a number of FDA-approved products on the market utilize PLGA as excipients to achieve sustained release of the active ingredient, such as e.g. Neutropin Depot®, Sandostatin LAR® or Trelstar Depot®. PLGA nanoparticles are used also as a carrier for AI-700 that is used for stress echo cardiological evaluation.

The efficiency of PLGA nanoparticles as a local drug delivery system (for example dexamethasone) to decrease neointimal proliferation, without having significant systemic effects was already shown in the rat carotid injury model after ballon injury (Andweson J. and Shive M. et al. 1997).

3.3.2.2 Chitosan nanoparticles

Chitosan (CS) is a linear aminopolysaccharide composed of approximately 20% 1,4-linked N-acetyl-D-glucosamine (GlcNAc) and approximately 80% β1, 4-linked D-glucosamine (GlcN) produced by partial deacetylation of chitin in hot alkali. As described in Yan W. 2005, CS has shown favourable biocompatibility characteristics (Knapczyk J. et al. 1989; Hirano S. et al. 1989, 1990) as well as the ability to increase membrane permeability, both *in vitro* (Aspden F. et al. 1997; Lehr C. et al. 1992; Dumitriu S. and Chornet E. 1998) and *in vivo* (Takeuchi H. et al. 1996). It is degradable by lysozyme, which circulates in the bloodstream, thus making it a suitable material for drug containing nanoparticles. Moreover the low toxicity (Aspden et al. 1995), ease chemical modification and excellent capacity for the protein-entrapment of CS make it a suitable candidate for use in gene- and drug-delivery systems (Calvo P. et al. 1997b, De la Fuente M. et al. 2008). CS has received increasing attention in the design and engineering of novel nanoparticulate drug delivery systems. Desirable properties of CS include biocompatibility, biodegradability, bio-and mucoadhesivity, as well as hydrophilic characteristics that facilitate administration and increase the bioavailability of poorly absorbable drugs across various epithelial barriers, such as corneal, nasal and intestinal mucosa (Alonso M. J. et al. 2007). An additional advantage of a CS based system is that it can be produced under aqueous and fairly mild conditions, thus being especially suitable to preserve the bioactive conformation of delicate macromolecules (e.g. proteins, hormones, antigens, pDNA, siRNA, growth factors, heparin, etc.) that otherwise would be prone to enzymatic degradation and hydrolysis (Janes K. et at. 2001; Csaba N. et al. 2006). The physicochemical, biomedical and pharmaceutical properties of chitosan have been described in detail in several articles (Skaugrud O. et al. 1999; Agnihotri S. et al. 2004; Ravi Kumar M. et al. 2004; George M. and Abraham T. 2006; Rinaudeo M. 2006, 2008). Drug delivery systems including chitosan-beta-cyclodextrin nanoparticles and nanocore-coated type capsules were

described in Alonso et al. (Alonso M. J. et al. 2007). Thus, Chitosan has been widely utilized in the development of potentially innovative drug delivery systems, tissue engineering and wound dressing systems over the last decade. Alonso M.J. and Goycolea F.M 2008 mentioned that over 50% of the total number of filed patents in 2006 that claim the use of this biopolymer as a substantial part of the invention are related to drug delivery, tissue engineering and wound healing (Higuera-Ciapara I. et al. 2007).

From a biopharmaceutical point of view, CS has the potential of serving as an absorption enhancer across intestinal epithelia, because of its mucoadhesive and permeability enhancing property (Janes K. et al. 2001). It has been shown that CS could enhance insulin absorption across human intestinal epithelial (Caco-2) cells without injuring them (Artursson P. et al. 1994; Schipper N. et al. 1996, 1997, 1999; Thanou M. et al. 2001). CS has been used in preparing films, beads, intragastric floating tablets, microspheres, and nanoparticles in the pharmaceutical field (Berthold et al. 1996; Felt O. et al. 1998; Giunchedi P. et al. 1998; Calvo P. et al. 1997a; Illum L. 1998; Wu et al. 2003). Previous results have also emphasized the importance of particle size and have demonstrated the advantages of nanoparticles (>1µm) over microspheres (Meclean S. et al. 1998). Their small size facilitates distribution via blood circulation throughout the body. Hydrophilic nanoparticles have in general a longer life time in the blood stream (Allemann E. et al. 1993).

In this thesis a very stringent immunisation protocol was developed for the production of polyclonal antibodies against a biocompatible and biodegradable polymer, such as PLGA (Chapter 6). A number of technical problems were solved regarding purification and testing of the titer, which proved difficult due to the rapid degradation of PLGA in the injected rabbits. The determination of PLGA Abs in immune sera was carried out using ELISA and Cluster Linked Immunosorbent Assay (CLISA). By direct purification of PLGA antibodies from immune rabbit sera, using affinity chromatography, a protein was isolated which interacts with PLGA. Analysis of this protein by means of native gel electrophoresis and mass spectrometry showed that this protein has a homology to albumins. Therefore, we investigated also the effect of human serum albumin on PLGA, and could prove its binding to PLGA nanoparticles.

According to these results, it is recommended to coat PLGA-NPs before injecting them into the human body to avoid unspecific coating with serum albumin.

Another drug delivery system based on chitosan nanoparticles was also studied in this thesis to show proof of principle for the novel developed implant material.

3.4 Specific aim 4

To provide a proof of principle testing of the interaction between the nanoparticles and their in biocompatible coating embedded binding site (Chapter 7)

3.4.1 Active coating

Active coating is based on the temporary binding of pharmacologically active substances onto implant material. Upon release, these substances can exert their intended pharmacological effect at the implant site. In this thesis we chose to use a drug delivery system was based on functionalized nanoparticles. These particles are capable of binding to molecular receptors embedded on the applied polymer coated implant material. Such a bio-recognitive system has to fulfill several requirements:

1) Any bio- or chemorecognitive interaction with other components than target molecules should be prevented;

2) The binding between receptor and particles has to be strong enough to keep the drug releasing nanoparticles locally and tightly bound;

3) The biorecognitive interaction between the polymer of the particles and the receptor should be strong enough, but weak with the emerging monomers (lactic- and glycolic acid in case of PLGA nanoparticles or 1, 4-linked N-acetyl-D-glucosamine (GlcNAc) and approximately 80% β1, 4-linked D-glucosamine (GlcN) in the case of chitosan nanoparticles) to allow reloading.

To meet these criteria, antibodies recognizing PLGA or chitosan, the polymers forming the nanoparticles, need to be bound to the implant surface. As the epitope recognized by an antibody comprises a sequence of 6-8 amino acids, it is expected that the binding of any residual monomer to the paratope is weak, and the monomer

is easily displaced by the PLGA- or chitosan-polymer. This effect was shown by classic binding competition assays (Elvin A. et al. 1960).

In this thesis the interaction between the nanoparticles and the implant was studied using fluorescently labeled particles visualized by fluorescence microscopy. It has been shown previously that different drugs can be embedded into biocompatible and biodegradable PLGA or chitosan nanoparticles. These nanoparticles then gradually release the drugs in a controllable manner. The release of encapsulated drugs can be tailored by modulating the molar ratio of the monomers, by the addition of auxiliary agents, and by the size of the particles (Ertl B. et al. 1999; Wolf M. et. al. 2003). This makes the nano-encapsulation of drugs in PLGA or chitosan nanoparticles an approved and safe technology for the long term delivery of therapeutics.

4 Applications of the novel implant material developed in this study

4.1 Reloadable drug delivery implant systems

Our proposed strategy for coating and drug delivery can provide the basis for a number of applications that require controlled, local drug delivery at the interface between an implant and healthy tissue. Upon the need for medication, drug-loaded nanoparticles can be injected into the human body as e.g. in case of heart stents or directly into e.g. specially constructed joint implants. These will subsequently bind to the particle-specific antibodies on the target implant, and release the drug. As nanoparticles can be loaded with different drugs, this technology allows easy adjustment to patient-specific medication needs, as well as the administration of compounds that would either degrade quickly, or that are difficult to dose, if given orally or intravenously (e.g. growth factors, immunosuppressives, thrombolytic enzymes made biocompatible by chemical engineering).

Bone implants: Depending on the application, nanoparticles can be loaded with various drugs to improve the recovery of the patient after implantation. For example, placement of an implant is always associated with the risk of microbial infection, and

the risk is considerably higher when used for fixing open-fractured bones (Tsukayama D. and Gustilo R. 1996) or in joint revision surgeries (Dunlop D. et al. 2002). This necessitates effective antimicrobial therapy immediately after surgery. However, systemic therapies are not optimal as there is poor accessibility to the implant site due to impaired blood circulation. In addition, after attachment to the implant surface pathogens can develop resistance to drugs, so that higher concentration of antibiotics need to be administered, increasing systemic doses may have a limit, because of toxicity (Wu P. et al. 2006). It can be envisaged that many of these implant-associated complications may be addressed far more efficiently through local delivery of pharmaceuticals as proposed in this project. For bone, implant therapies that stimulate the bone to bridge the gap between healthy tissue and the implant and even render it resistant to erosion are highly desired for a longer implant lifetime. In addition, osteosynthetic devices and artificial joints often face bones with a certain extent of osteoporosis. Restoring the bone mass locally may improve both the implant itself and the structure of the repaired bone. Bone regeneration can be strongly enhanced by growth factors. They promote replication, differentiation, protein synthesis and/or migration of respective cell types. These effects increase the osteoblastic activity of the bone tissue and therefore stimulate bone regeneration (Lieberman J. et al. 2002; Solheim E. 1998). Simple dip-coating of implants with certain growth factors significantly accelerates bone formation and gap bridging (Sumner D. et al. 1995). However, the efficacy of such physisorbed molecules is not optimal; because they are released quickly, and eluted molecules may cause side effects such as uncontrolled ossification at other sites. A number of important issues remain to be resolved, before local delivery systems for growth factors can be successfully implemented in the clinic. These are above all optimisation of dosing, release kinetics and release duration.

Cardiovascular stents: Drug delivering implants [e.g. drug eluting stents (DESs)] play already an important role in the treatment of cardiovascular diseases. These stents have been introduced as a new and promising technique to reduce restenosis after stent implantation. Several substances with immunosuppressive, antiproliferative and cytostatic activities have been tested as active stent coatings, and were shown to reduce restenosis in humans (Chieffo A. et al. 2002; Grube E. et al. 2004). Despite these advances, restenosis rates still remain substantial in patients

with high-risk for restenosis, such as patients with long lesions and complex lesion morphologies in bifurcations. The DESs currently in use contain only a certain amount of drugs, and restenosis may take place if this reservoir is used up. These limitations can be overcome through reloading the stent with drugs, as proposed in our study. Moreover nanoparticles can be loaded with different drugs that directly interfere with intima proliferation such as prostacyclin (PGI_2) which prevents thrombosis.

For patients with additional pathogenic problems, such as e.g. hypertension or diabetes additional appropriate drugs could be administered via nanoparticles. This would reduce discomfort for the patients by elimination the need for daily intake of pharmaceuticals and consequent reduction of their adverse side effects. The drug-loaded nanoparticles can be injected as required. Moreover if stent thrombosis occurs shortly after implantation and there is no possibility for acute coronary angiography, it is necessary to apply thrombolytic drugs. Conventionally these are administered systemically, with the disadvantage of adverse effects on the whole body, whereas the proposed targeted nanoparticles act only in the vicinity of the stent.

4.2 PLGA antibodies in clinical and research imaging

Targeting is the ability to direct the drug-delivery system to the site of interest. Nano-particles can be targeted actively, e.g. by conjugating them with specific antibodies against certain characteristic components of the tissue of interest. The preferential accumulation of chemotherapeutic agents in solid tumours as a result of the enhanced vascular permeability of tumour tissues, compared with healthy tissue, is an example of passive targeting. For active targeting PLGA antibodies can be used. A strategy for therapeutical and diagnostic applications is illustrated in Figures 3 and 4. A spacer molecule is coupled on one end with ligands that are selectively recognized by receptors on the surface of the cells of interest. The other end of the spacer molecule can bind to PLGA antibodies which interact specifically with PLGA nanoparticles that contain drugs for therapeutical purposes or visualisation dyes for diagnostic purposes or, e.g. gadolinium for MRI (magnetic resonance imaging) or PET (positron emission tomography) live imaging. This could allow for a more

precise targeting to the site of interest (for example in solid tumors) since ligand–receptor interactions can be highly selective.

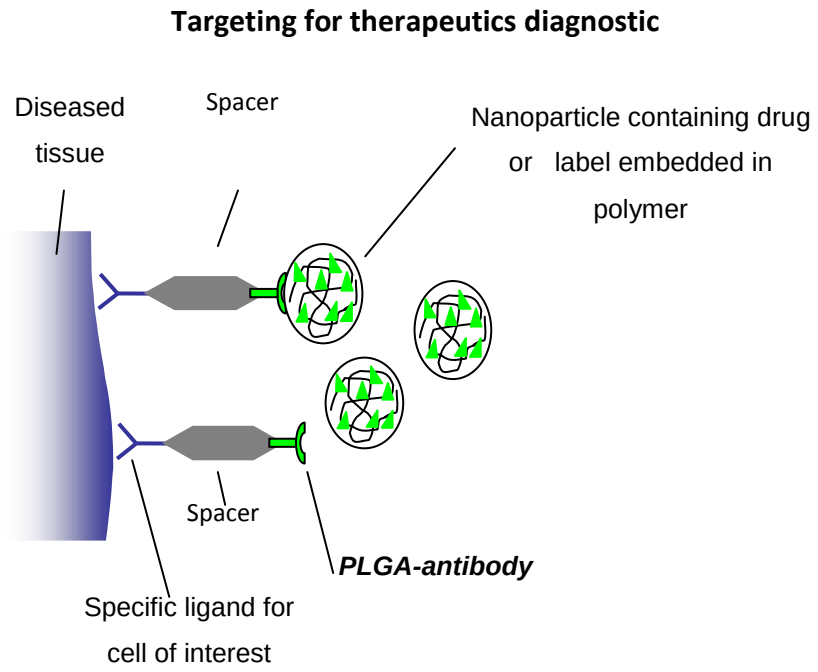


Figure 3. Therapeutic and diagnostic applications of PLGA antibodies.

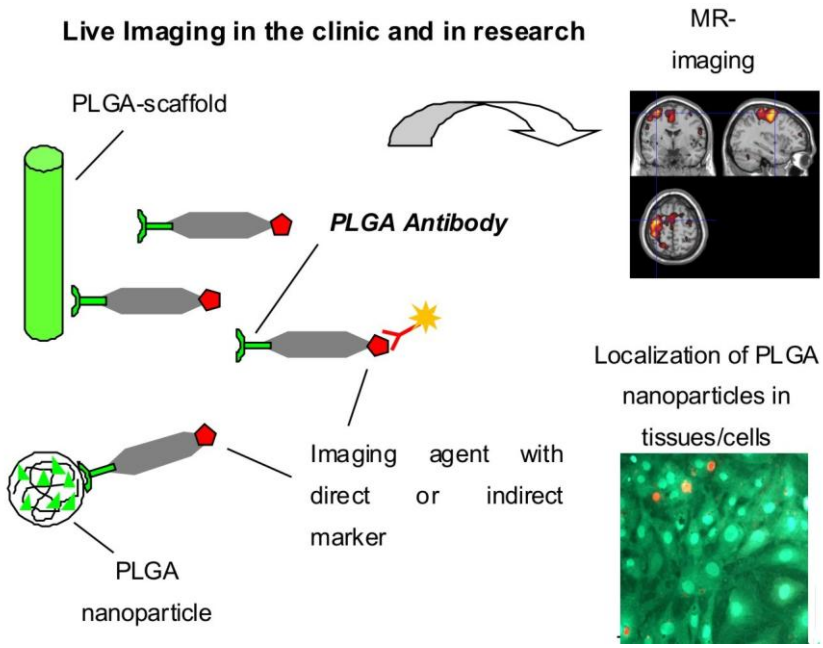


Figure 4. Diagnostic application of PLGA antibodies for tracing of the localisation of PLGA nanoparticles in the body using PET or MRI.

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Part 1

Covalent binding of a biocompatible coating and development of analytical tests for proof of a successful set-up

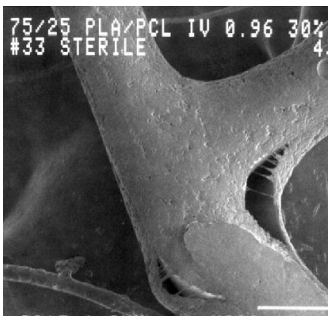
Chapter 1

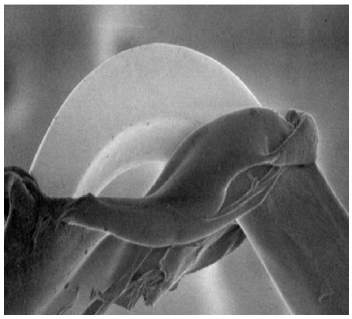
1. Analytical methods for detection of small amounts of amino groups on solid surfaces – a survey

2. Biocompatible coating of implant materials

Chapter 2

Cleavage of antibodies using immobilized dihydrolipoamide and anchoring of antibody fragments onto biocompatible-coated carrier





1. Analytical methods for detection of small amounts of amino groups on solid surfaces – a survey

This Chapter describes approaches developed for passive coating to reach the first specific aim in this thesis; ***to produce a covalently bound biocompatible coating for implant-materials***.

The implant materials used in this study were stainless-steel L316 and ceramics (ZrO_2). Passive coating for enhancement of biocompatibility through the so called "Stealth effect" was achieved by the use of partially functionalized, biocompatible derivatives of polyethylene glycol (PEG). The effective binding of PEG derivatives to stainless-steel L316, and ceramics was illustrated using analytical methods for detection of amino groups. Their absence or presence during the different steps of binding procedures provides a useful proof of successful reaction procedures. As the amount of amino groups on the surface of implant materials turned out to be very low, very sensitive analytical methods had to be used. Fluorescent staining with OPA (o-phthaldialdehyde) proved difficult to demonstrate such an effective setup on metal because of quenching of optical signals, as well as on ceramics, because of its uneven surface. For these purposes, silicate glass material was used. Silicate glass has similar chemical properties to ceramics and provides additional advantages such as availability, low price, even surface and does not interfere with optical signals when using fluorescence microscopy. Furthermore, it can be derivatized using the same procedures as metal or ceramics. The effective binding of PEG derivatives to stainless-steel L316, and ceramics and in parallel to silicate glass was shown using an enzymatic assay [alkaline phosphatase (AP)] specifically developed for our purpose using. The final binding of fluorescent labeled nanoparticles to coated surfaces was then shown on silicate glass. Different analytical methods were performed or adapted for detection of small amounts of amino groups on solid surfaces. Next to the quantification of available amino groups, the area-wide coating should be visualized with regard to biocompatible coating of medical relevant materials. Thereby, photometric and fluorometric methods were used as well as an enzymatic assay was developed and all compared to each other.

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1. Analytical methods for detection of small amounts of aminogroups on solid surfaces – a survey

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Abstract

Different analytical methods were performed or adapted for detection of small amounts of amino groups on solid surfaces. Next to the quantification of available amino groups, the area-wide coating should be visualized with regard to biocompatible coating of medical relevant materials. Thereby, photometric and fluorometric methods were used as well as an enzymatic assay was developed and all compared to each other.

Keywords

Biocompatible coating; TNBS assay; Kaiser test; Enzymatic assay; Sulfo-SDTP assay; OPA assay

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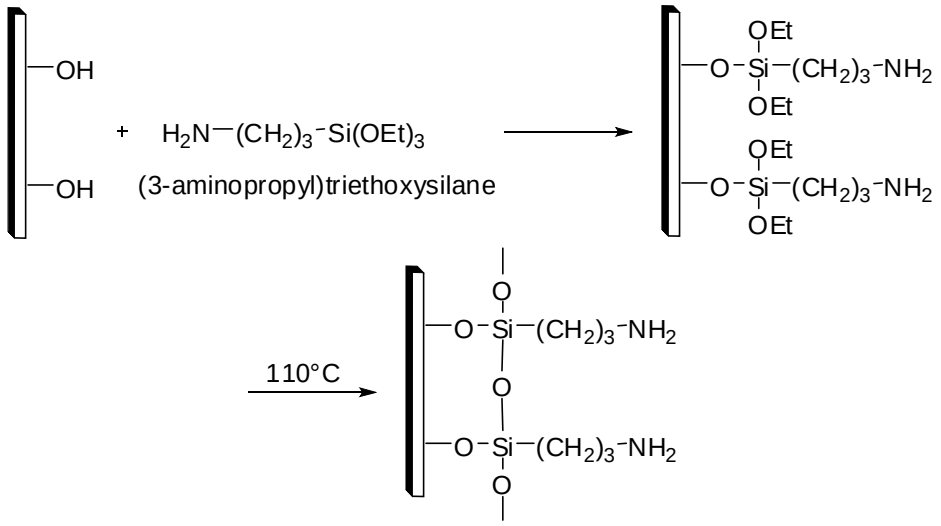
Introduction

For medical applications, the biocompatible coating of relevant materials is important for acceptance or rejection of the 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 (Williams D. et al. 2003). In the literature, Poly(ethylene glycol) (PEG) is one of the best described biocompatible polymers for non-fouling surfaces (Ratner B.D. et al. 2004). It is hydrophilic and therefore employed extensively in pharmaceutical and biomedical applications. For attachment of other compounds, it can be chemically modified.

Tests of biocompatibility are performed in animal experiments with porcine coronary artery as the most commonly used animal model or *in vitro* with the help of cell or organ culture. Thereby, the chemical, physical and structural properties of the biomaterial and the tissue responses to it are critical factors. Thus, next to culture studies (data not shown) the area-wide coating with the biocompatible bisamino-PEG (NH_2-(CH_2-CH_2-O)$_n$-NH2) was evaluated in this study by comparison of different analytical methods for the detection of small amounts of amino groups on solid surfaces.

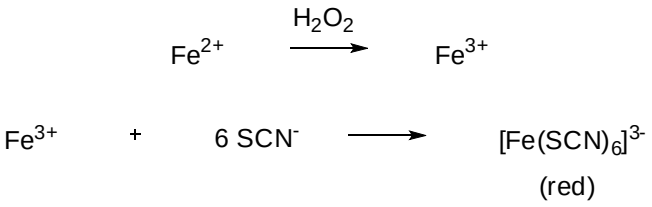
Coating procedures

As carriers, stainless steel 316L (12% Ni, 17% Cr, 2.2% Mo, 67% Fe, 1% Cu, 0.25% N) was used as standard of 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 or Gulsenit respectively were used. Gulsenit is an active magnesium silicate mineral having a particle size less than 10 µm and a density of about 3.2 kg/l. The first step of coating was conducted by amino-silanization with APTS (3-aminopropyltriethoxysilane), a popular organo-silane to create functional amino groups on inorganic surfaces (Scheme 1). The reaction can be performed either by aqueous or by organic solvent deposition. Heating to 110°C forms the siloxane bond resulting in a more stable coating.



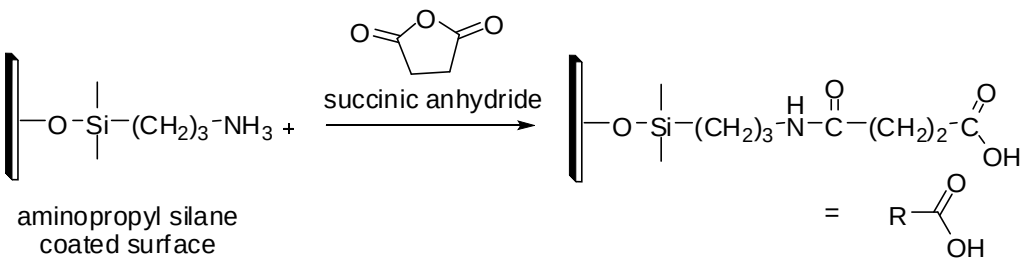
Scheme 1

For activation of 316L, the stainless steel was etched with 3% HNO_3, 0.5M HCl or 6M HCl for 10 min and soaked for two days in double distilled water (dd. H_2O) to obtain hydroxyl groups needed for the further amino-silanization as described before. The effective etching was verified by reaction of Fe^{3+} with NaSCN forming a red complex (Scheme 2).



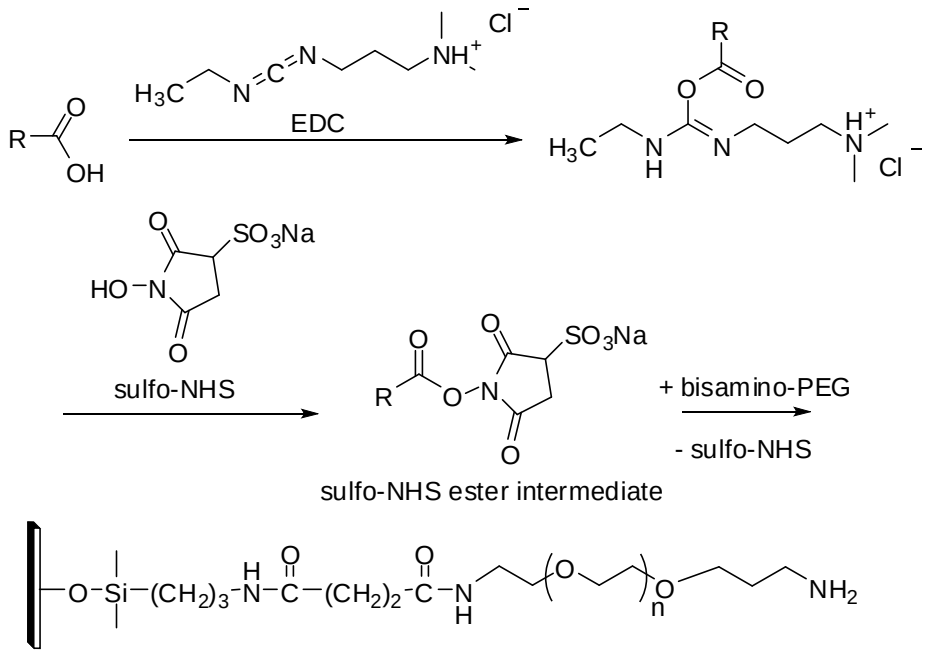
Scheme 2

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



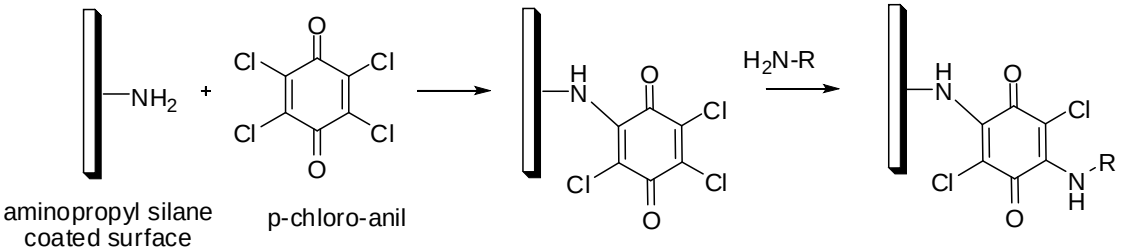
Scheme 3

EDC is a zero-length cross-linker and an often used carbodiimide for coupling of carboxylates with amines. Adding additional sulfo-NHS (sulfo-N-hydroxysulfosuccinimide) increases the yield of EDC-mediated reactions several times (Scheme 4) (Pittner F. 2002).

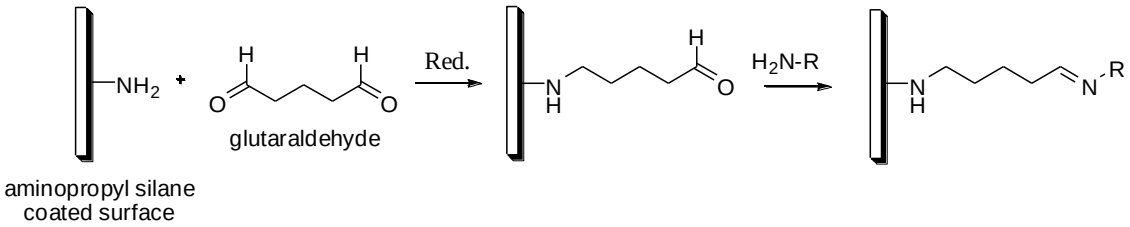


Scheme 4

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



Scheme 5



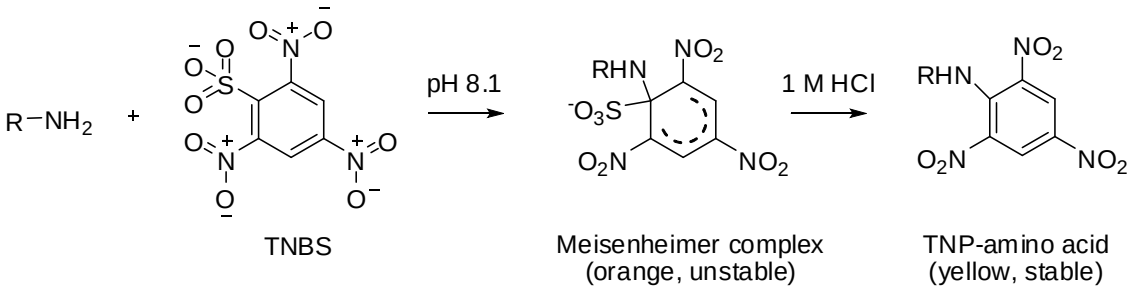
Schem6

Analytical methods for detection and quantification of amino groups

For detection of the amino groups of the amino-silanized or of the amino-PEGylated slides respectively, different analytical methods were compared. Thereby, the quantification of the amount of amino-groups available on the surface as well as the area-wide coating for optimal biocompatibility were studied. Here, the Kaiser-test, an enzymatic assay, assays employing TNBS (2,4,6-trinitrobenzenesulfonic acid solution) and sulfo-SDTP (sulfo-succinimidyl-4-O-(4-4´-dimethoxytrityl)-butyrate) and the fluorescent staining with OPA (o-phthaldialdehyde) are discussed.

TNBS assay

TNBS, a reagent introduced by Sateke in 1960 (Okuyama T. et al. 1960), reacts under relatively mild alkaline conditions with amines forming an unstable Meisenheimer complex – a highly chromogenic orange-colored derivative with an absorbance of 335nm. Subsequent acidification to pH 1 rapidly converts the orange unstable product to a yellow stable trinitrophenol (TNP) derivative (Scheme 7) (Palmer D. et al, 1969).

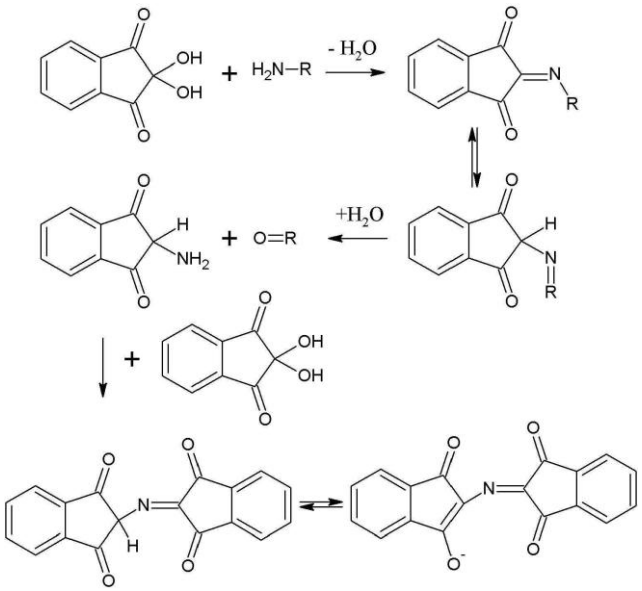


Scheme 7

Kaiser test

The ninhydrin reaction was developed by Moore and Stein in 1948 (Moore S. et al. 1948) and adapted for the detection of amino groups on solid phases by Kaiser et al.

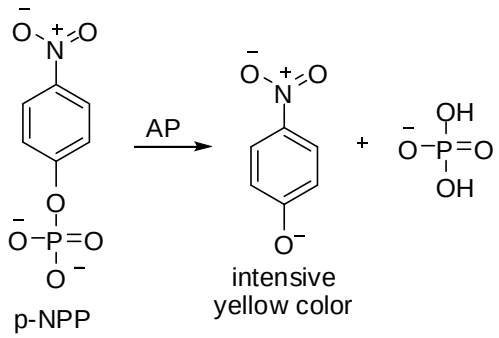
in 1970 (Kaiser E. et al. 1970). Ninhydrin reacts with primary amines on the surface forming Ruhemann's Blue (Scheme 8). As Ruhemann's blue is slightly soluble it can be found on the surface as well as in the solution, thus it is not useful for quantification.



Scheme 8

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 405nm (Scheme 9).

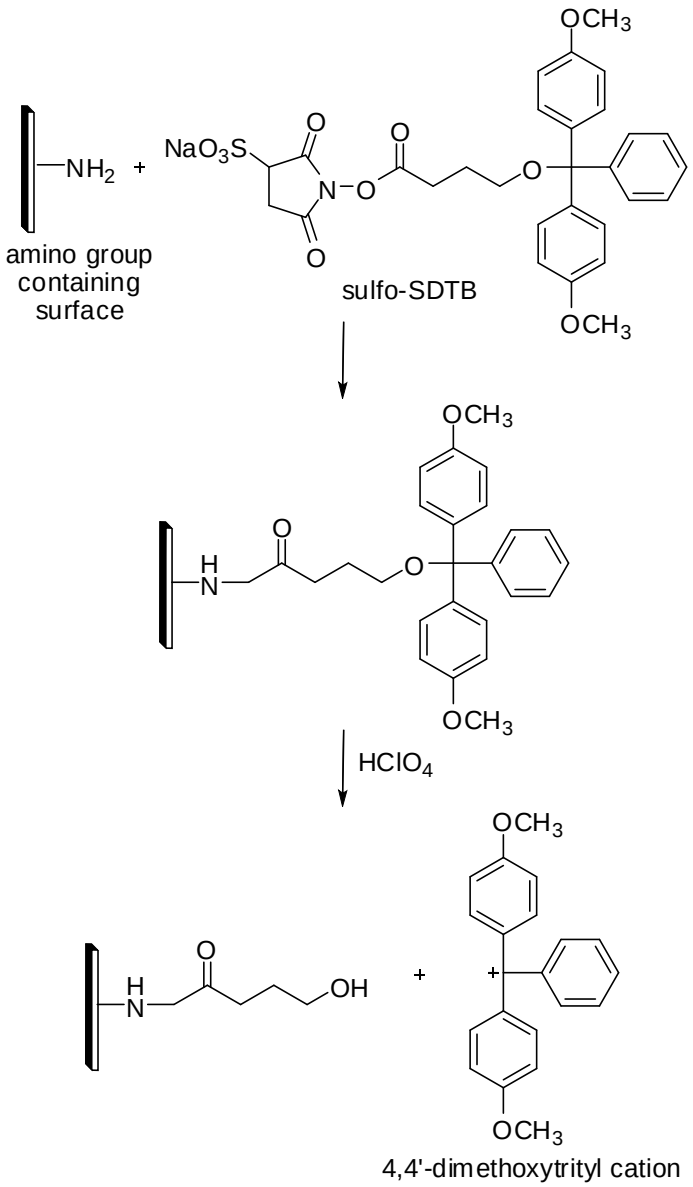


Scheme 9

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.

Sulfo-SDTP assay

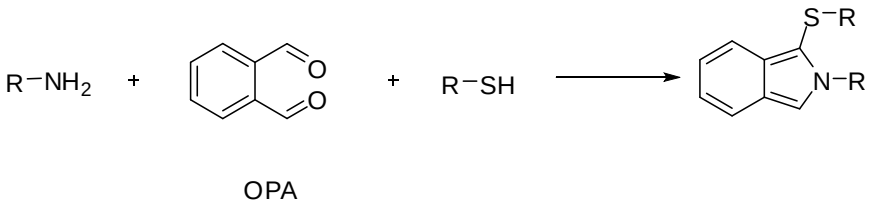
Sulfo-SDTB (sulfo-succinimidyl-4-O-(4,4´-dimethoxytrityl)-butyrate) is used for colorimetric assay and has a high sensitivity to amino groups on solid support (Gaur R. et al. 1989). Sulfo-SDTB reacts with amino groups in the presence of perchloric acid to release the 4.4´-dimethoxytrityl cation with a very high extinction coefficient (E_{498} = 70.000) (Scheme 10), which makes this method very sensitive (Alonzo C. et al. 1997). The released cation can be measured at a wavelength of 498nm.



Scheme 10

OPA assay

OPA (o-phthaldialdehyde) reacts with amino groups in presence of thiol-containing molecules like 2-mercaptoethanol generating a fluorescence product with an extinction wavelength of λ_{exc} = 360nm and an emission wavelength of λ_{em} = 455nm (Scheme 11). Detection limits for proteins in liquid are in the µg/ml range.



OPA

Scheme 11

Results and Discussion

TNBS assay

Only slides after amino-silanization by organic solvent deposition show a 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, it was not used further. As the much simpler OPA assay showed better results for these purposes, it was preferred.

Kaiser test

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

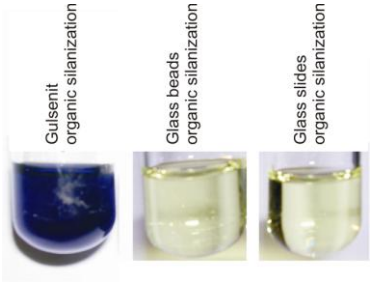


Figure 1. Detection of free amino groups with the Kaiser test. The intensity of the blue color is generated by the reaction of ninhydrin with free amino groups. Amino-silanized Gulsenit provides the most intensive blue color due to the high amount of free amino groups. However, the Kaiser test was of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

Enzymatic assay

Table 1 shows the results for amino-silanized carriers. Etching of 316L stainless steel with 0.5M HCl and 3% HNO_3 increases the amount of AP that could be immobilized about four fold compared to 6M HCl. For the non-etched 316L stainless steel, no AP activity was measured which demonstrates the importance of previous etching of stainless steel for subsequent amino-silanization. Thus, etching with 0.5M HCl or 3% HNO_3 was preferred for further immobilizations. The detected amount of amino groups on glass carriers is higher than 316L stainless steel despite the etching.

Sample description	Slope	Amount of coupled AP (ng/carrier)	Calculated amount of NH_2 [nmol/cm^2]	Appendix (1.2)
Not etched 316L	8.00E-05	-0.1	-1.929E-07	Figure 2A
316L etched with 0.5 M HCl	0.0006	2.5	4.823E-06	Figure 2B
316L 3% HNO_3	0.0005	2.0	3.858E-06	Figure 2C
Amino-silanized glass	0.0007	3.0	5.787E-06	Figure 2D

Table 1. Quantification of AP immobilized with glutaraldehyde onto amino-silanized carriers and the etching effect of stainless steel 316L (surface of the glass slides and 316L = 2.88 cm²). The amount of immobilized AP enzyme is calculated using a standard calibration curve (y = 0.0002x + 0.0001) as described in the section Experimental.

Table 2 shows the results of the 316L stainless steel after amino-PEGylation using EDC as a cross-linker at pH 5 and 10. The different etching methods of the 316L stainless steel and the different pH conditions for cross-linking with EDC show virtually similar results. However, the amount of immobilized AP is approximately about four fold less in comparison to the amino-silanized 316L stainless steel; the detected free amino groups on PEGylated glass at pH 5 is five times higher than

316L stain less steel and 4 fold higher compared to PEGylated glass at pH 10. This might be due to the fact that the terminal free amino groups of bisamino-PEG (MW 1500) are protonated and stretched better in acidic buffer than in alkaline buffer. Glass carriers showed the highest immobilization amount of amino groups. Therefore only glass carriers were used for detection of amino groups using sulfo-SDTB.

Sample description	Slope	Amount of coupled AP (ng/carrier)	Calculated amount of NH_2 [nmol/cm^2]	Appendix (1.3)
316L etched with 0.5 M HCl, PEGylated at pH 5	0.0002	0.5	9.645E-07	Figure 3, A
316L etched with 3% HNO_3, PEGylated at pH 5	0.0002	0.5	9.645E-07	Figure 3, B
316L etched with 0.5 M HCl, PEGylated at pH 10	0.0002	0.5	9,645E-07	Figure 3, C
316 L etched with 3% HNO_3, PEGylated at pH 10	0.0002	0.5	9.645E-07	Figure 3, D
Amino-silanized glass and amino-PEGylated at pH 5	0.0007	3	5.787E-06	Figure 3, E
Amino-silanized glass and amino-PEGylated at pH 10	0.0003	1	1.929E-06	Figure 3, F

Table 2. Quantification of amino groups via glutaraldehyde immobilized AP onto etched amino-silanized and amino-PEGylated carriers. See legend of Table 1 and section experimental methods describing the calculation of the detected amino groups.

Sulfo SDTB

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-chloro-anil 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-

chloro-anil. A similar amount of amino groups is detected after amino-PEGylation at pH 10 using EDC.

		Amino-PEGylated slides by means of			
	Amino-silanized slides	p-chloro-anil pH 5	p-chloro-anil pH 10	EDC/sulfo-NHS, pH 5	EDC/sulfo-NHS, pH 10
Absorption (498nm)	0.651	1.019	0.72	1.045	0.903
Concentration of sulfo-SDTB (µg /slide)	21.2	37.3	24.2	38.4	32.1
Calculated amount of NH_2 [nmol/cm^2]	15.45	27.24	16.93	28.07	24.90

Table 3. Detected amount of amino groups on amino-silanized and amino-PEGylated slides by means of either EDC/sulfo-NHS or p-chloro-anil at pH 5 or 10 using the standard curve for sulfo-SDTB. All coating experiments were done triplicate, the results given are the average amounts of amino groups on the solid surface for different coupling methods. The concentration of sulfo-SDTB/slide is calculated using the calibration curve of sulfo-SDTB giving the equation for standard function (y = 22.814x + 0.1685). The surface area of slides was 2.26 cm^2.

OPA assay

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 amino-silanization appears similar by staining with OPA whereas the TNBS assay demonstrates that 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 because it is a simpler and faster procedure.

A: Organic amino-silanization B: Aqueous amino-silanization C: Negative control

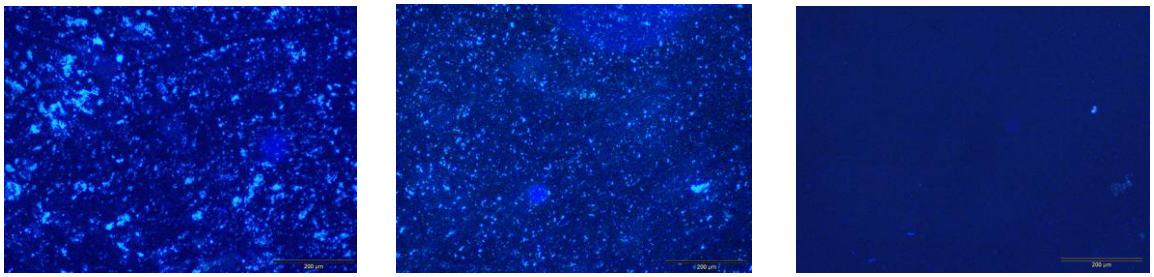


Figure 2. Visualizations of the fluorecent molecule OPA after reaction with amino groups after amino-silanization by A: organic solvent deposition, B: aqueous deposition, C: unsilanized slides (negative control).

The introduction of carboxylate groups onto the surface of amino-silanized surfaces was tested under different pH conditions (pH 6 and pH 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 have reacted.

A: *Succinic anhydride coupling at pH 6* B: *Succinic anhydride coupling at pH 8.4*

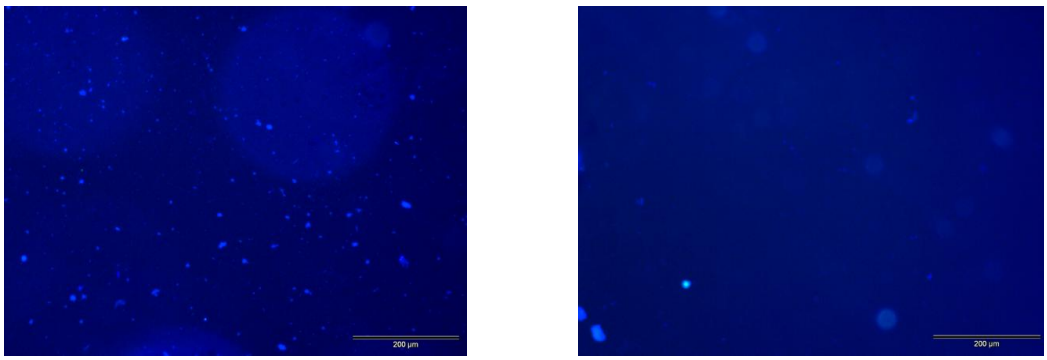


Figure 3. Effect of pH on coupling of succinic anhydride to amino groups. A: pH 6, B: pH 8.4, which is preferred because of better reactivity.

The immobilization of bis-amino-PEG or carboxyl-PEG (NH_2-PEG-CH_3) as negative controls 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. Sulfo-NHS is important to increase the reaction efficiency by several folds. However, no signal is observable with the carboxyl-PEGylated carrier.

A: negative control *B: EDC cross linker* *C: EDC/sulfo-NHS*

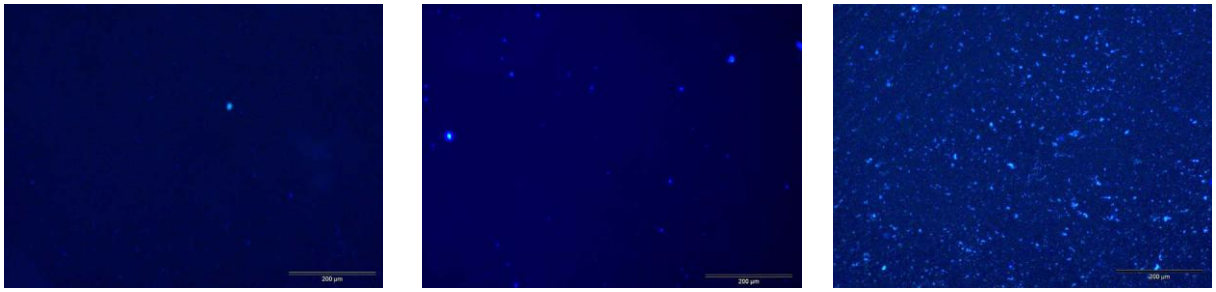


Figure 4. Reaction of the fluorescent molecule OPA with A: carboxl-PEGylated carrier (negative control), B: amino-PEGylated carrier linked only with EDC C: amino-PEGylated carrier linked with EDC plus sulfo-NHS.

Experimental Methods

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.

Coating

Etching of stainless steel 316L

To clean the surface from fatty material, the carriers were washed with isopropanol and rinsed several times with dd. H_2O. Etching occurs under mild conditions with 3% HNO_3. After repeated washing with dd. H_2O, the steel was soaked for two 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 ml of the supernatant of the 3% HNO_3 solution over steel 50 ml of 1% H_2O_2 were added. 5 µl of this solution were dotted onto the Whatman® filter paper. The color will turn to red in presence of Fe^{3+} ions (see supplementary materials Figure 5).

Amino-silanization

For amino-silanization by organic solvent or aqueous deposition, the glass slides were cleaned from fatty material with isopropanol and washed several times with dd. H_2O.

Amino-silanization by organic solvent deposition

The support materials (glass slides or stainless steel 316L) were shaken in a 5% (v/v) APTS (Fluka) solution in 95% EtOH at rt for 1h. After 3x of 5min washing with 95% EtOH, they were incubated over night at 110°C.

Amino-silanization by aqueous deposition

The support materials were shaken in a 10% (v/v) APTS solution in dd. H_2O at 75°C for 2h. After 3x of 5min washing with dd. H_2O, they were incubated over night at 110°C.

Coupling of succinic anhydride as a linker

1 g succinic anhydride (Merck) was suspended in 25 ml of phosphate buffered saline (PBS) pH6 (avoid buffers containing primary amino groups such as Tris) and the pH adjusted to either pH 6 or 8.4 with 1M NaOH. Even in buffered solutions, the pH should be monitored to prevent severe acidification of the reaction solution. This solution was added to the amino-silanized carriers and reacted at rt over night to assure complete blocking of all amino groups. After washing 3x 5min with buffer and 3x with dd. H_2O, the carrier can be activated with carbodiimides.

EDC combined with sulfo-NHS

400 mg EDC (Sigma) (0.1M) and sulfo-NHS (Pierce) (final concentration 5mM) were dissolved in 50 ml of dd. H_2O and the pH was adjusted to 10 with 1M NaOH. The carboxylated carriers prepared with succinic anhydride were incubated in this solution for about 1 to 2 h (maximum) at rt under gentle shaking and thereafter washed several times with dd. H_2O.

p-Chloro-anil as a linker

The amino-silanized carriers were washed 3x 5min with toluene under shaking and thereafter incubated in a 1% (w/v) solution of p-chloro-anil (Fluka) in toluene for 1h under gentle shaking. After washing 2x with toluene, acetone and dd. H_2O the carrier is ready for amino-PEGylation.

(Amino-)PEGylation

1 g O,O′-bis(3-aminopropyl)polyethylene glycol (bis-amino-PEG, MW ~1500) (Fluka) was dissolved in 50 ml carbonate-bicarbonate buffer, pH 10 or in 50 ml citric acid-sodium citrate buffer, pH 5. The slides prepared with EDC plus sulfo-NHS or with p-chloro-anil respectively were shaken in this solution over night and afterwards washed several times with dd. H_2O.

Analytical methods for detection and quantification of amino groups

TNBS assay

A 0.01% (w/v) TNBS–solution (Sigma) was prepared freshly by dilution of 20 µl 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 ml 10% SDS and 0.5 ml 1 M HCl added to each sample.

Kaiser assay

Solution A: Phenol (8 g) was dissolved in 2 ml EtOH. Solution B: KCN solution (10 mM) in dd. H_2O: 200 µl were diluted to a volume of 10 ml with pyridine. Solution C: 50 mg ninhydrin (Merck) was diluted to 1 g with EtOH and dissolved. Then 500 µl 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 ml 2.5% glutaraldehyde (Fluka) solution in 0.05M PBS buffer pH 7 for 1h and washed several times with ice water. 40 µl of AP solution in 300 µl 0.05M PBS pH 7 were dissolved and 100 µl of this solution added to each carrier and incubated over night in a humid chamber at rt. After washing the carriers with buffer (3x 5 min), the AP-activity can be measured as followes; 1) the carrier was placed in a disposal cuvette and filled up to 4 ml with 0.1M Tris buffered saline containing 0.15 M NaCl and 5 mM $MgCl_2$, pH 9.5 (TBS); The reaction was initiated by addition of 10 µl p-NPP (Koch Light LTD) solution (50 mg/ml dd. H_2O) and the absorption measured against a blank at 405nm every 30 sec. 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/ml in 0.1M TBS was prepared. Different aliquots (1.12, 2.24 and 4.49 µl) were added in a disposal cuvette and filled up to 4 ml with TBS and the reaction was initiated and mnoitored as described above.

The calibration curve was calculated by measuring the extinction of several concentrations of AP. The definite concentration of each AP and its slopes are used to give the equation for standard function (y = 0.0002x + 0.0001). The slope is proportional to the concentration of the enzyme, which should be somewhat equal to the number of amino groups on the surface of the carrier. Using the standard equation and the slope of each sample, the amount of immobilized amino groups can be calculated.

Sulfo-SDTP assay

A 2.33 mg/ml sulfo-SDTB (Pierce) stock solution (42 mg SDTB dissolved in 2 ml DMF and 16 ml 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/ml and the absorbance was 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 ml stock solution for 10 min, thereafter rinsed first with 1 ml dd. H_2O, and then soaked in 10 ml dd. H_2O to remove excess of sulfo-SDTB. 1 ml 35% perchloric acid was added to each carrier and reacted for 10 min. The observed orange color indicated the release of 4,4'-dimethoxytrityl cations. The 1 ml solution was then transferred to a cuvette and the absorbance measured as described above.

OPA Test

A solution of 1 ml 50 mM borate buffer, pH 9.2, 250 µl OPA (Fluka) (20mg/ml) and 250 µl 2-mercaptoethanol (Fluka) were added to the amino groups 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 the fluorescence microscope Olympus BX41 using an extinction wavelength of 360 nm and an emission wavelength of 436 nm. The photos were taken by ColorView Soft Imaging System (Olympus Soft Imaging Solutions, Münster, Germany) with a magnification of 10x (N.A 0.25) and edited with the Cell^D life science documentation software.

Supplementary Materials and Protocols

Etching of stainless steel 316L

Chemical etching with acids was carried out to activate stainless steel 316L introducing hydroxyl groups for e.g. amino-silanization. Figure 5 illustrates the fast spot test for detection of iron ions in the acids (3% HNO_3 and 0.5M HCl), which indicates successful etching of 316L stainless steel by showing red color due to $[Fe(SCN)_6]^{3-}$. Dotting acid solution only (negative control), shows no color change. Since both acids show a positive reaction, etching with 3% HNO_3 was used in further immobilization procedures.

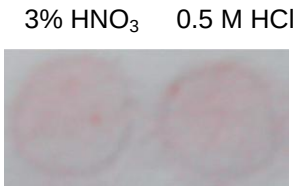


Figure 5. Spot test for the detection of iron ions after etching 316 L with 3% HNO_3 or 0.5M HCl. No color is detected with the negative control.

Quantification of amino groups on carriers

The Kaiser-test is of limited value for the detection of amino groups on most carriers and amino-silanized glass powder (see publication). Therefore, other detection assays were tested such as the TNBS-test, an enzymatic assay, using AP, a test using OPA and sulfo-SDTB.

TNBS-Test

The introduction of reactive chemical groups (amino-groups) onto carriers after amino-silanization was also tested using a TNBS-test. After addition of 1 ml 10% SDS and 0.5 ml 1 M HCl to organic and aqueous amino-silanized 316 L stainless steel and glass carriers, a light yellow color is observed only for the organic amino-silanized foil.

Tested carriers:

- Glass-slides after organic amino-silanization.
- Glass-slides after aqueous amino-silanization.

- Aqueous and organic amino-silanized glass-slides after amino-PEGylation using EDC- crosslinker and carbonate-bicarbonate buffer, pH 10.
- Aqueous and organic amino-silanized glass-slides after amino-PEGylation using EDC-crosslinker and citric acid-sodium citrate buffer, pH 5.

A faint yellow color was observed only on the carriers after organic amino-silanization, thus indicating the higher efficiency of organic amino-silanization. Therefore, it was chosen for further immobilizations. For the detection of free amino groups after amino-PEGylation, the TNBS test is not sensitive enough. Therefore other sensitive alternative tests as e.g. the enzymatic assay with AP were developed and used.

Enzymatic assay for the detection of amino groups, using AP

An enzymatic assay for the detection of amino groups was developed. Alkaline phosphatase was coupled to the amino groups on the carriers by means of glutaraldehyde cross-linking under carefully controlled conditions. Higher amounts of amino groups result in also higher amounts of bound AP, which can be quantified with p-NPP as a substrate. Table 4 shows the results for amino-silanized and amino-PEGylated ceramics (ZrO_2) carriers.

Sample description	Slope	Amount of coupled AP (ng/carrier)	Calculated amount of NH_2 [nmol/cm^2]	Appendix (1.2)
Amino-silanized ceramics	0.0002	0.5	9.259E-06	Figure 2F
Amino-PEGylated ceramics at pH 10	0.0002	0.5	9.259E-06	Figure 2H
Amino-PEGylated ceramics at pH 5	0.0002	0.5	9.259E-06	Figure 2G

Table 4**.** Quantification of AP immobilized with glutaraldehyde onto amino-silanized and amino-PEGylated ceramics (ZrO_2) carriers (surface of the ceramics 0.3 cm². See legend of Table 1 describing the calculation of the detected amino groups.

The results for 316 L stainless steel and glass are shown in Table 2 and for ceramics carriers (Table 4) after amino-PEGylation using EDC for crosslinking. The bis-amino-PEG-polymer immobilized onto an amino-silanized carrier contains free terminal amino groups, which can be used to couple AP by using GA (glutaraldehyde). The amount of immobilized AP on amino-silanized and amino-PEGylated ceramics was similar, but higher than observed with glass and 316 L stainless steel.

After immobilization of bis-amino PEG (NH_2-PEG-NH_2) at different pH (5 or 10) onto 316 L stainless steel carriers which previously activated through different etching methods then amino-silanized give almost identical amount of detected amino-groups.

After amino-PEGylation, the amount of immobilized AP is 6 times less (0.5 ng) compared to AP immobilization onto mere amino-silanized carriers (2.5-3 ng) (Tables 1, 2 and 4). However the free amino groups detected on PEGylated glass at pH 5 are five times higher than on 316L stainless steel and 4 fold higher compared to PEGylated glass at pH 10. This may be due to the fact that the conformation of immobilized polymers such as NH_2-PEG-NH_2 (MW 1500) is such that the terminal free amino group can be hidden. In acidic buffer the terminal free amino groups of NH_2-PEG-NH_2 (MW 1500) are protonated and point outward to the hydrophilic surrounding because of their electrical charge. Alternatively, both amino groups might react with the solid support. This effect should be reduced by using a large excess of NH_2-PEG-NH_2 for coupling.

The enzymatic assay using AP shows a similar amount of amino groups after amino-PEGylation on 316 L stainless steel at the different pH conditions (5 or 10). However in the case of glass carriers the enzymatic assay using AP was promising, we observed the highest amount of immobilized amino groups.

Sulfo- SDTB

The purpose of the sulfo-SDTB colorimetric assay was to measure quantitatively the available reactive amino groups on the surface of amino-silanized and amino-PEGylated carriers. Amino-PEGylation was carried out by means of either EDC/sulfo-NHS or p-chloro-anil. The coupling of NH_2-PEG-NH_2 was carried out at pH 5 as well as pH 10. The amount of reactive amino groups can be determined by means of a standard curve of sulfo-SDTB (Figure 6). The amount of 4,4´-dimethoxytrityl cations detected corresponds to the quantitative measurement of

reactive amino groups. The detected amount of 4, 4´-dimethoxytrityl cations from amino-silanized slides and amino-PEGylated slides using p-chloro-anil at pH 10 was similar. However a higher amount of detectable amino groups was observed for PEGylated slides at pH 5 for both activators (EDC/sulfo-NHS or p-chloro-anil) and a similar result was shown for amino-PEGylation at pH 10 using EDC/sulfo-NHS activation (see publication Table 3).

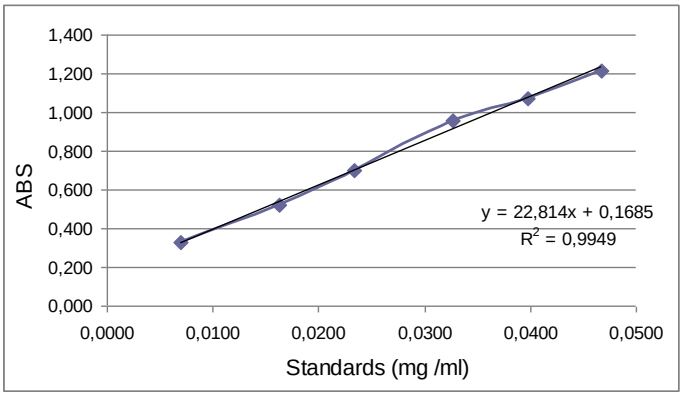


Figure 6. Standard curve of sulfo-SDTB (mg/ml).

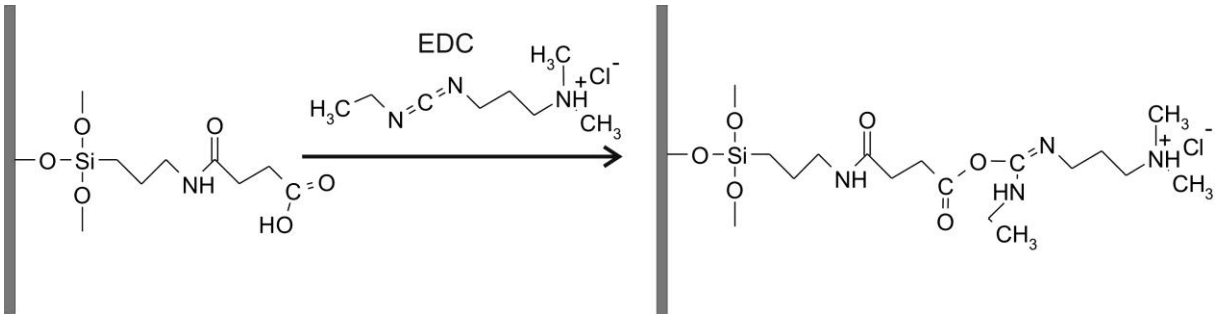
2. Biocompatible coating of implant materials

Binding of linkers suitable for coupling to PEG or proteins

To achieve biocompatibility of implant materials they need to be coated with PEG. The choice of procedure to couple molecules to the amino-groups on the material surface depends on the functional groups of the PEG derivative. For immobilization of bi-functional bis-amino-PEG (NH_2-PEG-NH_2), succinic anhydride is coupled first to the aminosilane and thereafter activated with EDC (Scheme 11) or EDC/sulfo-NHS (Scheme 4). Another method is applied for p-chloro-anil linkers (Scheme 5). Several proteins such as alkaline phosphatase or lactate dehydrogenase can be immobilized onto amino-silanized carriers using glutaraldehyde (Scheme 6) as a crosslinker (Hardy P. et al. 1969).

Activation of Carboxylate Groups via EDC

The carbodiimide EDC ((1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) is often used for coupling carboxylates to amines in bio conjugate procedures (Hoare D. and Koshland D. 1966; Chu B. et al. 1986; Ghosh S. et al. 1990). Most biological substances are water soluble and so are EDC and its reaction by-products. There is no need to use organic solvents or complicated buffer systems. EDC reacts with carboxylate groups to a highly reactive O-acylisourea (Schema 11). This intermediate reacts further with amino groups of NH_2-PEG-NH_2 by forming an amide bond. However, oligomerisation occurs if the molecule to be activated contains both a carboxylate and an amino group. In such systems EDC should not be used, and buffers containing carboxylates or free amines such as Tris or glycine have to be avoided. The reaction should be performed in an aqueous buffer system, preferably at pH 5. But even at pH 10 positive results are obtained (Pittner F. 2000).



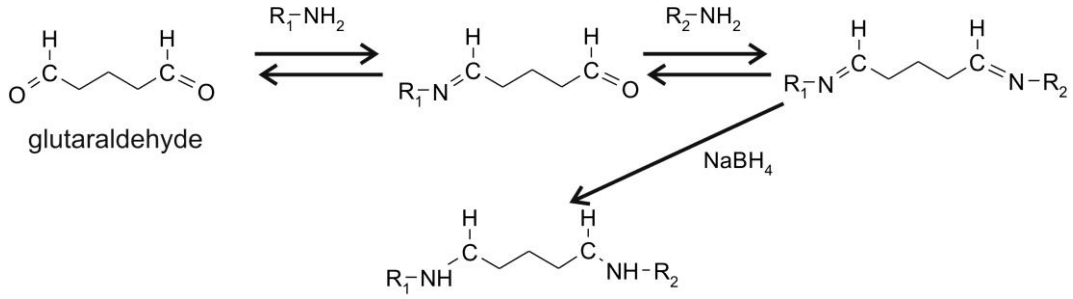
Scheme 11. The terminal carboxylate groups (created as shown in Scheme 3) react with EDC and form the highly reactive O-acylisourea. This intermediate reacts with primary amines of NH_2-PEG-NH_2 by forming an amide bond.

Procedure:

400 mg EDC was dissolved in 50 ml dd. H_2O and pH adjusted to 10 with 1M NaOH. The carboxylated carrier - prepared with succinic anhydride - was incubated in EDC solution for 1-2 h (not longer than 2h) at room temperature under gentle shaking. The activated carrier was washed several times with dd. H_2O. The carrier can subsequently be reacted with NH_2-PEG-NH_2.

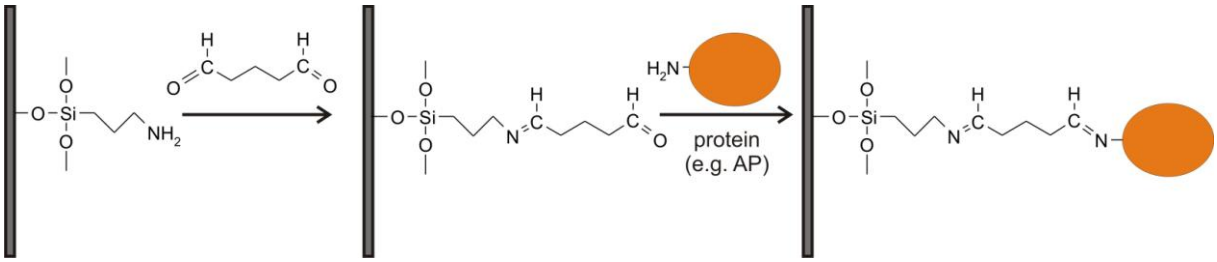
Coupling of Glutaraldehyde as a linker

Glutaraldehyde is a widely used homobifunctional cross-linker. The reactive aldehyde groups of glutaraldehyde form Schiff bases with proteins and other molecules containing amino groups (Hardy P. et al. 1969). Crosslinking via glutaraldehyde is difficult to reproduce due to variability between different glutaraldehyde solutions. Fresh glutaraldehyde often will not yield the same results as aged solutions (Pittner F. 2002). One problem stems from different degrees of “aging” by polymerization based on aldol condensation. Glutaraldehyde is an efficient crosslinker for proteins, but this is also the reason for its toxicity and fixative properties when incorporated into living cells. Reacting different amino groups in a predictable way is difficult, as both aldehyde groups of the same molecule can react with the first reagent, resulting in loop structures. This can be overcome by using a large excess of glutaraldehyde in the first reaction step. As in our case solid surfaces containing amino groups are reacted, the excess of the soluble glutaraldehyde can be rinsed off, increasing the possibility of binding another amino-group containing reagent (in our case AP) in the second step.



Scheme 12. Formation of Schiff bases through reaction of glutaraldehyde with primary amines. As is evident many byproducts may be formed. The reversible imine bonds may then be reduced selectively to the stable amines by means of borohydrides as sodium borohydride or sodium cyanoborohydride.

The immobilization of proteins such as AP (alkaline phosphatase) etc. on amino-silanized carriers is illustrated in Scheme 13.



Scheme 13. Glutaraldehyde as a crosslinker for binding of proteins

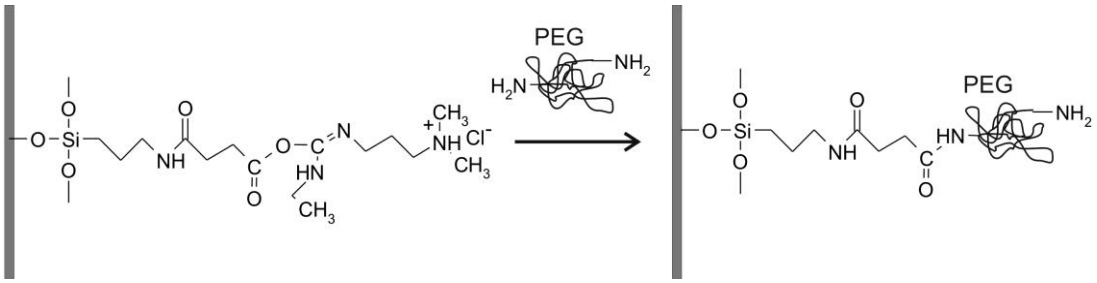
Procedure:

The amino-silanized carriers were activated in a 2.5 % glutaraldehyde solution (in 0.1M sodium phosphate, 0.15 M NaCl, pH 7.2) for one hour at room temperature under shaking. The carriers were washed with ice cold dd. H_2O. The enzyme AP (9 mg/ml) was diluted 1:10 in 0.1M sodium phosphate with 0.15M NaCl, pH 7.2 and 100µl of diluted AP added to each activated carrier. The reaction was carried out over night at 4°C. The carriers were washed with 0.1 M PBS and stored in 0.05M saline solution at 4°C.

PEGylation

Amino-PEGylation

Amino-PEGylation using NH_2-PEG-NH_2 can be performed either under alkaline or acidic conditions. Both methods were tried and, two different buffer systems (0.1M carbonate-bicarbonate buffer, pH 10 and 0.1M citric acid-sodium citrate buffer, pH 5) were used respectively.



Scheme 14. Amino-PEGylation via EDC activation of the succinylated carrier

Procedure:

1 g O,O′-bis (3-aminopropyl) polyethylene glycol (NH_2-PEG-NH_2, MW 1500) was dissolved in 50 ml of 0.1M carbonate-bicarbonate buffer, pH 10 or 0.1M citric acid-sodium citrate buffer, pH 5 respectively. The activated carrier [either with EDC (Scheme 14), EDC/Sulfo-NHS (Scheme 4) or p-chloro-anil (Scheme 5)] was incubated over night in acidic or alkaline buffer solutions respectively. Each PEGylated carrier was washed 3 times for 5 min under shaking in the respective buffers (depending on used buffer for immobilization) to remove unreacted PEG, and subsequently washed several times with dd. H_2O.

Carboxyl-PEGylation

Carboxyl-PEGylation using methoxy-carboxyl-PEG (MW 2000) is carried out on organic amino-silanized carriers using EDC at pH10. 0,887g Methoxy-carboxyl PEG, as synthesized according to Rumplayr K. 2006 (see appendix) was dissolved in 25 ml 0.1M carbonate-bicarbonate buffer, pH 10. 400 mg EDC (2 mM) was added to dissolved methoxy-carboxyl PEG and stirred for 30 min at room temperature. This solution was added to organic amino-silanized slides and incubated under shaking over night. The carboxyl-PEGylated carrier was washed 3 times with 0.1M carbonate-

bicarbonate buffer, pH 10 for 5 min under shaking to remove unreacted PEG, afterwards washed several times with dd. H_2O.

Detection of amino groups

Analytical methods are available for amino group quantification, but sometimes problems arise for the detection of very small amounts of amino groups on solid supports. The Kaiser test was sufficient for the detection of amino groups on carriers such as Gulsenit, however it had limitations in sensitivity when used for other carriers, such as glass, stainless steel 316L, and ceramics, because of the much smaller $–NH_2$ content. Other detection methods were investigated such as TNBS-test, OPA, an enzymatic assay using AP as well as sulfo-SDTB. The details of these methods were described in the publication above.

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Chapter 2

Cleavage of antibodies using immobilized dihydrolipoamide and anchoring of antibody fragments onto biocompatible-coated carrier

Specific Aim 2: ***To introduce binding sites for degradable, drug loaded nanoparticles into the biocompatible coating.***

In the course of this work it was necessary to anchor a suitable receptor system to bind drug containing biodegradable nanoparticles to mediate local pharmacological activity, the so called active coating. One of the challenges we had to address in this thesis was to find suitable biorecognitive systems for binding of the drug containing particles. The design of the receptor is of great importance to avoid and/or eliminate any bio- or chemorecognitive interactions with other components circulating in the blood. Furthermore, the binding between the receptor and the nanoparticles has to be strong enough to keep them tightly bound during their lifetime, but on the other hand allow reloading after final degradation. Antibodies (Abs) against the biocompatible nanoparticles were bound to the implant surface as biorecognitive site for reloading of the particles. As Abs against the drug carriers are very expensive in case of chitosan nanoparticles (CS-NPs, see Chapter 7), or only available in extremely small amounts for PLGA nanoparticles, binding conditions were optimized with HRP-IgG.

The Ab molecules were selectively cleaved with a reducing agent such as 2-MEA (2-mercaptoethanolamine), or the newly optimized method developed during this thesis using dihydrolipoamide immobilized to amino-silanized Gulsenit to create two Fab` fragments. The determination of Fab´ fragments immobilized to amino-silanized or amino-PEGylated glass carriers was shown using fluorescent staining with OPA–Test (o-phthaldialdehyde). This newly optimized method saves time, is economical and has additional advantages compared to the commonly used MEA method (described in Chapter 5): There is no need to remove the reducing agent from the sample solution, thus avoiding the time consuming gel filtration chromatography always prone to re-oxidation of sulfhydryls during this step. The reagent dihydrolipoamide can be easily removed from the sample solution via centrifugation and no contaminating by-products are produced. Furthermore, after regeneration of the immobilizate with $Na_2S_2O_4$ the reaction can be repeated several times with the same immobilizate.

Cleaving of antibody using dihydrolipoamide and anchoring of antibody fragment onto biocompatible coated carrier

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Abstract

This study demonstrates the possibility of anchoring from antibody fragment onto carrier surface with biocompatible coating. The antibody molecule is cleaved to two antibody fragment with immobilized dihydrolipoamide. The detection of the biocompatible coating and the immobilization of antibody fragment is shown using fluorescence microscopy. The biocompatibility coating of 316L stainless steel carrier is determined in cell culture with Caco-2 cells. This immobilization of antibody fragment can serve in further aspect as a suitable specific binding site for drug delivery systems.

Keywords

Lipoic acid; Polyethyleneglycol; Medical implant; Drug delivery

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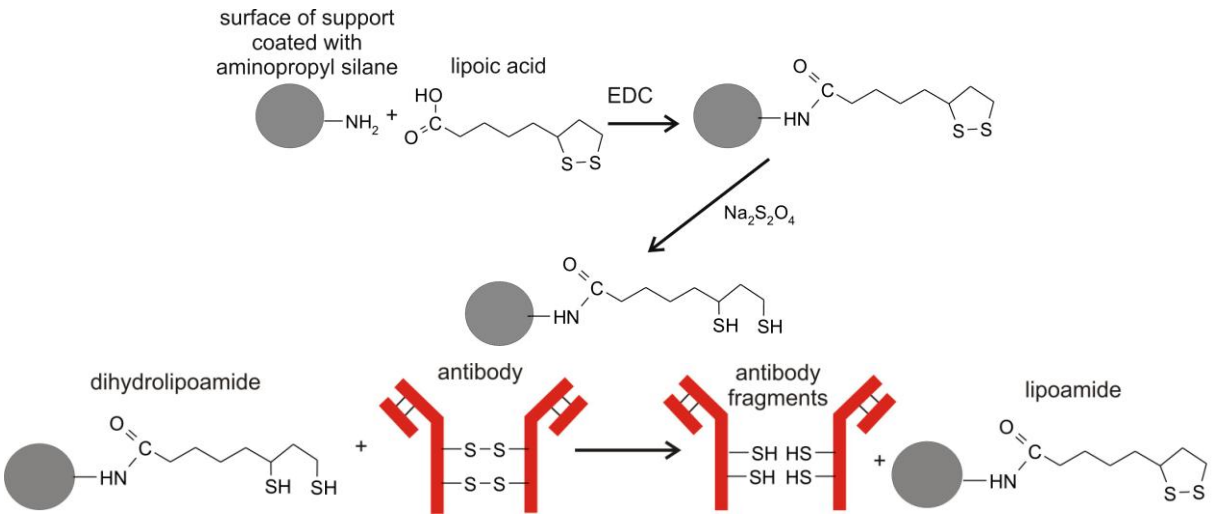
Introduction

The primary objective of this study is to develop carriers containing bio-compatible coatings and binding sites for bio-degradable nano- or micro-particles that may serve as reloadable drug delivery carriers by medical implant materials. Binding reaction /interactions for reloadable systems need to be sufficiently strong to keep the drug releasing nano- or micro-particles tightly bound for a certain period of time, but should also be weak enough to allow reloading after the gradual decomposition of the particles. In this study the specific binding site is chosen as an antibody fragment. As antibodies (Abs) against the drug carriers (biocompatible and biodegradable polymer) are not available, 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 onto a gel-material was used first by Gorecki and Patchornick in 1973. In our approach, lipoic acid (6, 8-dithiooctanoic acid) was immobilized to amino-silanized Gulsenit via the carboxylic acid group by means of the water soluble carbodiimide EDC. The efficiency of the immobilization was monitored using the Kaiser-test for free amino groups (Kaiser E. et al. 1970). Afterwards the cyclic disulfide of immobilized lipoic acid was reduced to thiols using sodium dithionite ($Na_2S_2O_4$) as a reducing agent (Scheme 1)



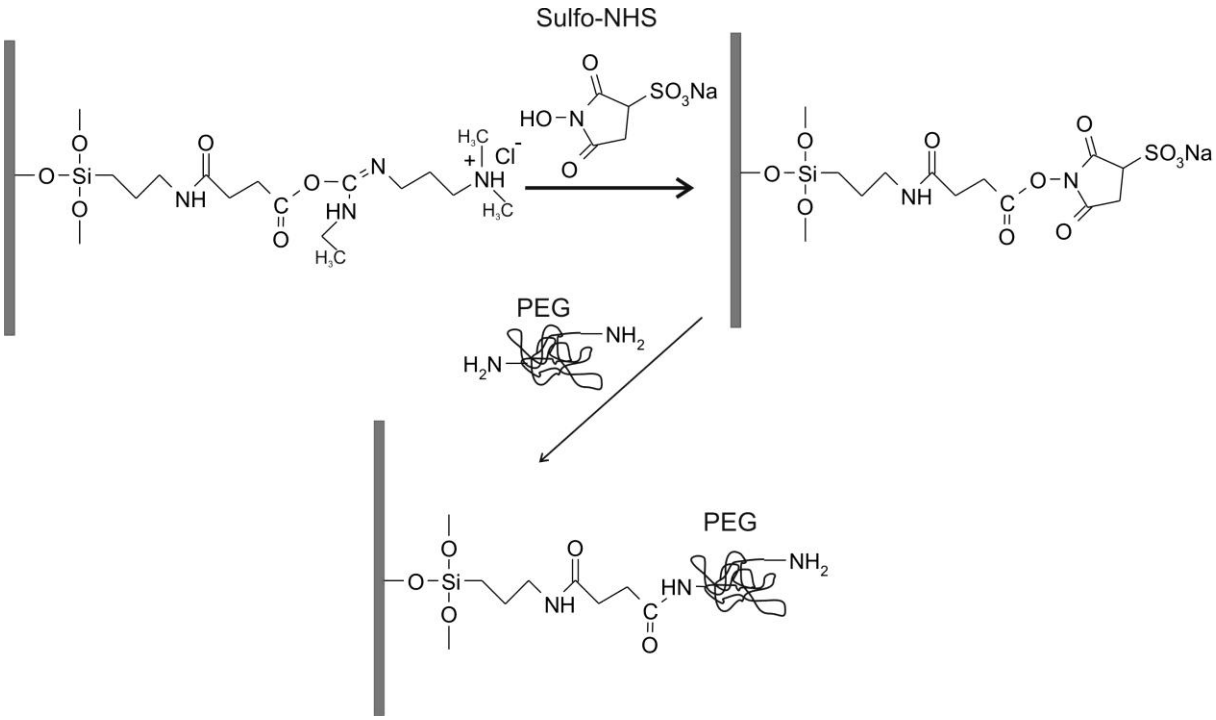
Scheme 1

These thiols are able to cleave disulfide bridges of Abs creating two Fab` fragments with free sulfhydryl groups. This method is time saving and economical. The reagent dihydrolipoamide can be easily removed from the sample solution via centrifugation and the Fab´ fragments are free of contaminating by-products. The efficiency of cleavage of Abs is shown by a positive Ellman's assay (Ellman C. et al. 1959). 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 is developed using polyethyleneglycol (PEG) derivatives. PEG is biocompatible, can act as a barrier and is widely used for this purpose due to its hydrophilic and also hydrophobic character (Ratner B.D. et al. 2004). In the course of this study, the previously cleaved antibody-fragments are anchored in the PEG-coating on the surface of the carrier material. The anchor of a suitable receptor system (Ab fragment against the polymer of biodegradable nano- or micro-particles) to bind drug containing biodegradable nano- or micro-particles can serve to mediate local pharmacological activity, the so called active coating. The nano- or micro-particles can be injected into patients after implantation. The nano- or micro-particles circulating in blood 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 an attractive means of drug administration on medical implants. Such coatings can be the basis for a number of applications that require controlled, local drug delivery at the interface between an implant and healthy tissue. As nano- or micro-particles can be loaded with different drugs, this technology allows easy adjustment to patient-specific medication needs as well as the administration of unstable compounds or drugs that are difficult to dose. Several studies have already shown the possibility to load and release different drugs nano- or micro-particles consisting of biodegradable and biocompatible polymers (Wolf M. et al. 2003, Alonso J.M. et al. 2008, Liu Y. et al. 2010).

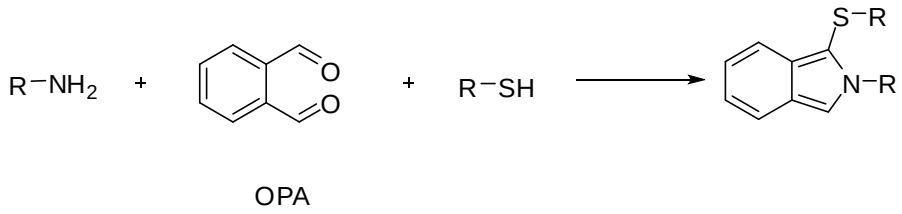
Results and Discussion

The surface of the carriers (316L stainless steel, silica glass) needs to be previously activated via 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) cross linker. For this purpose succinic anhydride is coupled first to the amino-silanized carrier and thereafter activated with EDC/sulfo-NHS (N-hydroxysulfosuccinimide) to immobilize NH_2-PEG-NH_2 (Scheme 2).



Scheme 2

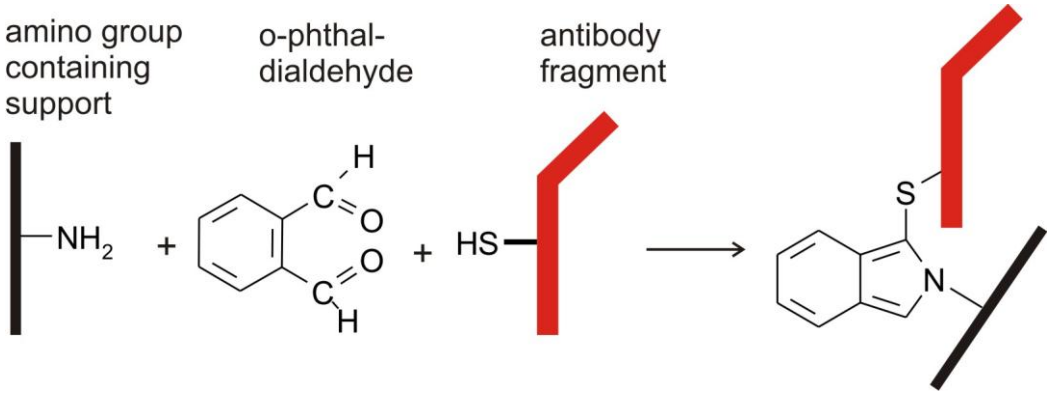
Fab´ fragments of polyclonal anti-peroxidase-Ab are immobilized onto amino-PEGylated carriers. The immobilization of Fab´ fragments onto amino-silanized and amino-PEGylated carriers is shown by OPA (o-phtaldialdehyde). OPA reacts with amino groups in the presence of thiol-containing molecules like ßME (ß-mercaptoethanol) generating a fluorescent product with an excitation wavelength of 360 nm and an emission wavelength of 455 nm (Scheme 3).



OPA

Scheme 3

In this approach, OPA is used as a crosslinker between amino groups on the carrier and sulfhydryl groups on the Fab´ fragments. For this purpose Fab´ fragments are used instead of ßME which is used normally for detection of amino groups by means of OPA (Scheme 4), thus allowing simultaneous detection of positive Ab cleavage as well as its immobilization onto amino-silanized or amino-PEGylated slides.



Scheme 4

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

Detection of amino-PEGylation and immobilization of Fab fragment by means of OPA-assay

The cleavage of HRP-Abs was carried out by means of dihydrolipoamide immobilized on amino-silanized Gulsenit. The efficiency of lipoic acid immobilization onto organic amino-silanized Gulsenit is shown by means of the Kaiser-test for amino group detection. The intensity of the 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 is detected with amino-silanized Gulsenit including lipoamide due to the absence of free thiol groups. OPA is used to crosslink the sulfhydryl of cleaved Abs to amino groups of NH_2-PEG-NH_2 on the surface of slides. This approach is very efficient as it allows immobilization onto amino-PEGylated slides and at the same time the detection of successful Ab cleavage. Anti peroxidase-IgG was cleaved with immobilized dihydrolipoamide to create two Fab` fragments containing free sulfhydryl groups in the hinge region. OPA crosslinks these Fab` fragments with amino-groups on amino-PEGylated slides. The immobilization of cleaved Abs to amino-PEGylated carriers is shown in Figure 1.

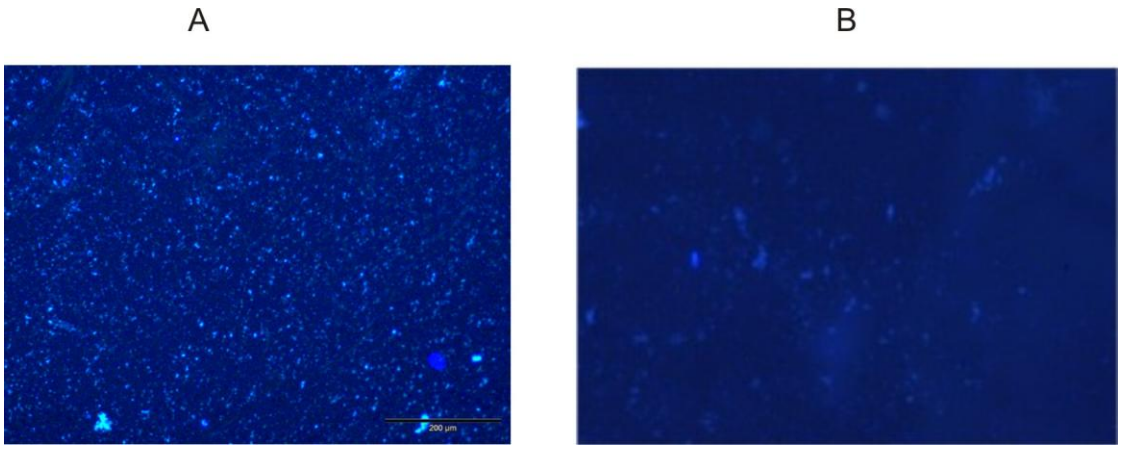


Figure 1. HRP-IgG Fab fragments immobilized by means of OPA onto silicate glass slides A: activated via EDC/sulfo-NHS and amino-PEGylated at pH 5, B: activated via EDC/sulfo-NHS (negative control).

Biocompatibility test of amino-PEGylated 316 L stainless steel using Caco2 cells

The biocompatibility of the plain and amino-PEG coated stainless steel was evaluated by cell proliferation studies, two and three days post seeding. Proliferation

revealed that both amino-PEG coated surfaces displayed tremendous impact on the proliferative activity of Caco-2 cells as shown in Figure 2.

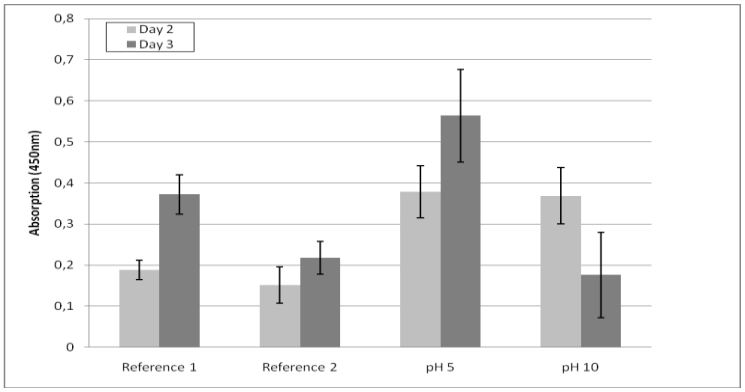


Figure 2. Proliferative activity of Caco-2 cells on day 2 and 3 post seeding for bis-amino-PEGylated stainless steel coated at pH 5 and pH 10. Reference 1 refers to proliferation on uncoated glass slides and Reference 2 is proliferation on plain high grade steel as growth support (n=6, mean ± SD)

A glass slide was used as reference substrate for cell proliferative activity and resulted a signal of 0.1881 AU (absorbance units) on day 2 and 0.3722 AU on day 3 post seeding, respectively. Plain high grade steel presented itself as possible growth substrate, with no toxicity. However, generally it resulted in lower cell proliferation 0.1516 AU on day 2 and 0.2179 AU and day 3 postseeding, which indicated a need for some improvements. These improvements were made by using amino-PEGylation. Both amino-PEG coated substrates represented suitable growth supports for Caco-2 cells with 2-fold proliferation compared to glass as a 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, the amino-PEG coating at pH 10 revealed lower (by two fold) proliferation then on day 2 compared to a glass reference on the same day.

The BrdU proliferation assay provided insight into the biocompatibility of amino-PEGylated high grade steel. Glass slides are commonly used as cell growth support (Mahelkova G. et al. 2009). On the second day, the initial adhesion and proliferation activity between glass and plain high grade steel is comparable. Coating with PEG is commonly known to reduce protein adsorption and cell adhesion (Johnson P.A. et al. 2009). On the contrary, amino-PEGylated stainless steel showed improved cell growth. The initial cell adhesion is highe; the cells show a stable attachment and keep proliferating at the same rate as on glass slides. These results suggested

amino-PEGylation at pH 5 as the best combination for biocompatible coating. Furthermore, this study confirmed amino-PEGylated high grade steel as biocompatible substrate that induces stable cell attachment and provides confident further cell growth.

Experimental Methods

Organic amino-silanization of inorganic carriers

The inorganic carriers amino-silanized in this study were stainless steel 316L (Goodfellow, Germany), silicate glass slides (Ø12 mm, Assistant, Austria), zirconia (ZrO_2-TZP-A BiO-HIP, Z-Systems AG) and Gulsenit (MAGINDAG, Austria). Gulsenit is an active magnesium silicate mineral having a particle size less than 10 µm and a density of about 3.2 kg/l. Stainless steel 316L and silicate 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. Silicate glass slides may be etched with 3% HNO_3 for 10 min or 1M NaOH for 30 min. The carriers were washed several times with dd. H_2O and then soaked for at least two days in dd. H_2O under gentle shaking. Replacement of dd. H_2O is highly recommended several times to enhance hydroxyl group formation. The surface of carriers is then modified by amino-silanization with 3-aminopropyl triethoxysilane (Pittner F. 2000) in order to immobilize NH_2-PEG-NH_2. The used carriers were covered with freshly prepared 5% (v/v) (3-aminopropyl) triethoxysilane solution (APTS, Sigma, Austria) in 95% EtOH and reacted for one hour under gentle shaking at room temperature. The carrier was washed with 95% (v/v) EtOH 3 times for 5 min under gentle shaking and incubated at 110°C over night.

Binding of succinic anhydride to the amino-silanized surface

Succinic anhydride is applied to introduce carboxylate groups onto the surface of amino-silanized material: 1 g of succinic anhydride (Merck, Austria) was suspended in 25 ml PBS buffer pH 8.4. The pH was monitored and adjusted to 8.4 with 1M NaOH to prevent severe acidification of the reaction solution, which could also damage the molecule to be coupled in the further step. This solution was added to the amino-silanized carrier and reacted at room temperature over night to assure complete blocking of all amino groups (control the pH over the first few hours of the

reaction). The carrier was then washed 3 times for 5 min with buffer followed 3 times with dd. H_2O.

EDC combined with sulfo-NHS

0.1mM EDC (Sigma, Austria) and Sulfo-NHS (Pierce, Switzerland) in a final concentration of 5 mM were dissolved in 50 ml dd. H_2O and the pH adjusted to 10 with 1M NaOH. The carboxylated carrier prepared with succinic anhydride was incubated in this solution for about 1 to 2 h (maximum) at room temperature under gentle shaking. The activated carrier was washed several times with dd. H_2O.

Amino-PEGylation

1 g O,O′-bis (3-aminopropyl) polyethylene glycol (NH_2-PEG-NH_2, MW 1500, Fluka, Austria) was dissolved in 50 ml of 0.1M citric acid-sodium citrate buffer pH 5 or 50 ml of 0.1M carbonate-bicarbonate buffer pH 10 respectively. The carrier, activated with EDC/Sulfo-NHS was incubated in the above prepared solutions separately at least overnight. Each amino-PEGylated carrier was washed 3 times for 5 min under shaking with the buffer to remove unreacted PEG and then washed several times with dd. H_2O.

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

To immobilize lipoic acid onto Gulsenit, 40 mg lipoic acid (Sigma) was dissolved in 20 ml EtOH and 50 mg EDC was dissolved in 10 ml EtOH. Dissolved lipoic acid and EDC were added to 5 g carrier (amino-silanized Gulsenit) and incubated overnight at room temperature under stirring. The carrier was rinsed 3 times with dd. H_2O then 3 times with EtOH; using a suction funnel, pore size 4 to remove solvent from carrier after every rinse. The Ellman's test was used to ensure that there wer no remaining free-SH groups. The efficiency of immobilization is determined with the Kaiser-test, which detected fewer amino groups in the immobilizate than with amino-silanized Gulsenit. The lipoamide bound to the carrier is reduced to dihydrolipoamide as following: 20 ml $Na_2S_2O_4$-solution (0.16 g/ ml dd. H_2O) was added to 5g of the carrier containing immobilized lipoic acid and reacted for 30 min at room temperature under shaking. The carrier was rinsed two times with dd. H_2O followed by two times 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. The 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 ml 0.1M PBS, pH 8 was added to 0.5g dihydrolipoamide immobilizate followed by 2 µl Ab (e.g. pc anti-peroxidase Ab, 41mg/ ml). The splitting reaction of the Ab was performed for 30 min under shaking at room temperature. The supernatant containing split Abs was isolated from traces of carrier by short centrifugation. 50 µl of Ellman's reagent was added to 100 µl of the centrifuged supernatant. If the test is positive, the rest of supernatant were then used for further experiments. 50 µl of Ellman's reagent was added to about 60 mg carrier. The test should give no color change. For regeneration of the carrier it is reduced with $Na_2S_2O_4$ (Sigma) as described above.

Ellman's test

The proof of the immobilization of lipoic acid onto the carrier is given by Ellman's test. 50 µl Ellman's reagent (DTNB (5, 5´-dithio-bis-(2-nitrobenzoic acid), Pierce) in a concentration of 4 mg/ml in 1 ml 0.1M PBS pH 8) was added to about 60 mg of carrier containing either immobilized lipoic acid or dihydrolipoamide (after reduction) respectively. 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 onto silanized or amino-PEGylaed carrier via OPA

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

Biocompatibility test of amino-PEGylated 316 L stainless steel using Caco2 cells

The BrdU cell proliferation ELISA test kit was obtained from Roche diagnostics GmbH, Vienna (Austria). All other chemicals were of analytical grade.

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/ml gentamycine in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were grown to 80 - 90% confluency and subcultured with TrypLE® select.

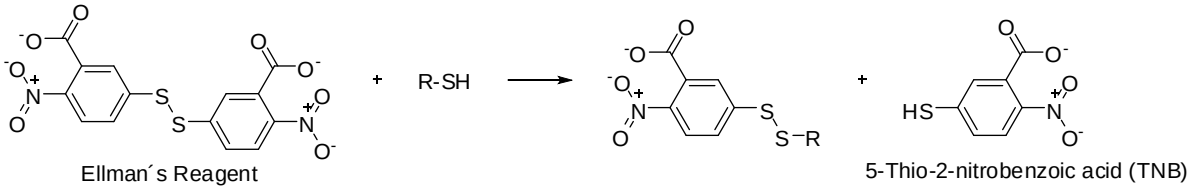
Proliferation studies

The proliferation rate was determined using BrdU cell proliferation ELISA test kit according to the manufacturer's instructions. The incorporation of 5-bromo-2-desoxyuridine (BrdU) into DNA of proliferating cells was quantified at 450 nm 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 slide and 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 (17000 cells/well) and the BrdU proliferation test was performed two and three days post-seeding.

Supplementary Materials and Protocols

Detection of SH-groups using the Ellman's assay

Scheme 5 shows the reaction of Ellman's reagent [DTNB (5,5´-dithio-bis-(2-nitrobenzoic acid)] with sulfhydryl containing molecules under slightly alkaline conditions to release the highly chromogenic compound TNB (5-thio-2-nitrobenzoic acid) (Ellman C. 1959; Riddles P. et at. 1979). The reagent contains a disulfide bond between two TNB groups and reacts with free sulfhydryls to create a mixed disulfide product. At pH 8, the release of one TNB group per available thiol provides a yellow colored product with an absorbance at 412 nm.



Scheme 5. The reaction of Ellman's reagent with a SH-group at pH 8 releases the yellow colored TNB anion with an absorbance at 412 nm.

As is illustrated in Scheme 5, the release of TNB anions of Ellman's reagent per available thiol yields a yellow colored product at pH 8. A positive Ellman’s assay reaction is observed with cleaved antibodies (2µl pc BSA-Ab/ml) prepared via immobilized dihydrolipoamide on Gulsenit, which is a better carrier than amino-silanized glass powder (Figure 2). Untreated Glass beads and Gulsenit show no signal due to the absence of free thiol groups in immobilized lipoamide.

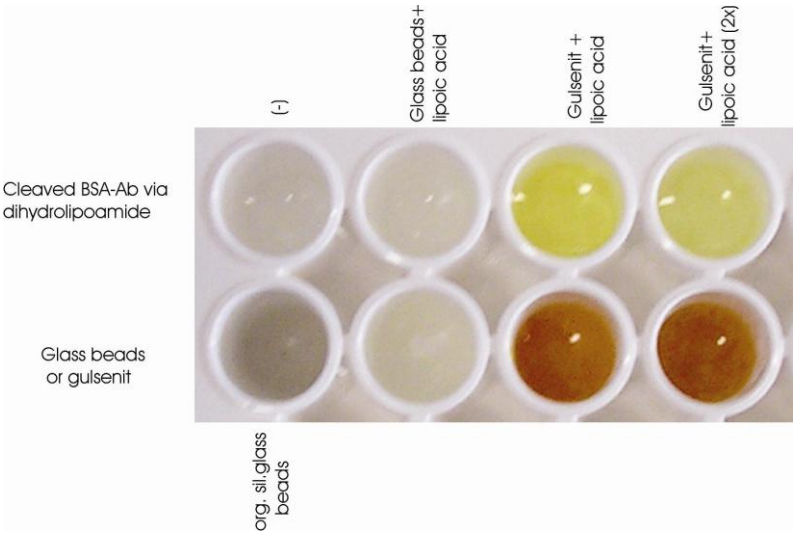


Figure 2. A positive signal of Ellman's assay is observed in the presence of sulfhydryl groups of Fab´fragments. Immobilized dihydrolipoamide on amino-silanized Gulsenit provides a darker yellow color than on amino-silanized glass powder. Ellman's assay shows no signal in the absence of sulfhydryl groups on Gulsenit or glass powder containing lipoamide. (-)= color of Ellman's reagent alone

Detection of NH_2-groups using the Kaiser test

The Kaiser test was efficient for the detection of free amino groups on amino-silica beads used for immunization (Chapter 6) and amino-silanized Gulsenit. Figure 3 shows the efficiency of lipoic acid immobilization onto organic amino-silanized Gulsenit. The intensity of the blue color decreased with increasing amounts of immobilized lipoic acid due to presence of only few free amino groups. As the immobilization of lipoic acid onto amino-silanized Gulsenit was more efficient than on amino-silanized glass beads, Gulsenit was chosen for immobilization of lipoic acid. However, the Kaiser-test is of limited value for the detection of amino groups on most carriers and on amino-silanized glass powder (see Chapter 1).

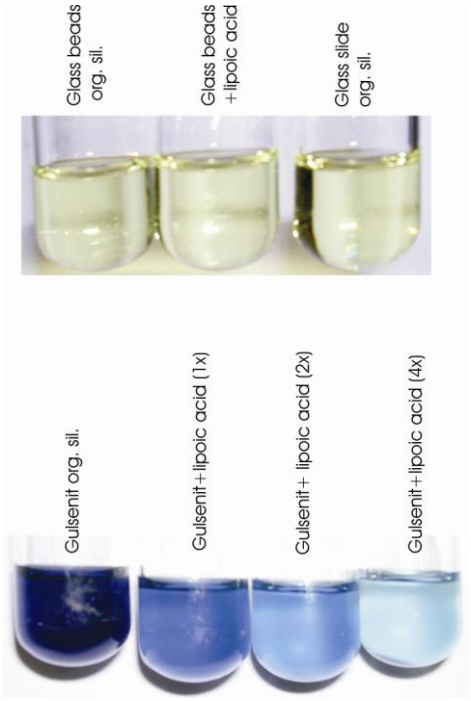


Figure 3. Detection of free amino groups by the Kaisertest. The intensity of the blue color is generated by reaction of ninhydrin with free amino groups. Amino-silanized Gulsenit provides the most intensive blue color due to the high amount of free amino groups, and was used as a binding site for lipoic acid. The color intensity decreases gradually with increasing amounts of immobilized lipoic acid. The Kaiser test was of limited value for the detection of free amino groups on amino-silanized glass slides or glass powder.

Detection of cleaved antibody immobilized onto amino-silanized or amino-PEGylated carrier using OPA-as a crosslinker and test

Sulfhydryl groups of ßME were applied for all previously described detections of amino groups using OPA (see Chapter 1). This section describes the use of OPA as a crosslinker to couple the sulfhydryl of cleaved Ab onto the surface of slides containing amino groups. HRP-IgG was cleaved with immobilized dihydrolipoamide to create two Fab` fragments containing free sulfhydryl groups in the hinge region. OPA was used as a crosslinker for these Fab` fragments with amino-groups on amino-silanized or amino-PEGylated slides (at either pH 5 or 10). Amino-PEGylation was carried out using EDC crosslinker or EDC/sulfo-NHS (see Chapter 1). Figure 4 shows that the EDC/sulfo-NHS activation apparently increases the efficiency of bis-amino-PEG coating. Due to the high cost of sulfo-NHS this method might not be suitable for an up-scaled industrial process, but for proof of principle as was required for this thesis it was the method used for all immobilization steps.

Figure	***Immobilization of* cleaved *HRP-Ab (via immobilized dihydrolipoamide) onto slides with:***
4 A	*Organic amino-silanization*
4 B	*Binding of succinic anhydride at pH 4.8, EDC-crosslinking at pH 10, coupling of bis-amino-PEG in carbonate-bicarbonate buffer at pH 10*
4 C	*Binding of succinic anhydride at pH 4.8, EDC/sulfo-NHS crosslinking at pH 10, coupling of bis-amino-PEG in carbonate-bicarbonate buffer pH 10*
4 D	*Binding of succinic anhydride* at *pH 4.8, EDC crosslinking at pH 10, coupling of bis-amino-PEG in citric acid-sodium citrate buffer pH 5*
4 E	*Binding of succinic anhydride at pH 4.8, EDC/sulfo-NHS crosslinking at pH 10, coupling of bis-amino-PEG in citric acid-sodium citrate buffer pH 5*
4 F	*Binding of succinic anhydride at pH 4.8, EDC crosslinking at pH 10*
4 G	*Binding of succinic anhydride at pH 4.8, EDC/sulfo-NHS crosslinking at pH 10*

Table 1. Set up conditions of carrier (glass slides) before immobilization of HRP- Fab`fragments by means of OPA. Figure 4 shows the fluorescence microscope images of each carrier.

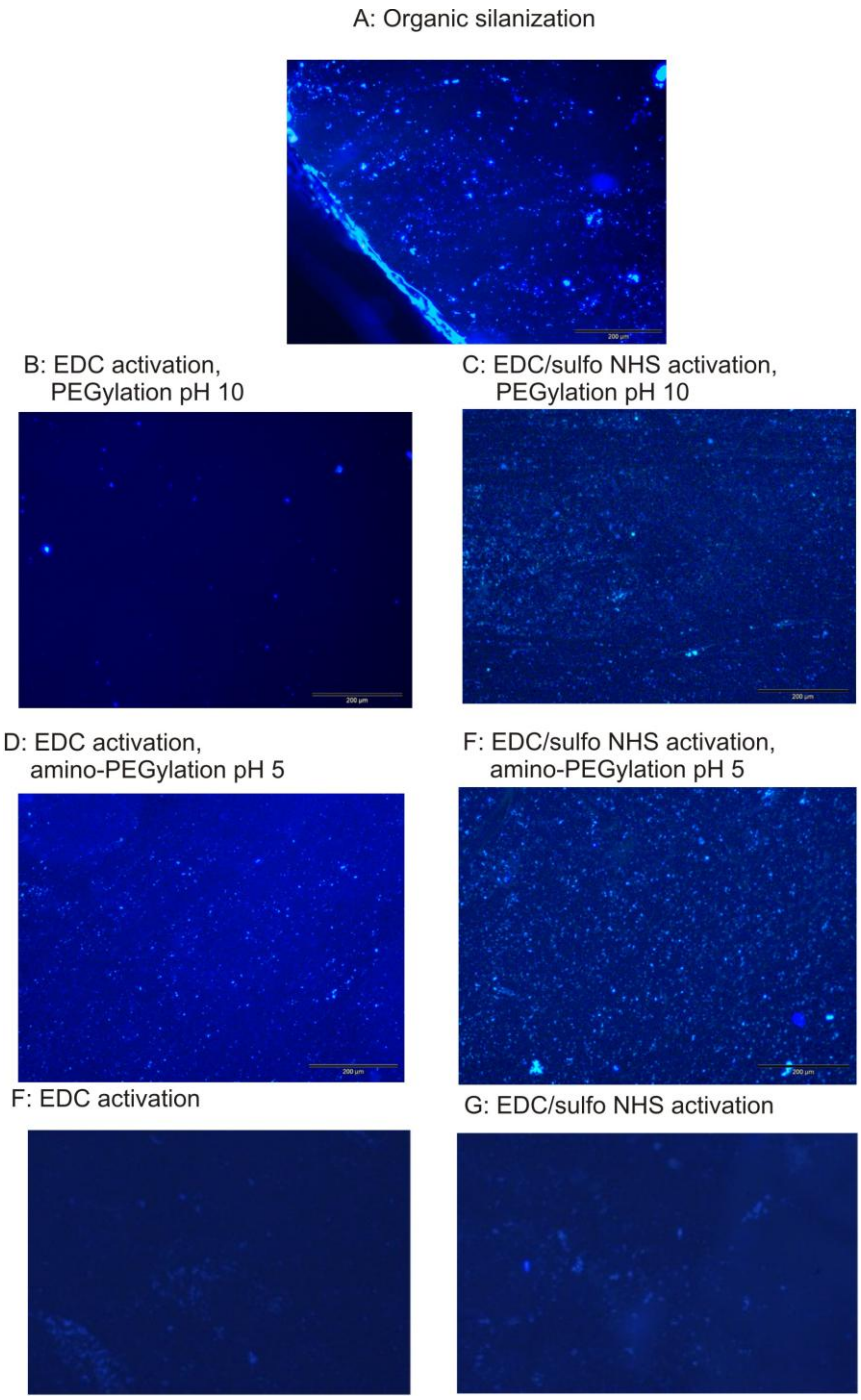


Figure 4. HRP-IgG Fab fragments immobilized by means of OPA onto following carriers (glass slides) A: organic amino-silanized, B: activated with EDC and amino-PEGylated at pH 10, C: activated with EDC/sulfo-NHS and amino-PEGylated at pH 10, D: activated with EDC and amino-PEGylated at pH 5, E: activated with EDC/sulfo-NHS and amino-PEGylated at pH 5, F: activated with EDC (negative control), G: activated with EDC/sulfo-NHS (negative control). Table 1 shows the details for set up conditions of each carrier.

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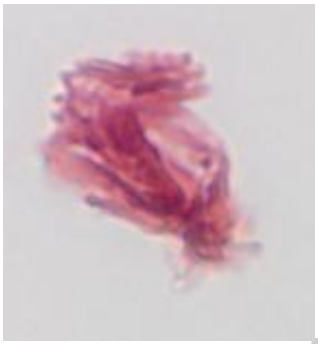
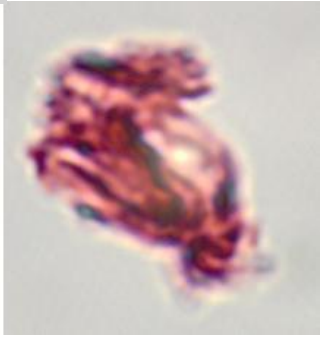
Part 2

Development of new analytical tools for demonstration of protein binding to solid surfaces and binding of PLGA-nanoparticles to the receptor on the implant surface

Chapter 3
A dot-blot test using gold colloid cluster technology as a miniaturizable alternative to ELISA and hapten inhibition tests

Chapter 4
Immunosorbent assay using gold colloid cluster technology for determination of IgEs in patient sera





Chapter 3

A dot-blot test using gold colloid cluster technology as a miniaturizable alternative to ELISA and hapten inhibition tests

The third aim of this thesis focuses on our effort ***to search and develop a biorecognitive and reloadable binding system [antibody or enzyme e.g. LDH (*lactat dehydrogenase)] *for the drug delivery system (e.g. PLGA-nanoparticles).*** For this purpose pc anti PLGA antibodies were produced in rabbits (no. 52 und 53). The determination of PLGA Abs in rabbit's immune sera was rather difficult as PLGA easily hydrolyzes and a completely new system for detection (CLISA – Cluster Linked Immunosorbent Assay) had to be established as is described in this Chapter. This method was first optimized using several antigen/antibody interactions and then used for determination of PLGA-Abs in rabbit's immune sera (Chapter 5). During the course of this development, we realized the potential of applying it for detection of allergens and also for antibodies in sera of allergic patients (Chapter 4). The binding specifity of LDH to PLGA is described in more details in Chapter 5.

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A dot-blot test using gold colloid cluster technology as a miniaturizable alternative to ELISA and hapten inhibition tests

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Abstract

A novel miniaturizable method to quantify antigens is described in form of a Cluster Linked Immunosorbent Assay (CLISA), using inexpensive nitrocellulose (NC) membranes as a support for dot-blotting the antigens. Antibodies for detection are labeled with gold-colloid clusters (GCC). After blocking of the membrane with non reacting protein and application of the GCC labeled antibodies the signal is detectable by visual colorimetry and can be compared to a color scale prepared from a dilution series of known sample concentrations. The color reaction product is stable for a very long time and does not fade. The sensitivity of the method is comparable to that of ELISA if not better and furthermore needs only small amounts of antibody for detection or for GCC labeling. This method provides an alternative to the use of expensive enzyme-conjugated antibodies for a number of applications, such as tracking of antibodies during purification or hapten inhibition tests.

Keywords

Antibody determination; Protein determination; CLISA; Immunoassay

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Introduction

Immunoassays are based on specific antibody-antigen reactions (Wild D. 2001) and quantification of the antigen is generally achieved by measuring the amount of a label molecule bound covalently to the antibody, which can then be detected due to its radioactivity, enzyme reaction, fluorescence, chemiluminescence, bioluminescence, or electrical conductivity (Wild D. 2001; Rossier J.S. et al. 2001; Santandrew M. et al. 1999). However, these labels share a common drawback, as they are not suitable for long-term preservation (Wild D. 2001). While some isotopes have half-lives that would allow long-term applications, the use of radioactivity has in general become less desirable, because of issues concerning waste disposal and potential harm to human health. Furthermore, most fluorescence based assays suffer from fading signal intensity due to photo-bleaching (Longin A. et al. 1993). Recently, nanoparticles, in particular gold and silver particles, were used successfully for labeling, as their size can be controlled easily, they are stable over long periods of time, and they are compatible with biological macromolecules, including proteins and nucleic acids (Daniel M.C. et al. 2004; Jain KK. 2003). Quantification by visual inspection with the naked eye may help reduce costs and makes the test also applicable for field studies, as most other available techniques require specialized detection equipment, software, and specific sample preparation before the detection step.

The procedure for preparing gold colloid clusters (GCC) is well established. Colloidal gold has an orange, red or red-violet color, depending on the size of the particles which can be adjusted by the modifying the method of preparation. Colloidal gold is prepared by reducing gold salts with different reagents, the most common one being used in this research is tri-sodium-citrate and others are include tannic acid, ascorbic acid, and phosphorus. Depending on the method used, GCC preparations vary in particle size (expressed as the average particle diameter) and size variability [expressed as the coefficient of variation (CV) of the gold particle diameter in a sol]. Such a sol is called monodisperse when the CV is less than 15% (Schalkhammer T. 2003). The GCC preparation used in this research is carried out according to the method described by Frens 1973. This method provides monodisperse colloidal gold with a size range from 14 to 50 nm. The size of the clusters can be adjusted by adding different amounts of reducing agent (tri-sodium- citrate).

GCC with an average diameter of 17 nm can be prepared by reduction of $HAuCl_4$ with tri-sodium-citrate. Immediately after adding the reducing agent, aggregation of gold atoms forms central icosahedral gold cores of eleven atoms at nucleation sites. The formation of nucleation sites proceeds very rapidly. The remaining gold atoms in solution bind to the nucleation sites, until all atoms are removed from the solution. As a larger number of nucleation sites for a given amount of gold chloride in solution results in smaller final size of each gold particle, the size of the particles is determined by the amount of reducing agent. To obtain stable gold colloids, they have to be surrounded by a charged layer preferably negative, formed by the residual negative ions in the solution. This negatively charged layer, forming the so called zeta potential, causes the gold particles to repel each other and to stay suspended. Proteins such as antibodies (Abs) bind very stably to the surfaces of these gold particles.

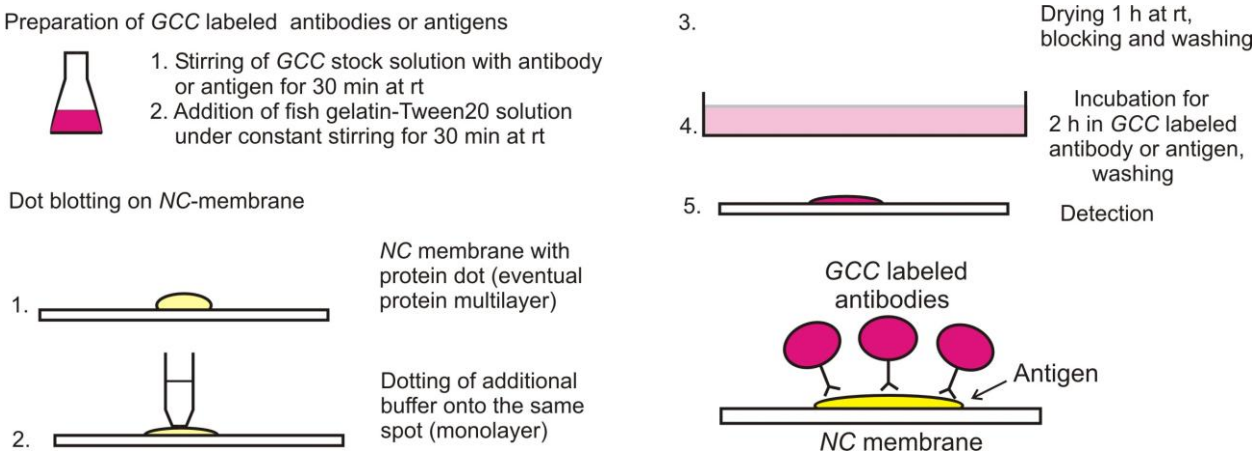

Figure 1. Setup of the dot blot test compared to experimental results using BSA (or HSA) as an antigen and GCC labeled polyclonal-BSA-antibodies (or monoclonal-HSA- antibodies) for detection.

This phenomenon is utilized to quantify antigens dotted onto the NC membrane in form of a Cluster Linked Immunosorbent Assay (CLISA, see Figure 1). Here we demonstrate some examples of successful applications of this principle: Firstly, the detection of bovine serum albumin (BSA) samples dotted onto NC membrane with polyclonal BSA-antibodies tagged with GCC. Secondly, the interaction between human serum albumin (HSA) samples dotted on NC membrane and monoclonal HSA-antibodies tagged with GCC. Thirdly, using an inverse setup, peroxidase-Ab dotted onto NC membrane and peroxidase tagged to GCC.

The sensitivity of the method makes it comparable to ELISA and allows for a rapid quantification of antigens at least semi quantitatively. This approach opens a wide field for applications such as testing for antibody-antigen binding specificity or tracing the purification of antibodies.

Results and Discussion

The dot-blot tests presented here can be used for various purposes; e.g. semi quantitative estimation of antigens by binding to GCC conjugated antibodies or testing specificity of antibodies. In order to achieve specific binding of the GCC conjugates to the respective samples on the NC membrane, either the antigen or the antibody is coupled to the GCC. Antibodies bind with their Fc-domain to the clusters and with the Fab-domain to the antigen on the NC membrane. In this study, 1% aqueous fish gelatin containing 0.1% Tween 20 is used as a stabilizing and blocking reagent for GCC as it lacks HS-groups.

Semi quantitative determination of antigens - BSA and HSA dot blot tests

To test for applicability of our approach in a broader field, BSA as well as HSA and their respective GCC labeled antibodies were used as test substances for semi quantitative estimations (Figure 2). A signal between BSA or HSA samples on the membrane and GCC labeled polyclonal BSA or monoclonal HSA-AB respectively is observed. The color of the reaction signal depends on sample concentration.

For BSA as an antigen the weakest color is observed at 0.025µg/ml and almost no signal can be detected at 0.001µg/ml. The lower limit of detection in this test is considered to be at 0.025µg/ml (Figure 2a). With increasing concentrations the color gradually shifts towards a dark red. At higher concentrations adding even more sample does not lead to a further increase in color as stacking of protein on the dot will occur. In case of HSA decreased color intensity is observed at 0.1µg/ml and no signal can be detected at 0.05µg/ml (Figure 2c).

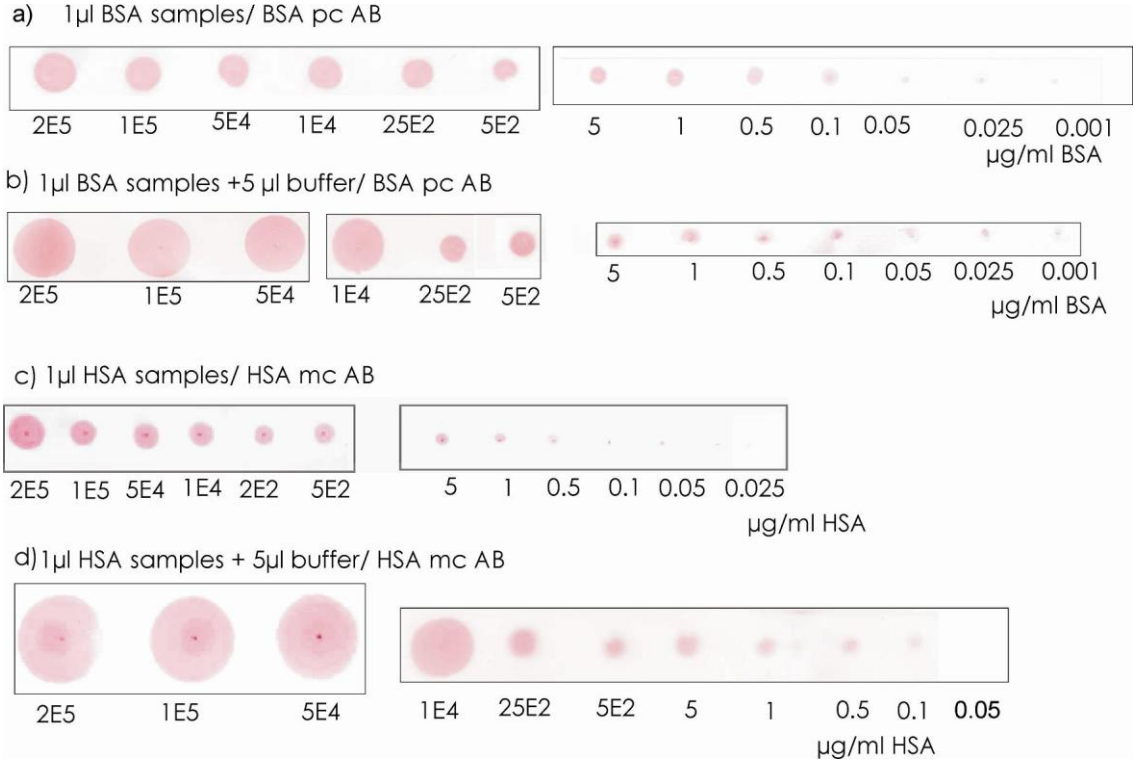

Figure 2. **Semi quantitative dot blot test for BSA and HSA samples:**

a) BSA samples (concentrations in the range between 200mg/ml to 0.001µg/ml) dotted onto NC-membrane without dotting of additional buffer onto the same spot.

b) BSA samples dotted onto NC-membrane and dotting additional buffer onto the same spot, to minimize multilayers of protein.

c) HSA samples (concentrations in the range between 200mg/ml to 0.025µg/ml) dotted onto NC-membrane without dotting of additional buffer onto the same spot.

d) HSA samples dotted onto NC-membrane and dotting additional buffer onto the same spot, to minimize multilayers of protein.

The test can be improved substantially by dotting a certain amount of buffer onto the spot immediately afterwards, resulting in much bigger spots (Figure 2b and 2d). As the affinity of NC membrane and protein is very high, only protein not already bound directly to NC can be dissolved by the buffer solution migrating to free NC surface areas, where it is immediately bound thus resulting in quite even covering of the NC on much bigger spots. As shown in Figure 3, the amount of bound antigen can then be calculated from the diameter or more precisely from the surface area of the detected spots. In the lower range of sensitivity the diameters of the spots are the same as they are determined by the diameter of the tips used for dotting. Here the amount of antigen may be estimated by color intensity of the spot.

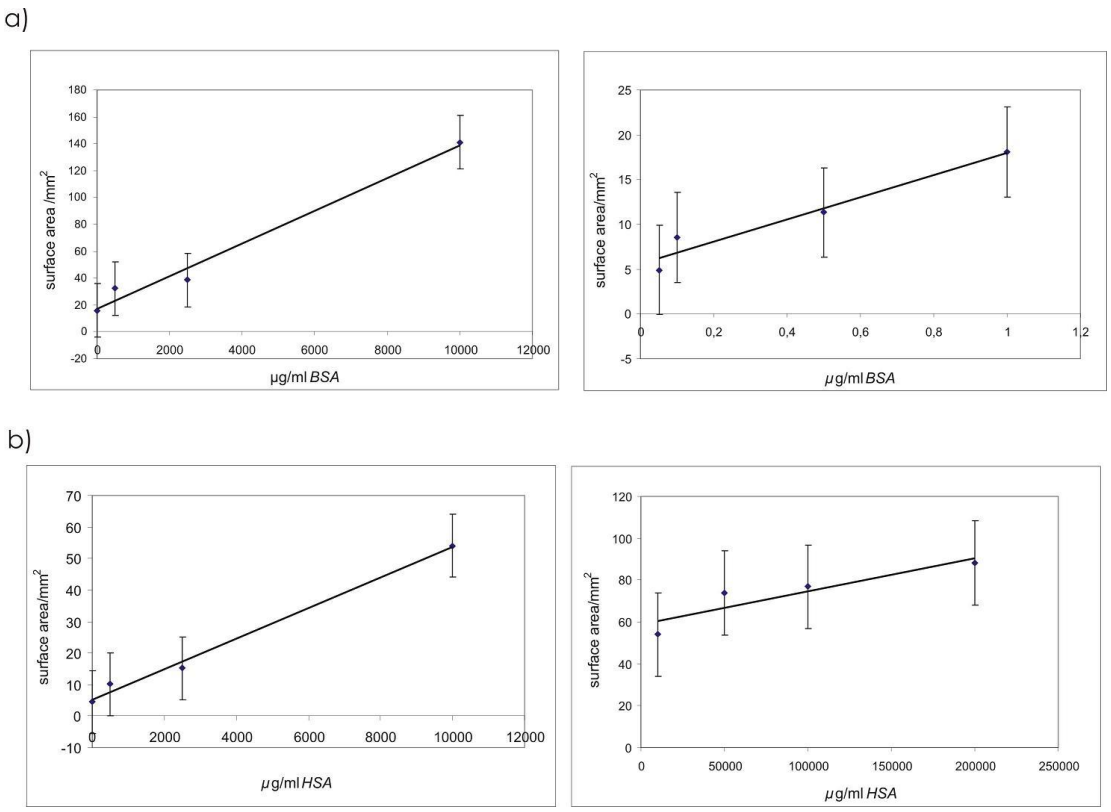

Figure 3. Calculation of antigen concentration from surface area of spot after addition of 5 µl buffer: The developed dot blot membranes were scanned, the pictures transferred to a graphics program (Corel Draw 11) and the diameter of the spots then measured (using the zoom tool for small spots). Accuracy of the values on y-axis is dependent on the quality of the scanner.

a) BSA samples in various concentrations (left: 5 µg/ml to 10mg/ml and right: 0.05 to 1 µg/ml)

b) HSA samples in various concentrations (left: 5 µg/ml to 10mg/ml and right: 10mg/ml-200mg/ml

For negative controls, BSA and HSA samples in the same concentrations as described above were incubated for 2 hours in the same solutions omitting the antibodies and resulting in no detectable signals. As a second negative control, antigen samples were incubated in GCC labeled with non binding antibodies (monoclonal HSA-antibody for BSA samples and β-galactosidase-antibody for HSA samples) also resulting in no color reaction (see Figure 4 b, c).

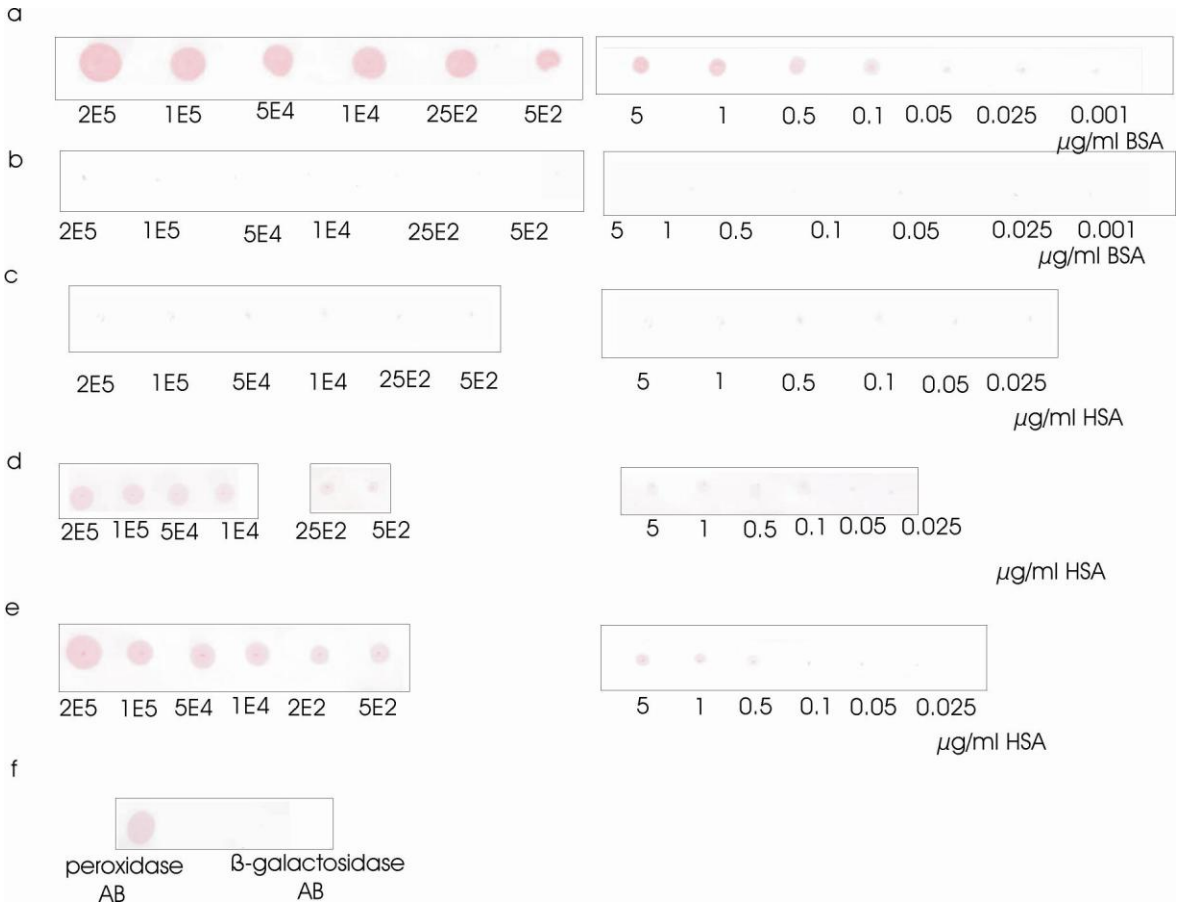

Figure 4. Semi quantitative dot blot test for BSA, HSA and peroxidase including negative controls and cross reactivity

a) BSA samples reacted with GCC labeled polyclonal BSA antibodies

b) BSA samples reacted with GCC labeled monoclonal HSA antibodies (negative control)

c) HSA samples reacted with GCC labeled β-galactosidase antibody (negative control)

d) HSA samples reacted with GCC labeled polyclonal BSA antibodies (cross-reactivity)

e) HSA samples reacted with GCC labeled monoclonal HSA antibodies

f) Peroxidase antibody reacted with labeled Peroxidase but not with negative control (ß-galactosidase antibody)

Testing for specificity of antibodies

Incubation of HSA samples with GCC labeled polyclonal anti BSA-antibodies showed a certain degree of cross-reactivity due to some degree of homology between human and bovine serum albumins as can be seen from Figure 4d. This shows that the test presented here could serve as substitute for the more time consuming and inconvenient hapten inhibition tests. Various antigens can be dotted onto the same NC membrane and incubated with the GCC labeled antibody to be tested for specificity. This setup can also be used for microarrays and automatisation.

Inverse dot blot setups

In some cases GCC tend to aggregate after addition of certain antibodies, which are positively charged at the test conditions as is the case for e.g. peroxidase antibody. In this case an inverse setup is recommended.

Peroxidase antibody dot blot: Peroxidase antibody (1µl 41 mg/ml, Sigma Aldrich) and also monoclonal ß-galactosidase antibody (1µl 2-2.5 mg/ml, Promega) as a negative control are dotted onto the NC membrane. Blocking and washing procedures and also incubation with the antigen GCC labeled peroxidase (40µg peroxidase used for labeling, Fluka) was done according to the previously mentioned protocol and showed only specific binding to the peroxidase antibody on the membrane (see Figure 4 f). As an additional negative control, GCC without peroxidase were reacted with a peroxidase antibody dotted membrane as above, showing no color, indicating no unspecific binding.

Experimental Methods

Synthesis of 17 nm gold colloid clusters (GCC) according to Frens

Frens et al. described procedures for producing mono-disperse colloidal gold with a size range from 14 to 50 nm. Preparation of 17 nm gold clusters: In a very clean Erlenmeyer flask rinsed several times with doubly distilled water (dd. H_2O) and covered with aluminum foil, 10 mg $HAuCl_4.3H_2O$ (Sigma Aldrich) is added to 100 ml dd. H_2O. The solution is heated to boiling under vigorous stirring. Then 4 ml of sterile filtered 1% (w/v) tri-sodium-citrate (Riedel-de Haen) is added very quickly (the solution is blown out of a 5-ml glass pipette). The initiation of nucleation can be seen after approximately 25 sec when the slightly yellowish solution will turn into a faint blue color. After approximately 4 min, the blue color changes into cherry-red, indicating the formation of monodisperse spherical particles. The solution is boiled for an additional 10 min to complete the reduction of the gold chloride under constant stirring. Spectrophotometric analysis at excitation wavelengths from 300 to 800 nm shows the plasmonic absorption as a single peak with a maximum at 520 nm. The GCC solution can be stored at 4°C for prolonged period and used until a color change is observable.

Preparation of GCC-labeled antibodies

In order to achieve specific binding of the labeled GCC to the samples dotted on NC membrane, either the antigen or the antibody has to be bound or adsorbed to the GCC. Stock solution (10 ml) of GCC (17 nm) is mixed with 1 µl antibody under gentle stirring for 30 min at room temperature (rt) to generate the labeled GCC-antibody conjugate. Polyclonal anti-rabbit bovine serum albumin (BSA) antibody (Sigma Aldrich, 3.6 mg/ml), and monoclonal anti-human serum albumin (HAS) antibody (Sigma Aldrich, 7.8 mg/ml) were used in this study. Then 1 ml aqueous 1% fish gelatin (Sigma Aldrich) including 0.1% Tween 20 (Sigma Aldrich) is added under stirring for 30 min. Fish gelatin stabilizes GCCs during the following incubation steps and blocks free binding sites of GCCs avoiding their direct binding to detected antigen on the NC membrane. The concentration of fish gelatin was assessed using a salt flocculation test for GCC. 10 µl of 1% fish gelatin dissolved in dd. H_2O stabilizes 100 µl 17nm GCC.

Preparation of GCC-labeled antigen

The protocol for labeling antigens used in this study is carried out according to the protocol for the preparation of GCC labeled antibodiesPeroxidase-GCC conjugate 10 ml of 17 nm synthesized GCC stock solution is mixed with 40 µg peroxidase (Fluka, 502U/mg) to generate the conjugate. The following incubation step of peroxidase-GCC conjugate in fish gelatin and Tween 20 is carried out as described above.

Dot blotting of test- samples

A test sample (1µl, antigen or antibody) is dotted onto the surface of a NC membrane (pore size 0.45 μm, Protran BA 85, Schleicher & Schuell). For semi quantitative tests 5 µl Tris-HCl buffer (50 mM Tris-HCl, 0.15 NaCl pH 7.3) is then dotted onto the same sample spot to increase the diameter of the protein monolayer avoiding multilayers of protein on the sample spot (Figure 1a). This step can be omitted if only testing for antigen- specificity is desired. The spots are dried for 1h at rt. Then the membrane is immersed in blocking solution containing 2% fish gelatin (Sigma Aldrich), 0.1% Tween 20 (polyethylene glycol sorbitan monolaurate, Sigma Aldrich) and Tris-HCl buffer for 20 min at rt under gentle shaking. Fish gelatin is used for eliminating unspecific background staining as it does not contain free reactive HS–groups that would form a tight chemical bond with the GCC. Therefore, blocking with HS-group

containing reagents, such as BSA or milk, is not recommended. Blocking is further enhanced by the addition of the non-ionic detergent Tween 20. After blocking, the membrane is treated 2x5 min in excess washing solution (Tris-HCl buffer containing 0.5% Tween 20) and 3x1 min in dd H_2O to remove residual NaCl, which otherwise causes flocculation of GCCs. Flocculation occurs immediately if the gold sol is unstable and the red color of GCC turns to violet and blue. The washed membrane is then incubated for 2 hours in the GCC-labeled antibody or antigen solution (inverse setup) at rt under gentle shaking, followed by washing for 3x10 min under gentle shaking at rt in the above mentioned solution.

Dilution series of antigen samples

BSA dilution series in Tris/HCl buffer pH 7.3 were made in a concentration range between 20 mg/ml and 0.001 µg/ml, HSA dilution series in Tris/HCl buffer pH 7.3 in the range from 20 mg/ml and 0.025 µg/ml.

Salt flocculation test

Gold particles in a gold colloid are surrounded by a layer of negative charges, deriving from negatively charged ions present in the reaction mixture (tri-sodium citrate) during synthesis. Due to this charged layer the gold particles repel each other and stay dispersed. During labeling with GCC the colloidal gold sols become unstable in the presence of electrolytes. Addition of electrolytes turns the red color of GCC into violet or blue, and clusters flocculate. However, when poly-electrolytes are added under appropriate conditions, these adsorb spontaneously to the gold particles, and sols are rendered hydrophilic and stable in the presence of added salt. The binding of poly-electrolytes to gold is irreversible in most cases (Walter H. 1999). In this study the GCC is stabilized using fish gelatin. The salt flocculation test is carried out according to Walter and Bauer (Walter H. and Bauer G. 2000)

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Chapter 4

Immunosorbent assay using gold colloid cluster technology for determination of IgEs in patient sera

The demand for rapid, sensitive and low-cost alternatives immunoassay-tests for a wide variety of applications in several fields (such as allergies, tumor marker and serological tests) will likely increase. For this purpose a novel immunoassay-method for detection of antibody or antigen in form of a dot-blot test on microtiterplate or nitrocellose (NC) membrane using gold nanoparticles was developed in this study. This Chapter describes our efforts to develop this method using several interactions between different antigen/antibody and solutions for GCC-flocculation (e.g. using Protein G) and application of CLISA for other analytic purposes such as detection of allergens in patient sera.

The gold colloid cluster technology - developed for determination of successful binding of antibodies to the implant surface and also for use in long term tests concerning degradation of bound nanoparticles to these surfaces as well as possible reloading - proved successful also as a general immunochemical test tool. Therefore –as a byproduct to the main goal of this thesis – this technology was also used for testing sera of allergic patients and compared to conventional ELISA tests. The stability of GCC-labeled allergens makes them potentially applicable in a kit format. A GCC based allergy test could promise more simplification and patient comfort compared to the currently available allergy tests (such as skin-prick tests). The advantages of this rapid method are; 1) the procedure is not time consuming (the whole procedure takes four hours) and economic; 2) the GCC detection method is very sensitive and therefore requires only very low amounts of Abs; 3) there is no need of enzyme conjugated secondary antibodies and their substrates; 4) the signal detection of the cherry red color of GCCs can be observed by the naked eye and does not require any instruments, which makes it useful also for analytic field studies, e.g. profiling patient's potential allergies.

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Immunosorbent assay using gold colloid cluster technology for determination of IgEs in patient sera

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Abstract

A sensitive and simple immunosorbent assay using gold colloidal cluster labeling is developed for determination of proteins such as antigen or antibody. This method is can be carried out on nitrocellose membrane or on microtiterplate as a dot blot support. The positive interaction between proteins can be detectable by naked eye and produced colored reaction product is stable for prolonged periods. In this study is shown that the CLISA-method (Cluster Linked Immunosorbent Assay) is an alternative to ELISA. Several interactions between different antigen/antibody and solutions for GCC-flocculation (e.g. using Protein G) are studied. CLISA was also applied for other analytic purposes such as detection of allergens in patient sera.

Keywords

ELISA; Allergen; Patient sera, CLISA; Immunoassay; β-Lactoglobulin.

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Introduction

The demand for rapid, sensitive and low-cost alternatives immunoassay-tests for a wide variety of applications in several fields (such as allergies, tumor marker and serological tests) will likely increase. For this purpose a novel immunoassay-method for detection of antibody or antigen in form of a dot-blot test on microtiter plate or nitrocellose (NC) membrane using gold nanoparticles was developed in this study. Antibodies (Abs) for detection are labeled with gold-colloid clusters (GCCs). Abs bind with their Fc-domain to the clusters and with the Fab-domain to the antigen on the NC membrane or microtiter plate as a support for dot-blotting (Figure 1). The signal of positive interaction between GCC-labeled Abs and its dotted antigen (Ag) is detectable by naked eye and can be compared to a color scale prepared from a dilution series of known sample concentrations. The color intensity of the signal is proportional to the sample concentration. The colored reaction product is stable for prolonged periods and does not fade, thus making this method a simple, fast and convenient means for detection of antigen/antibody biorecognitions.

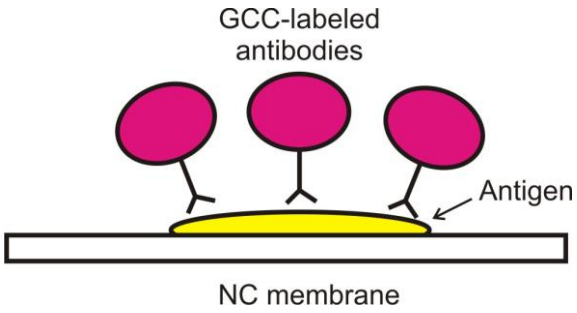

Figure 1. Principle of a Cluster Linked Immunosorbent Assay (CLISA) in form of a dot-blot test using GCC-labeled Abs which bind with their Fc-domain to the clusters and with the Fab-domain to the antigen on the NC membrane or microtiter plate as a support for dot-blotting.

Proteins such as antibodies can bind very stably to the surfaces of these gold particles via intercalation of their sulfur atoms with the gold lattice. A “good” conjugate of gold particles and protein is one where the protein is adsorbed passively onto the surface of the gold (Chandler J. et al. 2000). Antibodies attach firmly to gold via the Fc region and leave the Fab region jutting through the double-ionic layer surrounding the gold particle and are thus able to bind the analyte (Figure 2).

Adsorption of proteins to gold particles occurs within seconds through the following mechanisms:

1. Charge attraction of the negative gold particle to the positively charged amino acids within the protein (e.g. lysine).
2. Hydrophobic adsorption of the protein to the gold particle surface, through certain amino acid residues including tryptophan.
3. Dative binding between the gold particle and sulphur residues on the protein (from cysteine residues)

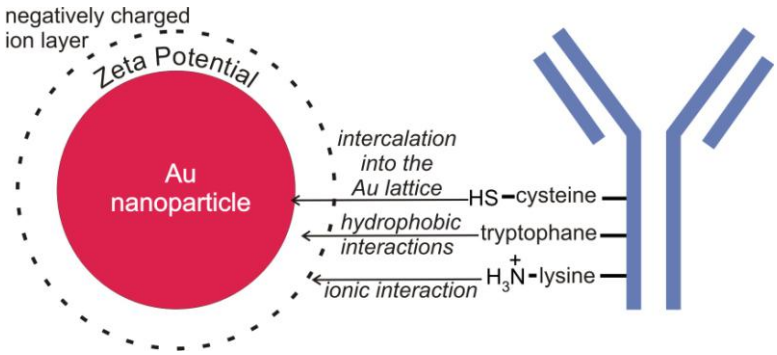

Figure 2. Binding forces between an antibody and a gold particle.

These binding interactions were utilized in a Cluster Linked Immunosorbent Assay (CLISA) to quantify antigens such as, BSA, HSA, ß-lactoglobulin (ß-LG), and IgEs in sera that were dotted onto a NC membrane or microtiterplate.

Some proteins such as pc anti-peroxidase Abs led to the precipitation of GCC as observed by its color turning from cherry red to dark violet or black, which indicate the formation of larger gold aggregates. To avoid this problem, either Protein G was used to stabilize the GCC solution or the Ab preparations were diluted further. Another possibility is the use of an inverse setup, where pc anti-peroxidase Abs are dotted onto the NC membrane and peroxidase is tagged to GCC (Al-Dubai H. et al. 2008). Protein G binds to the surface of gold particles and protects them from precipitation after addition of proteins. In this case, the additional proteins (Abs) bind to Protein G (Figure 3). The sensitivity of the method is comparable with ELISA. Furthermore this GCC-labeling method may also be applicable for other analytic applications, such as the detection of allergens in patient sera.

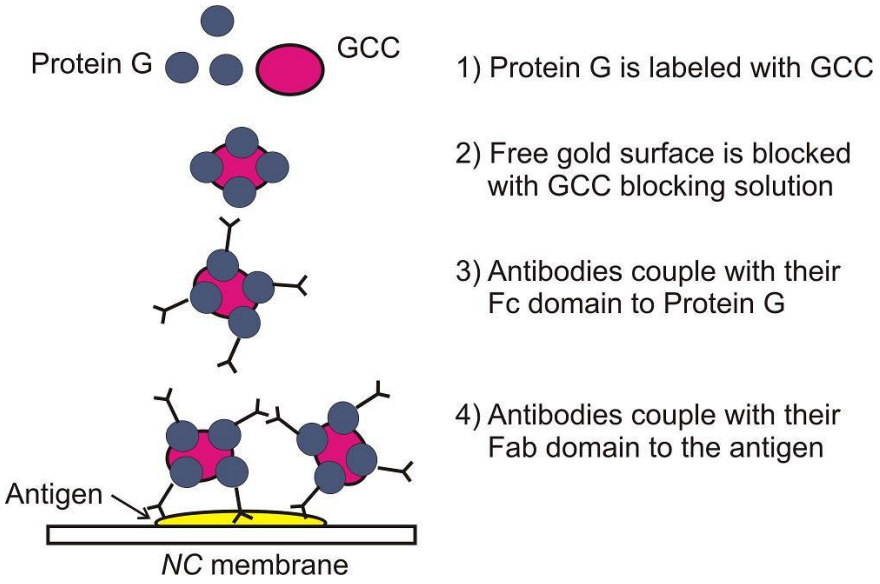

Figure 3. Scheme of stabilization of GCC using Protein G.

The GCC preparation in this research was carried out according to the protocol reported by Frens G. (1973). This method generates monodisperse colloidal gold with a size range from 14 to 50 nm. The size of the clusters can be adjusted by the regulating the amount of reducing agent (tri-sodium- citrate) used. GCC with an average diameter of 17 nm were prepared by reduction of gold tetrachloric acid ($HAuCl_4$) with tri-sodium citrate. Immediately after adding the reducing agent, aggregation of gold atoms leads to the formation of central icosahedral gold cores of eleven atoms that serve as nucleation sites (Figure 4). The formation of nucleation sites proceeds very quickly. The remaining gold atoms in solution bind to the nucleation sites, until all atoms are deposited. As a larger number of nucleation sites for a given amount of gold chloride in solution results in smaller final size of each gold particle, the size of the particles is determined by the amount of reducing agent.

Gold tetrachloric acid + reducing agent → gold colloid

$$(HAuCl_4 + 3\ e^- \rightarrow Au^0 + H^+ + 4\ Cl^-)$$

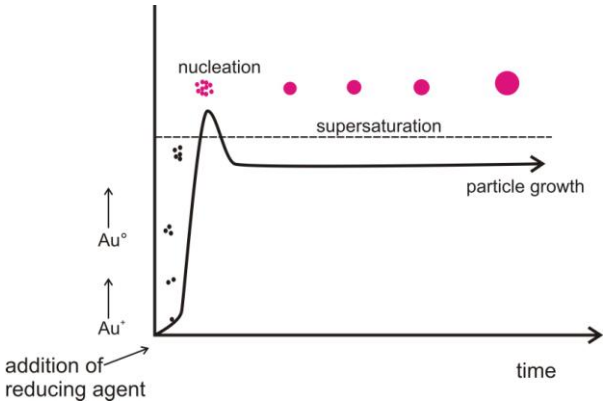

Figure 4. Reduction of gold ions to form gold particles.

Results and Discussion

In order to achieve specific binding of the GCC conjugates to the respective samples on the NC membrane, either the Ag (antigen) or the Ab (antibody) is coupled to the GCC. Abs bind with their free –SH groups on the Fc-domain to the GCCs, and with the Fab-domain to the antigen on either the NC membrane or microtiterplate.

Imaging of GCC particles

The 17 nm synthesized GCC particles were visualized by transmitted light microscopy (Leitz-Reichert Univar) magnification 40 x (Figure 5).

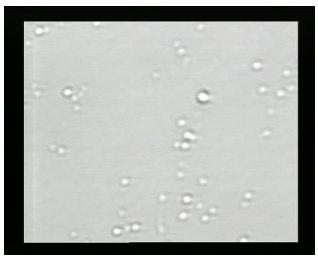

Figure 5. Images of sole 17 nm GCC particles taken on a Univar microscope with a binary video camera and 40 x magnification.

The color of synthesized GCCs is cherry red (Figure 6 a) and the intensity of the color increases after addition of protein (Figure 6 b, c). However, the concentration of added protein is critical due to the tendency of GCCs to flocculate immediately above a certain protein concentration (Figure 6 d, e).

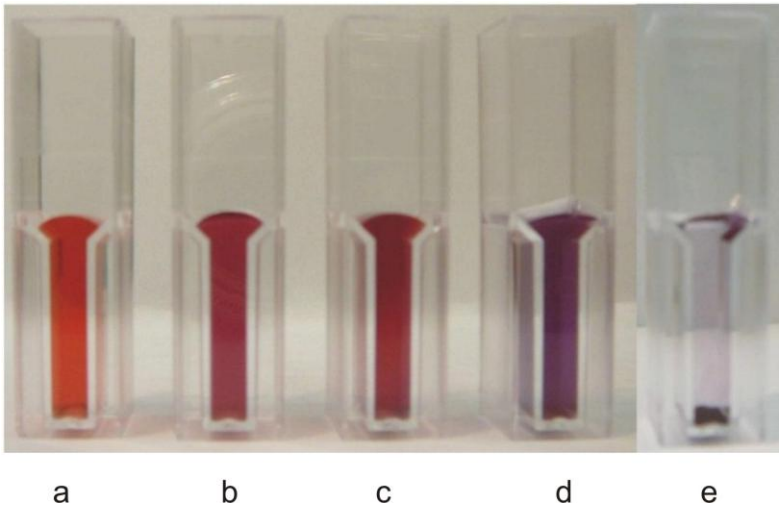

Figure 6. a) Color of GCC solution; b) after adding 1µl pc anti-BSA Ab to 10 ml GCC solution; c) after adding 10 µl pc anti-BSA Ab to 10 ml GCC solution; d) GCC flocculates after adding 1 µl pc anti-peroxidase Ab to 10 ml GCC-solution and its red color turns into violet then blue; e) black by total precipitation of GCC solution.

Transmitted light microcopy images in Figure 7 show that individual GCC particles bind to Abs, and form dark, cherry red aggregates. The color and size of the aggregates depend on the concentration of added Abs (Figure 7 a, c). The change in color intensity of GCC-labeled Ab solutions was shown with either 10 µl or 1 µl pc anti-HSA Ab by detection of 1 µl HSA sample (200 mg/ml) dotted onto the NC membrane (Figure 7 b, d). This phenomenon illustrates that the detection of protein depends on the color, size and type of aggregate. However, the GCCs tolerate only a certain amount of protein added, which needs to be tested for each protein separately. Adding too much of a protein turns the red color of GCCs into violet, blue, and finally black due to flocculation of GCCs (Figure 6 d, e).

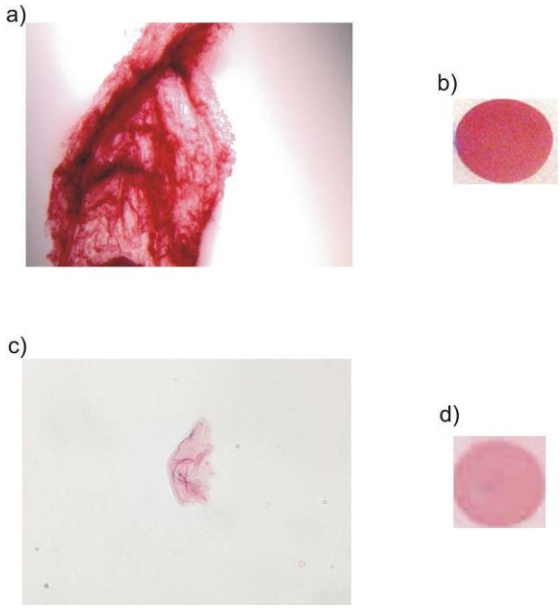

Figure 7. Detection of 1µl HSA (200 mg/ml) dotted onto NC membrane after incubation in GCCs (10ml) including different amounts (b; 10 µl, d; 1 µl) of labeled mc anti-HSA Ab and images of these GCC-labeled Ab solution (a; 10 µl, c; 1 µl) at 40x magnification.

Antigen detection using GCC labeled Abs and ELISA – a comparison

For evaluation of the here presented GCC-labeling method, it was compared to ELISA for the HAS protein, as standard ELISA tests are available for this protein. Detection of HSA antigens by means of GCC-labeling was carried out in a microtiterplate format and compared to detection by ELISA.

The GCC-labeling method provides the following advantages:

- The procedure is not time consuming (the whole procedure takes four hours) and economic.
- The GCC detection method is very sensitive and therefore requires only very low amounts of Abs.
- There is no need of enzyme conjugated secondary antibodies and their substrates.
- The signal detection of the cherry red color of GCCs can be observed by the naked eye and does not require any instruments.
- This makes it useful also for analytic field studies

The detection of HSA samples using CLISA (GCCs labeled with mc anti-HSA Ab) was similar to ELISA and the lower limit of detection was observed at 25 ng/ml (Figure 8a, row A). After incubation of coated HSA-samples on the microtiterplate

with GCC including only blocking solution no signal was observed (Figure 8a, row B negative control). No color reaction was observed for the negative controls after incubation of coated samples in GCC labeled with non binding Ab (mc anti-β-galactosidase Ab, Figure 8a, row C) and after incubation of GCC-labeled Ab in blocked and washed wells without coated HSA-samples (Figure 8a, row D). A certain degree of cross-reactivity was observed after incubation of coated HSA-samples in GCC-labeled with pc anti-BSA Ab due to some degree of homology between human and bovine serum albumins as can be seen in Figure 8a, row E. This cross-reactivity was also detected with ELISA. The importance of blocking GCC particles using fish gelatine is illustrated in Figure 8a, row F. A positive unspecific signal was observed after incubation of coated HSA samples (see materials and methods) in GCC solution due to the high affinity of GCC to bind albumins.

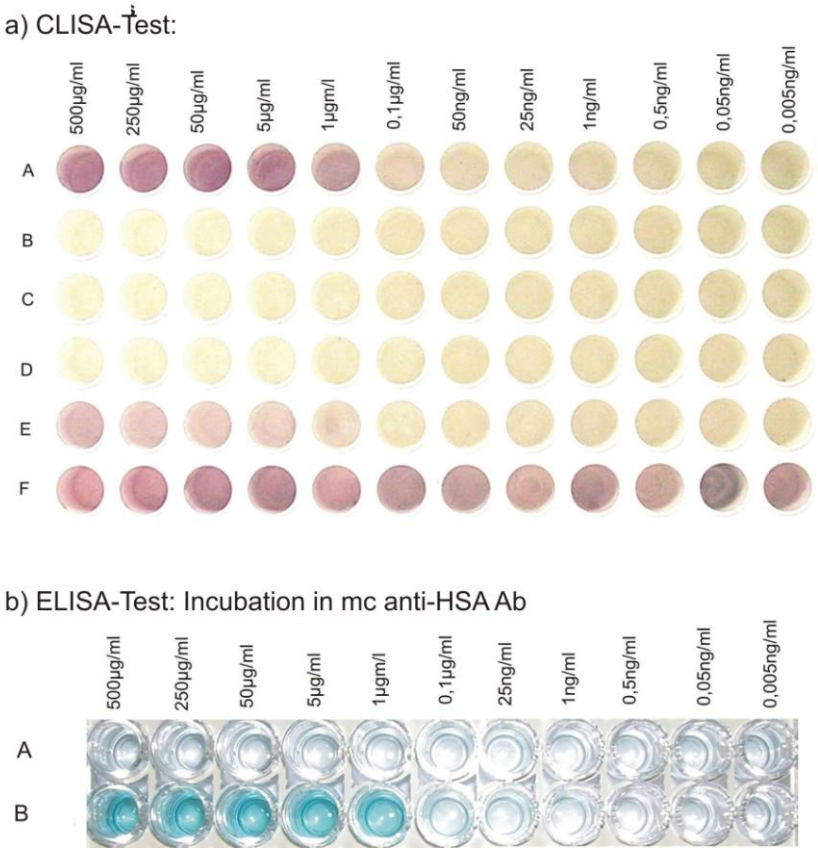

Figure 8. a) CLISA Test: Wells of microtiterplates were coated with HSA- samples and incubated in:
A: GCC- labeled with mc anti-HSA Ab including GCC blocking solution.
B: GCC solution including GCC blocking solution (negative control).
C: GCC-labeled with mc anti-ß-galactosidase Ab including GCC blocking solution (negative control).

D: GCC-labeled with mc anti-HAS Ab including GCC blocking solution incubated in blocked, washed and without coated HSA-sample wells (negative control).

E: GCC-labeled with pc anti-BSA Ab including GCC blocking solution (for testing the specificity of Ab).

F: Incubation of GCC solution (without GCC blocking solution) in wells coated with samples (to show the importance of blocking GCC particles using GCC blocking solution).

b) ELISA Test: incubated in:

A: Wells of microtiterplates were coated with Tris/HCl puffer and incubated in mc anti-HSA Ab solution (negative control).

B: Wells of microtiterplates were coated with HSA- samples and incubated in mc anti-HSA Ab.

Avoiding of GCC flocculation during reaction with certain proteins

GCCs tend to flocculate after addition of certain proteins. To avoid the GCC flocculation appearing immediately after addition of some proteins such as anti-peroxidase Ab, either Protein G is added to GCC solution or its amount was increased. Figure 9 A and B illustrate the positive signal after incubation of GCC-labeled Ab (pc peroxidase- Ab) with dotted peroxidase after increasing the amount of GCC solution (20 ml incubated in 1 µl peroxidase Ab) and additional use of Protein G. Protein G was incubated with GCC solution and bound to the surface of gold particles. This prevented premature precipitation of gold particles after addition of proteins.

Figure 9. Incubation of dotted peroxidase with GCC-labeled with peroxidase Ab. GCC flocculation ater addiding of peroxidase Ab can be avoided by; A) increasing the amount of GCC solution, which was incubated with Ab to produce GCC-labeled Ab or by; B) addition of Protein G to the GCC solution before incubation with the Ab. No color reaction was observed by dotted ß-LG protein (negative control).

This effect is shown in Figure 10 and illustrated again by detection of beta-lactoglobulin (ß-LG), the major allergen in milk. The detected signal of ß-LG using

GCC-labeling is still quite intense at 0.001 mg/ml. No signal was observed after the incubation of NC membrane containing dotted ß-LG samples in GCC solution including Protein G and GCC blocking solution in the absence of Ab (negative control).

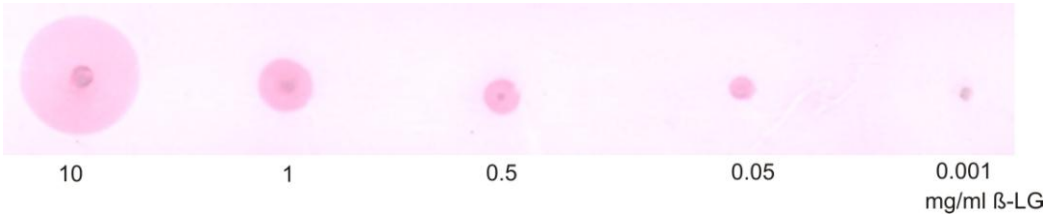

Figure 10. Positive signal of detected ß-LG using the GCC labeling method.

Application of GCC-labeling for detection of allergens in patients sera

Microtiterplate wells containing the dotted sera of allergic patients were incubated with GCC-labeled allergen solutions over night at 4°C. Positive color reactions were obtained (Figure 11) by specific interactions of specific IgE in allergic patients serum to the suspected allergen labeled with GCC. Table 1 gives an overview of the results.

The allergy of patient A was suspected to be against grasses, rye, birch tree, ash tree and ragweed. The GCC-labeling test indicates specific IgEs against grasses/cereals, *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus* and ash tree in his serum. The quality of used ragweed allergen seemed to be not sensitive as no color signal was observed for patient A who has been tested allergic against ragweed before.

Patient B was previously tested positive for allergy against grasses and trees. The GCC-test procedure gave corresponding positive allergy results for grasses and ash tree. Patient C suffered from allergy against different pollen, foodstuff and different animals. GCC-labeled tests demonstrated a positive color reaction for grasses/cereals, ash tree, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*. Allergen cross-reactivity occurs when IgE antibodies originally raised against one allergen recognizes or binds a similar protein from another source (Aalberse RC. et al. 2001; Aalberse RC. et al. 2000; Weber RW. 2003). A cross reactivity between *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* allergens to crustaceans and sea food is known (Ayuso R. et al. 2002; Sidenius KE. et al. 2001; Jeong KY. et al. 2006). This could be the reason for higher color intensity of the detected signal in patient C compared to patients A and B. The signal intensity

for grasses/cereals allergens was low in patient B, due to allergy against only grasses, however patient A had allergy against grasses and rye and patient C against food and different pollen and therefore the signal intensity was higher. These findings show that cross reactivity with other antigens could increase the total signal in one sample.

It is remarkable that the sera of patients A and B gave a positive color reaction with *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* allergen, although this was not indicated for those patients. It might be derived from an immune response of patients developed against both allergens and the respective IgGs were detected in their serum. To increase the specificity of this test for the detection of allergens, purification of sera is necessary for the isolation of IgEs. No signal was observed with the other supplied allergens against which the patient sera did not contain specific IgEs, such as fungi I, mugworth (*Artemisia vulgaris*). Moreover the specificity of the GCC based test is highly dependent on the allergen concentration and purification.

As is shown in Figure 11 and 12 (well 9) positive color reaction was obtained by incubation of the dotted patient sera in GCC-solution including GCC blocking solution. An explanation might be that the concentration of added GCC blocking solution was too low for blocking in the presence of high concentrations of albumins. HSA is the most abundant protein in human blood plasma and albumin reacts with GCC with high affinity. HSA contains free reactive SH–groups that form a tight chemical bond with the GCC. However, adding of allergens and GCC blocking solution was sufficient to block the whole surface of GCC particles, therefore not all the used GCC labeled with allergens show a signal with dotted serum. These aggregates were then able to bind specifically to the IgEs in serum and gave a positive signal on the surface of the microtiterplate and no color reaction in the absence of suspected allergens. The negative control (Figure11 row D) shows no color reaction after incubation of blocked and washed wells with the different prepared GCC-labeled allergens.

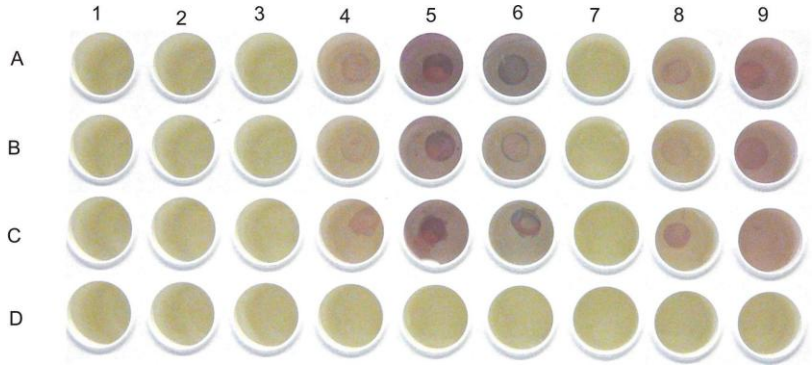

Figure 11. Detection of IgE in patient sera shows cherry red color signals. Columns: Allergens: 1: 044 fungi I (380 µg protein/ml); 2: 106 mugwort (standardised concerning biological activity); 3: 154 short ragweed (320 µg/ml); 4: 015 grasses/cereals (630 µg/ml); 5: 708 *Dermatophagoides farinae* (130 µg/ml) (dust mite, Ed.); 6: 725 *Dermatophagoides pteronyssinus* (standardised concerning biological activity) (house dust mite, Ed.); 7: 108 birch tree (standardised concerning biological activity); 8: 116 ash tree (320 µg/ml); 9: GCCs-solution including GCC blocking solution as a negative control – see Section Results and Discussion. Wells of rows A, B, C were incubated with patients-serum. Wells of row D were blocked, washed and used as negative control. See Table 1 for overview of the results.

Patient: allergy	**4 GCC& grasses/cereals**	**5 GCC& *Dermatophagoides farinae***	**6 GCC& *Dermatophagoides pteronyssinus***	**8 GCC& ash tree**
A:Grasses, ragweed, birch-&ash-tree, rye	+	+	+	+
B: Grasses & Trees	+	+	+	+
C: Pollen-& animal mix, foodstuff	+	+	+	+

Table 1. Overview of positive color reaction (+) of allergic patient sera to the suspected allergen by means of GCC-labeling method (Figure 11, 12)

Furthermore the same GCC based detection was carried out with 21 days old GCC-labeled allergen to test the stability of labeled allergens after storage. Figure 12 presents the same allergens as were tested in Figure 11, but using GCC-labeled allergen that has been stored for 21 days before use by 4°C. The stability of GCC-labeled allergens makes them potentially applicable in a kit format, as long time storage of components is a prerequisite for kit development. A GCC based allergy test could promise more simplification and patient comfort compared to the currently available allergy tests (such as skin-prick tests).

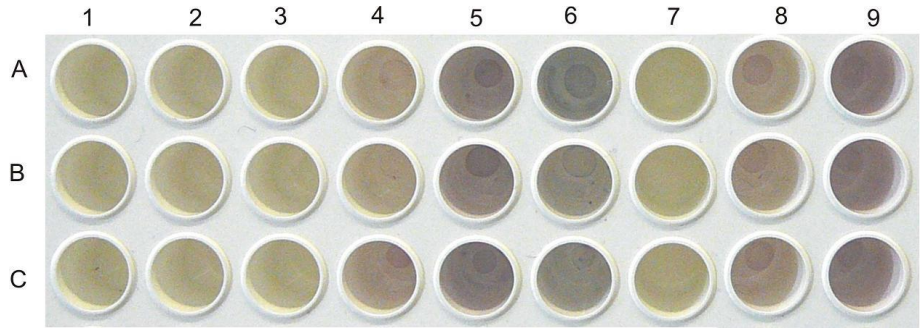

Figure 12. Test for stability of GCC-allergen conjugates: Repetition of the same experiment as illustrated in Figure 11 but here using 21 days old GCC-labeled allergens (stored at 4°C).

Experimental Methods

Synthesis of 17 nm gold colloid clusters (GCC) according to Frens:

The production of mono-disperse colloidal gold with a size range from 14 to 50 nm was prepared according to the method developed by Frens G. (1973). A detailed protocol for the preparation is described by Al-Dubai H. et al. 2008.

Preparation of GCC-labeled antibodies

For a specific test on the NC membrane or the microtiterplate, either the antigen (Ag) or the antibody (Ab) has to be bound or adsorbed to the GCC and the aggregate then stabilized with fish gelatine. Fish gelatine stabilizes gold clusters during the following incubation steps and blocks free binding sites of GCCs avoiding their direct binding to detected antigen on the NC membrane. 10 ml stock solution of GCC was mixed with 1µl pc anti-BSA Ab 3.6 mg/ml (Sigma Aldrich) or mc anti-HSA Ab 7.8 mg/ml (Sigma Aldrich) under gentle stirring for 30 min at room temperature to generate the GCC–Ab conjugate. 1ml GCC-blocking solution (1% (w/v) fish gelatine (Sigma

Aldrich) dissolved in dd. H_2O including 0.1% (v/v) Tween 20, Sigma Aldrich) was added under further stirring for 30 min 10 µl of 1% (v/v) fish gelatine dissolved in dd. H_2O stabilizes 100 µl 17nm GCC (Al-Dubai H. et al. 2008; Walter H. and Bauer G. 2000). As a negative control 10 ml stock solution of GCC was mixed with 1 ml GCC-blocking solution under stirring for 30 min. The GCC labeled Abs solution is checked by microscopy (Olympus and Leica-Reichert Univar respectively) concerning their aggregation status.

Qualitative determination of antigens on NC membranes

For antigen-sample preparation a dilution series in Tris/HCl buffer (50 mM Tris/HCl pH 7.3) were made in a concentration range between 10 mg/ml and 1 µg/ml ß-LG (Sigma Adrich) and 100 mg/ml peroxides-sample (502 U/ mg, Fluka). A test sample (1µl antigen) was dotted onto the surface of a NC membrane (pore size 0.45 µm, Protran BA 85, Schleicher & Schuell). The dotted sample was dried for 1 hour at room temperature. The membrane was then immersed in blocking solution containing 2% (w/v) fish gelatine, 0.1% (v/v) Tween 20 and Tris-HCl buffer for 20 min at room temperature under gentle shaking. Fish gelatine was used for eliminating unspecific background staining as it is free of reactive –SH groups that would form a tight chemical bond with the gold cluster. Therefore, blocking with -SHgroup containing reagents, such as BSA or milk, is not recommended. Blocking is further enhanced by addition of the non-ionic detergent Tween 20. The membrane was treated 2 times for 5 min in excess washing solution [Tris-HCl buffer containing 0.5% (v/v) Tween 20] and 3 times rinsed in dd. H_2O to remove residual NaCl, which otherwise causes flocculation of GCCs. Flocculation occurs immediately if the gold sol is unstable and the red color of gold clusters turns into violet and blue. The washed membrane was then incubated for 2 hours in the GCC-labeled Ab solution at room temperature under gentle shaking. For preparation of GCC-labeled Ab solution GCC stock solution was mixed with Protein G (1mg/ml, Fluka, dissolved in 50 mM Tris/HCl buffer pH 7.3) under gentle stirring for 20 min at room temperature. Then Ab solution [either pc anti-ß-LG (1mg/ml, Bethyl) or pc-anti peroxidase (41 mg/ml, Sigma Aldrich)] was added and stirred for further 30 min. Finally GCC-blocking solution was added under further stirring for 30 min. Table 2 shows quantities and concentrations of the solutions used.

The NC membrane was washed 3 times for 10 min under gentle shaking at room temperature in the washing solution. The detection of a positive signal (red color of gold clusters) was observed with the naked eye and does not require any instruments.

GCC solution	Protein G (1mg/ml)	Antibody	GCC-blocking solution
5 ml	5 µl	5µl pc anti-ß-LG (1mg/ml)	0.5 ml
20 ml	10 µl	1µl anti peroxidase (41 mg/ml)	2 ml

Table 2. The solutions used to produce labeled-GCC Ab including Protein G.

Detection of HSA-antigen using GCC-labeling (CLISA)

The selection of the microtiterplates used is important to reduce the background of negative controls and enhance the color reaction of positive control. The DYNATECH Microtiter™ 2 plates, DYNATECH LABORATORIES, INC (U.S.A), exhibit these desired properties and were used in our studies. They are arranged in a standard 96 well format, have a flat bottom made of polystyrene and additional materials and are also treated to remove electrostatic charge at the end of the manufacturing process. For antigen-sample preparation a dilution series in Tris/HCl buffer (50 mM Tris/HCl pH 7.3) were made in a concentration range between 200 mg/ml and 1 µg/ml HSA (Sigma Aldrich). The wells of the microtiterplate (Microlitetiter™ 2, DYNATECH) were filled with sample (60 µl per well of HSA-dilutions in 50 mM Tris/HCl pH 7.3) for one hour at room temperature. The microtiterplate was emptied by shake off and blocking solution was added to the wells for 20 min then washed 3 times for 5 min in washing solution under shaking, followed by rinsing 2 times in dd. H_2O. The samples on the microtiterplate were incubated in 350 µl GCC-labeled Ab solution per well for 2 hours at room temperature under shaking. The GCC-labeled Ab solutions were prepared by adding of 1 µl Ab [mc anti-HSA Ab (7.8 mg/ml, Sigma Aldrich) or mc-anti ß-galactosidase Ab (2.5 mg/ml, Promega) or pc anti-BSA Ab (3.6 mg/ml, Sigma Aldrich) separately] to 10 ml GCC stock solution under stirring for 30 min at room temperature followed by the addition of 1ml GCC-blocking solution under continued stirring for additional 30 min. The samples were washed 3 times in washing solution and then 3 times rinsed in dd. H_2O. The detection of a positive signal (red color of gold clusters) was observed with the naked eye.

Detection of HSA-antigen using ELISA

The wells of the microtiterplate (Maxisorp, NUNC) were coated with 60 µl of samples per well over night at 4°C (wells of negative control were coated only with Tris/HCl buffer). The samples were blocked and washed as described before. The dotted samples were incubated in 100 µl Ab solution per well (1:5000 diluted in 0.01M PBS pH 7.4) for 2 hours at room temperature under shaking. The samples were washed 3 times with washing solution for 5 min. The microtiterplates were incubated for 1 hour with 100 µl per well of the conjugated HRP secondary Ab (anti-mouse IgG, Sigma Aldrich) solution (diluted 1:10.000 in 0.01M PBS pH 7.4) at room temperature under shaking. The microtiterplate was washed 3 times with washing solution for 5 min 100 µl TMB-substrate (Sciotec, Austria) was added per well and incubated for 10 min at room temperature. The color of TMB-substrate changes to blue in presence of antigen. The color intensity is proportional to the amount of detected protein. The reaction was stopped with 50 µl 1M H_2SO_4. The color intensity of individual wells was quantified at 450 nm with a Microplate Reader Model 450, Bio-Rad. The coated wells were also incubated with pc-anti-BSA-Ab solution (prepared in analogy to mc-anti-HSA-Ab solution described above) for testing of antibody specifity.

Detection of allergens in patient sera using gold cluster labeling

GCC solutions were labeled with several allergens (allergy-causing substance) from Prick-/Scratch testing solutions. The scratch test or skin prick test is one of the most commonly used methods of allergy testing on the skin. It is performed by placing a small amount of the suspected allergen on the skin. The allergic skin shows a physiological reaction as swelling or redness at the scratch/prick site. The prepared GCC-labeled allergen solutions were incubated in the dotted allergic patient sera on the microtiterplate. The specific interaction of the IgE in the allergic patient serum and the suspected allergen, labeled with GCC, shows a positive color signal on the surface of microtiterplate. 2 µl serum of each allergic patient was dotted onto one row (A-C) of a microtiterplate (Microlite^{TM} 2, DYNATECH) from well 1 to 9 and incubated overnight at 4°C. Wells of row D on the microtiterplate were blocked, washed and used as negative control.

The patients were allergic to:

Patient A: Grasses, rye, birch tree, ash tree, ragweed

Patient B: Grasses and trees

Patient C: Pollen mix, foodstuff and animal mix

All wells of the microtiterplate were blocked and washed in 100 µl per well washing solution (Tris-HCl buffer containing 0.5% (v/v) Tween 20) under stirring for 5 min under shaking. Each washed well (1-8 of rows A-D) was incubated in 350 µl of GCCs-labeled allergen solutions, each row including one of the following allergens (1-8 see below), overnight at 4°C. The ninth well was incubated in GCCs-solution including GCC blocking solution as a negative control. Eight different GCC-labeled allergen solutions were separately prepared by stirring 10 ml GCC stock solution with 500 µl of the following allergen solutions (fungi I 300µl) for 30 min at room temperature. 1 ml GCC-blocking solution was added to each GCCs- labeled allergen solutions under stirring for 30 min. The GCCs-solution including GCC blocking solution as negative control was prepared analogues to GCC-labeled solution omitting allergen.

Allergens:

1) 044 fungi I (380 µg protein/ml)
2) 106 mugwort (standardised concerning biological activity)
3) 154 short ragweed (320 µg/ml)
4) 015 grasses/cereals (630 µg/ml)
5) 708 *Dermatophagoides farinae* (130 µg/ml) (dust mite, Ed.)
6) 725 *Dermatophagoides pteronyssinus* (standardised concerning biological activity) (house dust mite, Ed.)
7) 108 birch tree (standardised concerning biological activity)
8) 116 ash tree (320 µg/ml)

The wells were rinsed with dd. H_2O and a positive signal was observed with the naked eye. For testing the stability of the GCC labeled antigen solutions the same experiment was repeated after 21 days using the same solutions of GCC-labeled allergens that had been stored at 4°C. The GCC-labeled allergens were stable and showed the same results as freshly prepared GCC-labeled allergens (Figure 11, 12). These preliminary results show that CLISA could be a useful test principle to develop further into a kit format allergy test

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Part 3

Proof of binding of nanoparticles serving as drug carriers to receptors onto the biocompatible-coated implant surfaces and long term test showing degradation of nanoparticles and reloadability

Chapter 5

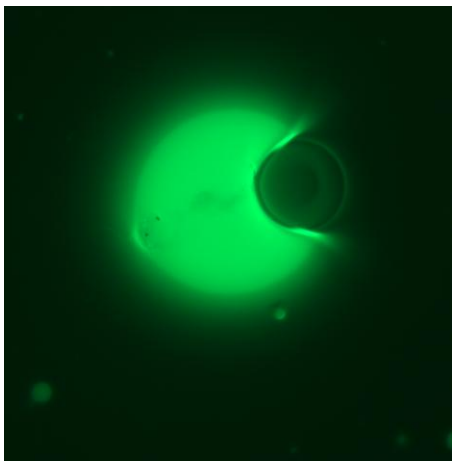
CLISA as a tool for detection of successful immunization of rabbit's in order to produce PLGA Abs and testing binding of PLGA NPs to the coated implant surface for reloadability studies.

Chapter 6

Production of polyclonal antibodies against the biocompatible polymer PLGA using silica amino microspheres as a carrier

Chapter 7

Production of polyclonal antibodies against the biocompatible polymer PLGA using silica amino microspheres as a carrier



Chapter 5

CLISA as a tool for detection of successful immunization of rabbit's in order to produce PLGA Abs and testing binding of PLGA NPs to the coated implant surface for reloadability studies.

Chapter 5

An important aim of the thesis was to search and develop a biorecognitive and reloadable binding system [antibody or enzyme e.g. LDH (Lactate Dehydrogenase)] for PLGA nanoparticles as a drug delivery system. For this purpose pc anti PLGA antibodies were produced in rabbits. The determination of PLGA Abs in rabbit's immune sera was detected by means of CLISA that was established using several antigen/antibody interactions as is described in Chapters 3 and 4. The search for biorecognitive and reloadable binding system for PLGA-nanoparticles was investigated also for other proteins such as LDH. The binding specificity of PLGA to LDH is discussed in this Chapter and also a degradation time assay of PLGA-NPs after their specific binding to their immobilized recognitive site (PLGA-Abs) on the surface of the carrier using CLISA.

Abstract

The degradation time assay of PLGA-NPs was carried out with immobilized, non purified immune sera of rabbit 52 and 53. The carriers were loaded with PLGA NPs. A positive interaction between immobilized immune sera and PLGA NPs was then detected visually by means of a CLISA assay. To assess degradation of PLGA NPs over time, the carriers were then incubated in Ringer´s solution at 37°C. During two months of incubation the color of the carriers faded. Then the carriers were incubated with fresh PLGA NPs, and reloading was detected again using the CLISA method. The carriers showed a marked increase in color but the color intensity was lower than after the initial loading. After one year incubation of carriers in Ringer's solution, the reloading procedure with PLGA NPs and detection using CLISA were repeated. But this time color intensity could not be enhanced. This shows that the immobilized immune sera after one year incubation did not bind specifically to PLGA NPs.

The purified proteins from immune-sera by means of affinity chromatography using PLGA loaded columns showed no characteristics of PLGA-Abs. These proteins were analyzed by tryptic digestion and peptide mapping using mass spectrometry and rather revealed sequences for proteins that bear strong homology to albumins. This means that the immunisation of rabbit 52 and 53 was not efficient and a more stringent immunization strategy had to be carried out (Chapter 6). Due to this unspecific interaction between albumin and PLGA a new targeting system using another biocompatible polymer (chitosan, Chapter 7) was developed.

Introduction

To produce PLGA antibodies, two rabbits (number 52 and 53) were immunized with PLGA nanoparticles suspended in an adjuvant. The immunization procedure was performed in collaboration with a certified laboratory. Nanoparticles (NPs) were produced using the water-in-oil-in-water (w/o/w) solvent–evaporation technique (Weissenböck A. et al. 2004) in the presence of Pluronic F-68. Pluronics are a class of nonionic copolymers called Poloxamers that contain a central hydrophobic polyoxypropylene chain and two hydrophilic polyoxyethylene chains. Depending on the length of the side chains their surface activities can be modulated and used to tune the hydrophilicity-lipophilicity balance of solutions and reaction mixtures. The success of pc PLGA-Ab production was proven using GCC-labeling as well as ELISA for the sera of rabbits 52 and 53 (see Chapter 6). The CLISA method developed in this thesis for the detection of PLGA Abs produced in immune sera of immunized rabbits (rabbit number 52 and 53) was also previously optimized for other Abs to show the versatility of this assay and are described in Chapter 3 and 4.

Determination of PLGA Abs in rabbit's immune sera (no. 52 und 53) using CLISA

As described in Chapter 3 and 4, flocculation occurs immediately after addition of some proteins such as anti-peroxidase Ab. To avoid this problem, several methods were established in addition to the described inverse dot blot method described in Chapter 3. The gold sol of labeled GCC was stabilized either by using Protein G or by increasing the amount of labeled GCC (Chapter 4). Protein G binds to the surface of gold particles and protects them from precipitation when the protein analytes are added (see Chapter 4, Figure 4). The success of labeled GCC stabilization by means of Protein G has been demonstrated in several examples such as labeling of GCC with pc anti-peroxidase Ab, pc anti-ß-LG Ab (Chapter 4). In this part of work Protein G was used for stabilization of GCC-solution before addition of either LDH (Lactate Dehydrogenase) or sera of rabbit's number 52 and 53 to avoid flocculation.

Degradation time assay for the degradation and reloading of PLGA nanoparticles

The same CLISA method was also used for a visual long term assay for the verification of degradation of PLGA nanoparticles bound to the receptor on the biocompatibly coated implant surface and a successful reloading with fresh nanoparticles. The principle of the degradation time assay is based on the coupling of PLGA-Ab in non purified immune sera (of rabbit 52 and 53) onto amino-silanized or amino-PEGylated carriers. The disulfides in the hinge region of the Abs in immune sera, which connect the heavy chains, were selectively reduced with the reducing agent 2-MEA (2-mercaptoethanolamine) to create two Fab` fragments, each of which contains one antigen binding site (Palmer J.L. and Nissmoff A. 1963; Sun M C. et al. 2005). The generated free sulfhydryl groups can be used for immobilization by means of thiol-reactive probes or crosslinkers such as Sulfo-SMCC. As conjugation takes place via -SH groups in the hinge region of the Ab, the antigen binding regions retain their activity. The antibody fragments were immobilized onto amino-silanized or amino-PEGylated carriers using sulfo-SMCC crosslinker. Sulfo-SMCC (Sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate) is a water soluble heterobifunctional crosslinker whereas SMCC [Succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate] is soluble only in organic solvents. Both can be used to immobilize either the whole antibody molecule or an Ab-fragment (Fab´) by reacting with primary amines (Figure 1). The NHS-ester as part of the cross linker reacts with primary amines, whereas the maleimide group reacts with the sulfhydryls of the cleaved Ab fragments.

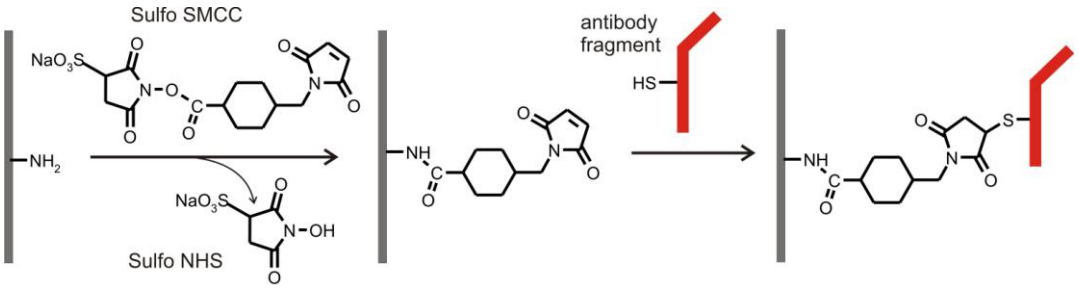


Figure 1. Immobilization of antibody fragments onto carriers containing amino groups via sulfo-SMCC activation.

These carriers bind specifically to PLGA NPs. The binding between immobilized PLGA-Ab on the carrier and PLGA NPs was detected using CLISA. For this procedure, gold colloid cluster (GCC) conjugate was used to label PLGA-Abs in the immune sera. Subsequently, the carriers that were loaded with PLGA NPs were incubated with GCC-PLGA-Abs. The binding of PLGA NPs to the carriers could then be detected visually as the GCC-labeled PLGA-Abs bound onto PLGA NPs on the carrier.

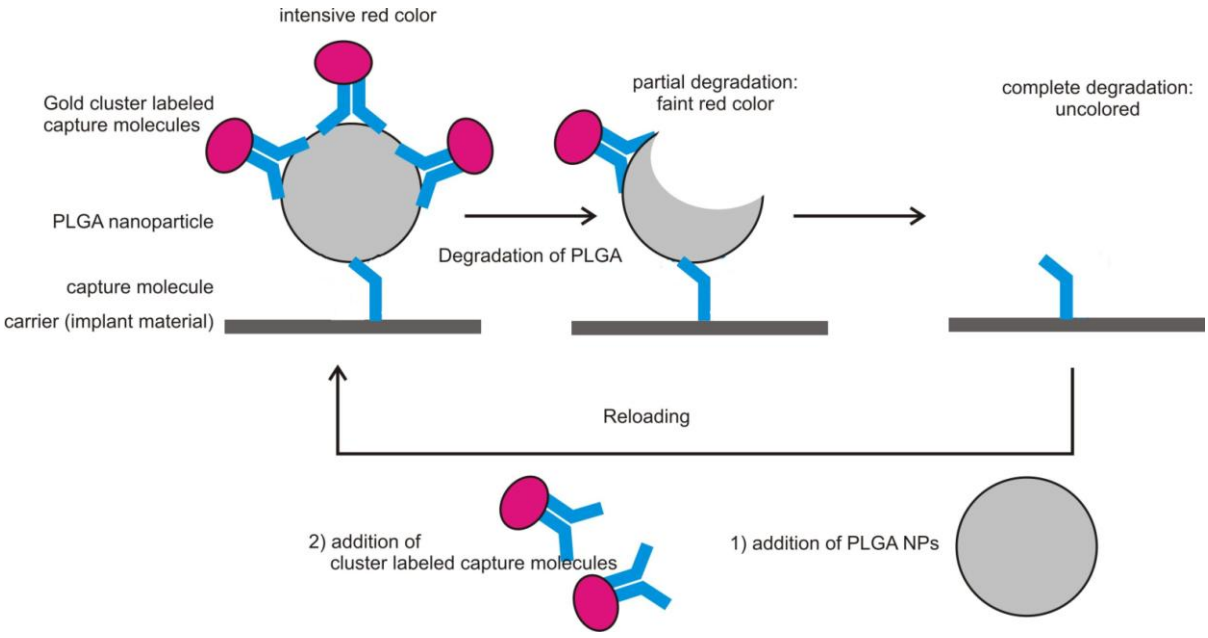


Figure 2. Schematic representation of the principle for testing of reloadability of coated implant materials.

To assess degradation of PLGA NPs over time, the carriers were then incubated in Ringer´s solution at 37°C mimicking the chemical composition of body fluids surrounding a putative implant. After complete degradation of PLGA NPs, the binding site (immobilized Abs on the carrier surface) becomes free again to bind PLGA NPs (Figure 2). Consistent with this mechanism is the decrease of the intensity of red color that results from GCC labeled PLGA-Abs, which can be directly visualized. After complete degradation of PLGA-NPs, the GCC labeled PLGA Abs dissociated from the carrier as the binding partner (PLGA-NPs) was missing, resulting in a colorless carrier. After repeated incubation of the carrier with PLGA NPs the color intensity then increased again, as GCC labeled Abs were able to bind again.

Purification of IgGs from rabbit 52 and 53 immune serum using Dynabeads® M-280 Sheep anti-Rabbit IgG or Protein A

To produce PLGA-Abs that may serve as a biorecognitive binding site for PLGA NPs on implant surface material, rabbit's no. 52 and 53 were immunized. As mentioned earlier, the immunization procedure was performed by a certified laboratory and was not a part of this thesis. However, several purification methods for serum to isolate PLGA-Abs had to be developed in this thesis. Immunoglobulins G (IgGs) in sera of rabbit no. 52 and 53 were isolated using Dynabeads® M-280 Sheep anti-Rabbit, or Protein A Antibody Purification Kit. Affinity chromatography was carried out to purify PLGA-Abs from isolated IgGs with either Dynabeads® M-450, Epoxy containing immobilized PLGA, or in a different approach by Gulsenit conjugated with PLGA as a column material. Moreover, the effect of direct isolation of PLGA-Ab using affinity chromatography was investigated.

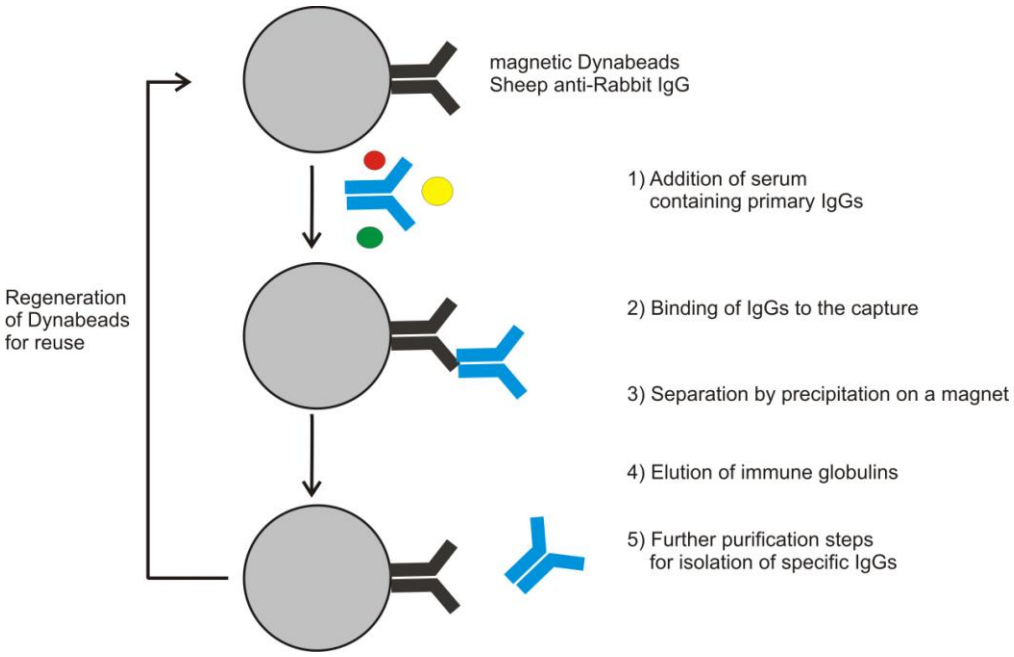


Figure 3. Schematic principle of purification of IgGs using Dynabeads® M-280 Sheep anti-Rabbit.

Dynabeads® M-280 Sheep anti-Rabbit IgG

Dynabeads are uniform superparamagnetic polystyrene beads with a surface suitable for physical and chemical binding of antibodies (Abs), Antigens (Ags) or other biomolecules. Dynabeads provide a versatile tool for isolation of both cells and non-cellular targets (such as proteins and other biomolecules). Using a magnet

(Dynal® MPC™, Magnetic Particle Concentrator), the beads can be easily separated and the target molecules can then be handled in a highly specific manner. The beads can be added directly to the sample containing the target Ab/Ag. Dynabeads® M-280 Sheep anti-Rabbit (2.8 µm) are designed as a solid support for simple and efficient binding of all rabbit IgGs via a rabbit primary Ab (Figure 3). All IgGs in sera of rabbit no. 52 and 53 were isolated via Dynabeads® M-280 Sheep anti-Rabbit.

Affinity chromatography of purified IgGs of rabbit 52 and 53 to isolate PLGA-Abs

PLGA IgGs were isolated from the purified IgG fraction of rabbit sera via Dynabeads® M-280 Sheep anti-Rabbit or a protein A column. The carriers for affinity chromatography (either Dynabeads® M-450Epoxy or Gulsenit) were immobilized with PLGA. In a second approach the PLGA-Abs were isolated directly from immune sera (rabbits no. 52, 53) without previous purification of IgGs by means of PLGA immobilized to Gulsenit. As a control, this affinity chromatography was also carried out with pre-immune serum of rabbit and human serum. Dynabeads® M-450 Epoxy (4.5 µm diameter) are hydrophobic and covered with surface epoxy groups, these may be used for immobilization of ligands containing amino, thiol or hydroxyl groups. No further activation is necessary and irreversible binding of the ligands to the epoxy groups is achieved over a wide temperature range and at a neutral to alkaline pH.
PLGA (Resomer RG 502 H) was coupled onto Dynabeads® M-450 Epoxy (Figure 4) and used as a material for affinity chromatography to bind PLGA-Abs from previously isolated IgGs of sera (rabbit's no. 52, 53).

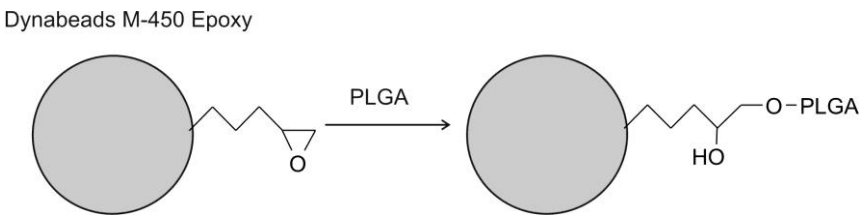


Figure 4. Immobilization of PLGA onto Dynabeads® M-450 Epoxy

Results and Discussion

Detection of PLGA Ab in sera of rabbit 52 and 53

The immunization of rabbits 52 and 53 was carried out by injection of PLGA-NPs, which contained a small amount of Pluronic. Therefore, the immune sera were tested for PLGA and Pluronic Abs. Several dilution series of PLGA and Pluronic were incubated in GCC solution including Protein G and labeled with immune serum of rabbit 52 or 53 respectively. Both immune sera of Rabbit 52 and 53 gave similar results, whereas the negative controls (Pluronic and PLGA samples) showed no signal when incubated in GCC solution containing Protein G and GCC blocking solution (Figure 5). PLGA samples incubated in GCC-labeled solution showed a faint color signal at an immune serum dilution of 4:10.000 and at PLGA concentrations in the range between 10 mg/ ml and 1 mg/ml. At PLGA concentrations from 0.5 to 0.005 mg/ml, the faintest color signal was seen at 5:10.000 immune serum dilution. The color intensity of PLGA in the concentration range between 10 to 1 mg/ml and between 0.5 to 0.005 mg/ml was identical (Figure 5). This shift of color intensity between the two different ranges (10 to 1 mg/ml and 0.5 to 0.005 mg/ml) by either low concentrations of PLGA or immune sera is indicative of a protein (possibly a PLGA Ab) present in the immune sera that binds specifically to PLGA.

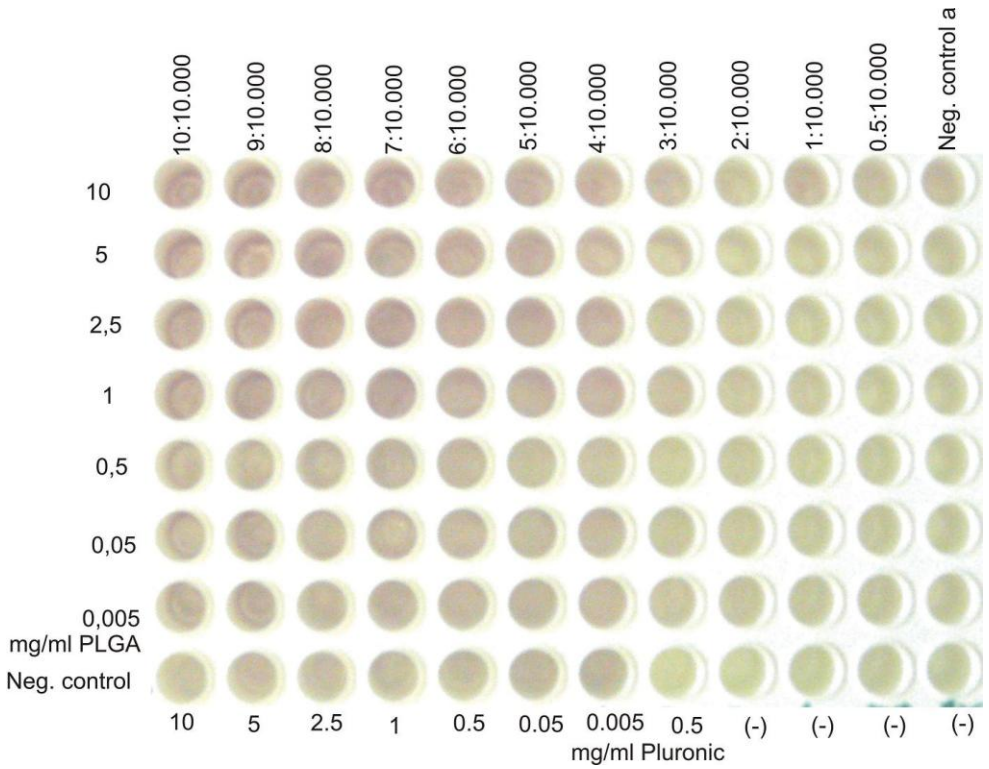


Figure 5. Detection of PLGA Ab in immune serum of rabbit 52 using GCC-labeling. A positive color signal was observed when incubating different PLGA concentrations in different dilutions of GCC-labeled with immune serum. The color intensity was depending on the concentration of both, PLGA and dilution of immune serum. No signal was detected by incubation with Pluronic in different dilutions of GCC-labeled with immune serum (negative control). In the negative control for PLGA Abs PLGA samples were incubated in GCC solution without Abs, but with Protein G and GCC blocking solutio; no signal was detected.

Degradation time assay for the degradation and reloading of PLGA nanoparticles

The degradation time assay was carried out with immune sera of rabbit 52 and 53, omitting purification to test the avidity of whole immune sera and benefit from the total amount of PLGA Ab in the immune serum of rabbit 52 and 53. A positive interaction between immobilized immune sera on amino-silanized glass carriers and PLGA NPs was visualized directly by color reaction (Figure 6 A; 1, 2) using CLISA (GCC-labeled with immune serum of rabbit 52 or 53 separately). The carrier for the negative control was prepared by omitting the immobilization of immune sera and showed a less intensive color reaction (Figure 6; A 3) due to some unspecific interaction between labeled GCC sera and amino-silanized carrier. All carriers were incubated in Ringer's solution, which was exchanged repeatedly, at 37°C under

gentle shaking for one month. The unspecific background of the negative control disappears after incubation for one day in Ringer's solution. However, the color of carriers containing immobilized immune sera faded slower during two months incubation (Figure 6 B; 1, 2, 3). After the two months of incubation, the carriers were reloaded with fresh PLGA NPs, and reloading was detected again using the CLISA method as described previously. The carriers with immobilized immune sera showed a marked increase in color (Figure 6 C; 1, 2). However, color intensity was lower than that observed after the initial loading (Figure 6 A). Carriers were also incubated in Ringer's solution for a period of one year, and the reloading with PLGA NPs and detection using CLISA were repeated. This time the color intensity remained constant as that observed prior to reloading with PLGA (Figure 6 D; 1, 2). This means that the immobilized immune sera, after incubation for one year, did not bind specifically to PLGA NPs. Therefore, PLGA-Abs were purified from immune rabbit sera in order to immobilize a high amount of PLGA Abs.

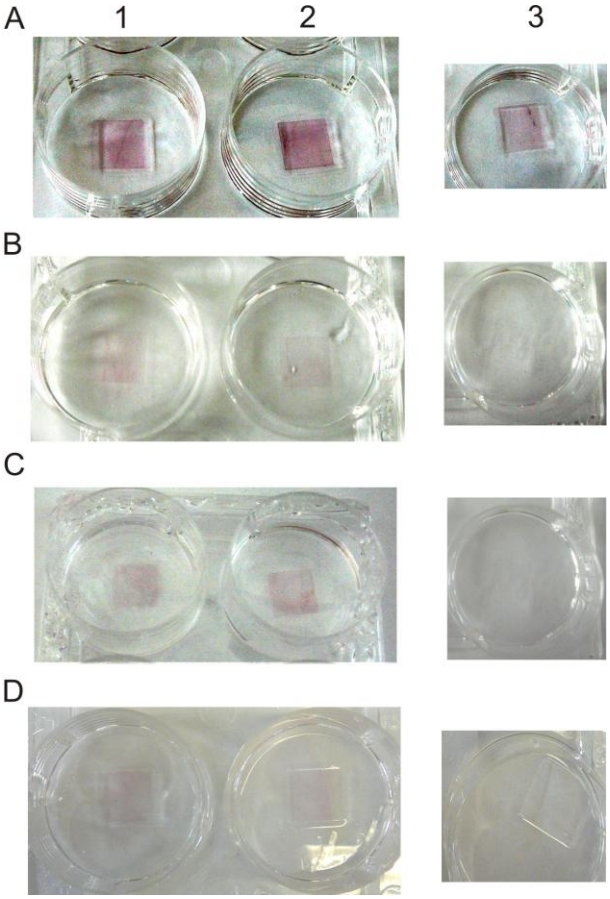


Figure 6. Long time testing for reloadability of carriers using CLISA for the detection of the biorecognitive interaction between immobilized immune sera 1: rabbit 52, 2: rabbit 53, 3: negative control) on carriers containing bound PLGA-NPs (A). B: After 2 months of incubation in Ringers solution at 37°C. C: After reloading of PLGA NPs. D: After one year of incubation the color remained constant even after repeated loading with PLGA NPs. Negative control (3) omitting immobilization of immune serum showed a faint color signal (A) and this color disappears after one day incubation.

Moreover, the degradation time assay was carried out using different amino-PEGylated materials [stainless-steel L316 and ceramics (ZrO_2)] and compared with glass. The color intensity for ceramics and glass was similar and therefore only glass carriers were incubated in Ringer solution for one year as described before. The amino-PEGylation was carried out at pH 5 and 10. Both pH conditions showed positive signals (Figure 7). The negative controls showed a faint color reaction as was already discussed before.

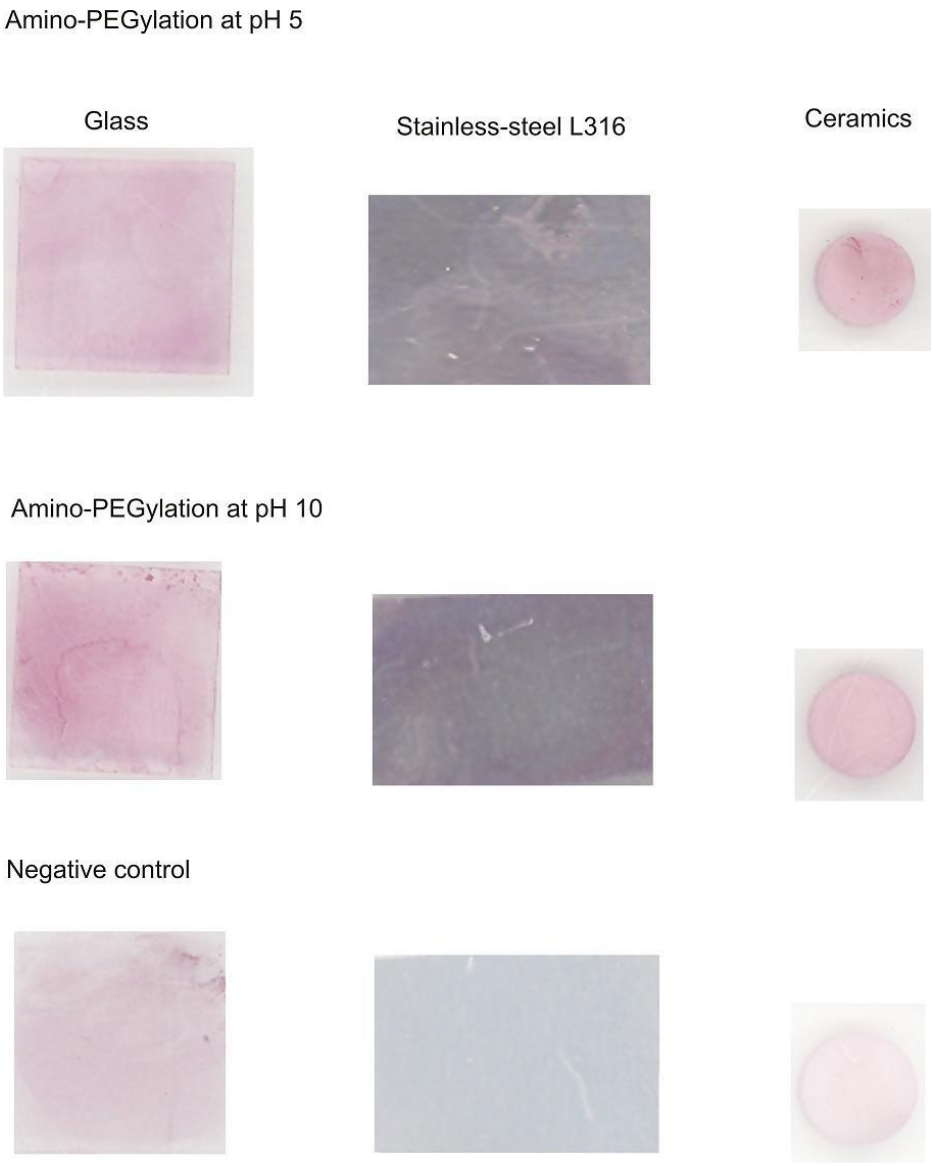


Figure 7. CLISA on different amino-PEGylated carriers loaded with PLGA NPs shows a positive signal and negative controls without PLGA NPs show a much fainter color.

Purification of immune serum

In this section, some of the issues that proved difficult to resolve and impacted on the proof of principle for using PLGA Abs as capture molecules for PLGA NPs will be discussed. CLISA showed a positive interaction between PLGA and GCC-labeled with immune sera of rabbits 52 and 53. In this section CLISA was carried out using GCC labeled with preimmune serum of rabbits that should have served as a negative control to compare with immune sera. Surprisingly, even the preimmune serum either fresh, or old (stored for one year at -20°C) showed a positive interaction with PLGA dotted onto a microtiter plate (Figure 8). Thus, the question arose as to which protein

is present regularly in the serum of rabbits and binds to PLGA. One of the possible candidates was L-Lactate dehydrogenase (LDH). LDH is an ubiquitous enzyme in all vertebrate species and catalyzes the final reaction step of glycolysis, the formation of L-(+)-lactic acid. The enzyme activity that is found in serum or plasma usually originates from the liver, the heart, and skeletal muscle, and erythrocytes as well as from tumors (Kopperschläger G. et al. 1996). Haemolysis may increase lactate dehydrogenase-1 in the serum (Leung F. et al. 1981). Therefore, to assess whether LDH could be a possible, ubiquitous unspecific binding protein, LDH from different species (pork, cow and rabbit) was also labeled with GCC solution and incubated in microtiterplates dotted with PLGA. CLISA tests showed no interaction between PLGA and LDH (Figure 8). Therefore, LDH can be ruled out as the protein binding unspecifically to PLGA.

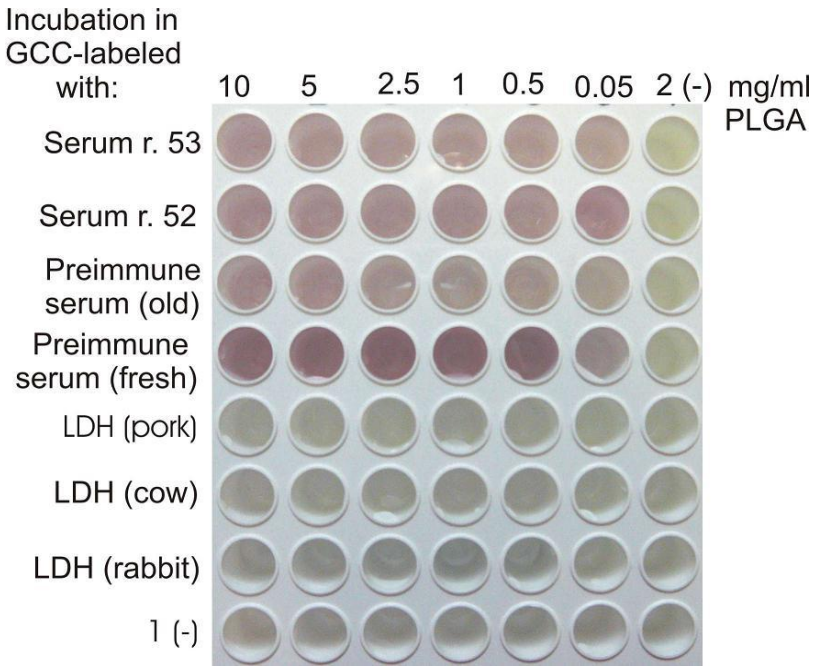


Figure 8. CLISA of preimmune sera (fresh, old) and immune-sera of rabbit (r.) 52 and 53. All sera showed a positive interaction between sera and PLGA dotted onto the microtiterplate. The highest color intensity was observed in fresh preimmune sera. No color signal was observed in LDH of different species and in the 1st and 2nd negative control (-). Negative control 1 shows the incubation of GCC solution containing Protein G and GCC blocking solution in the wells coated with different concentrations of PLGA. In negative control 2 the wells were blocked and washed then incubated with GCC-labeled with sera or LDH.

On the basis of these results, preimmune serum and immune-serum was purified by means of affinity chromatography using PLGA loaded columns to identify which protein in serum interacts with PLGA. This protein could then be used as a specific binding site for PLGA-NPs instead of PLGA-Abs. For this purpose, PLGA was immobilized onto amino-silanized Gulsenit. The efficiency of immobilization was determined with the Kaiser-test (Figure 9) described in Chapter 1.

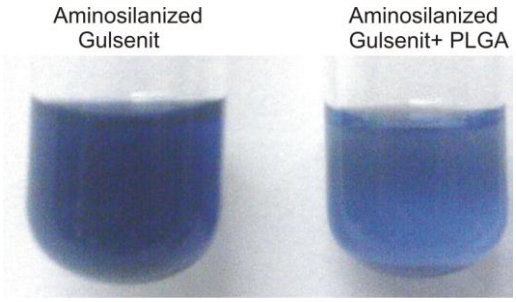


Figure 9. Kaiser test of amino-silanized Gulsenit before and after immobilization of PLGA. The lighter blue colour indicates the lower amount of free amino groups in amino-silanized Gulsenit after coupling of PLGA.

Table 1 shows that the concentration of purified protein using Gulsenit coupled with PLGA (affinity chromatography columns material) from preimmune serum is lower than that of immune serum of rabbit 52 and 53 measured by Qubit™ Fluorometer. These proteins were analyzed by native gel electrophoresis (Figure 10) and western blot (Figure 11).

Sample	Method	Protein concentration µg/ml
Rabbit's preimmune serum	Affinity chromatography Gulsenit	15.1
Immune serum of rabbit 52	Affinity chromatography Gulsenit	18.5
Immune serum of rabbit 53	Affinity chromatography Gulsenit	17.2
Rabbit's preimmune serum	Dynabeads® M-280 Sheep anti-Rabbit	16.88
Immune serum of rabbit 52	Dynabeads® M-280 Sheep anti-Rabbit	17.3
Immune serum of rabbit 53	Dynabeads® M-280 Sheep anti-Rabbit	16.5
Preimmune serum of rabbit	Protein A	13.6
Immune serum of rabbit 52	Protein A	15.6
Immune serum of rabbit 53	Protein A	14.5

Table 1. Concentration of proteins isolated from preimmune serum and immune serum using different methods and measured by means of a Qubit™ Fluorometer.

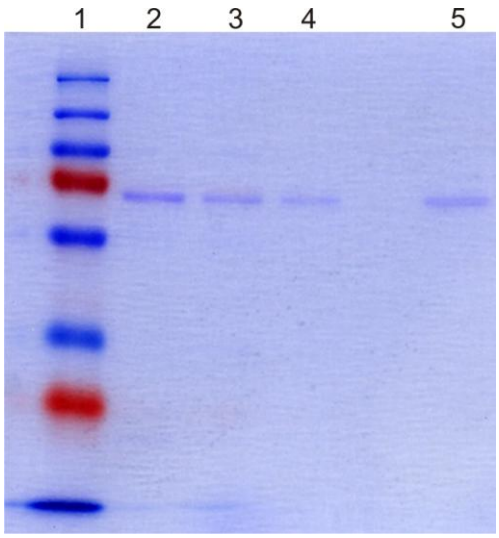
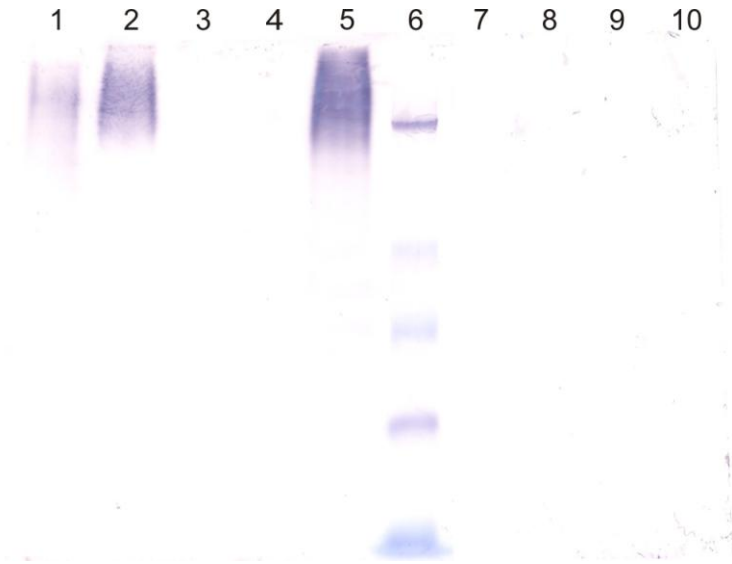


Figure 10. Native gel after CBB-stain of purified sera using affinity chromatography. 1: protein ladder Rage Ruler TM, 2: human serum, 3: immune serum of rabbit 53, 4: rabbit preimmune serum 5; immune serum of rabbit 52.

Even the isolation of pure IgGs from immune sera, either using Dynabeads® M-280 Sheep anti-Rabbit, or Protein A columns, confirmed that the preimmune serum contained a lower concentration of IgGs than the immune sera. After affinity chromatography of the pure IgGs, no more protein could be detected using Qubit™ Fluorometer measurement. These results were also confirmed by analysis of isolated IgGs from all sera after native gel electrophoresis using ELISA and AP detection; a black smear was observed (Figure 11: lanes 1, 2, 5) which referees to the presence of different kinds of IgGs in the sample. However no signals were observed after affinity chromatography (using Dynabeads® M-450 Epoxy with coupled PLGA) of the isolated IgGs (Figure 11). These experiments confirm that the immune sera of rabbits 52 and 53 do not contain PLGA IgGs.



1	purified IgGs from preimmune serum isolated with Dynabeads® M-280 Sheep anti-Rabbit
2	purified IgGs of preimmune serum isolated via protein A followed by affinity chromatography using Gulsenit with PLGA isolated via Dynabeads® M-280 Sheep anti-Rabbit
3	1 after affinity chromatography using Dynabeads® M-450 Epoxy with coupled PLGA
4	2 after affinity chromatography using Dynabeads® M-450 Epoxy with coupled PLGA
5	purified IgGs of rabbit 52 of serum immune isolated with Dynabeads® M-280 Sheep anti-Rabbit
6	protein ladder Page Ruler TM
7	5 after affinity chromatography using Dynabeads® M-450 Epoxy with coupled PLGA
8	purified IgGs of preimmune serum isolated via protein A and after affinity chromatography using Gulsenit with PLGA
9	purified IgGs of rabbit 53 isolated via protein A and after affinity chromatography using Gulsenit with PLGA
10	purified IgGs of rabbit 52 isolated via protein A and after affinity chromatography using Gulsenit with PLGA

Figure 11. Detection of purified proteins from preimmune and immune serum after native gel electrophoresis by means of ELISA and AP. The black smear in lanes 1 and 2 shows the presence of different kinds of IgGs in the sample and empty lanes demonstrate that the immune serum of rabbit 52 and 53 do not contain PLGA IgGs.

The purified proteins from preimmune- and immune-sera, by means of affinity chromatography using PLGA loaded columns, were analyzed by native gel electrophoresis. As shown in Figure 10, a single band is obtained. This band was

analyzed by means of tryptic digestion and peptide mapping using mass spectrometry, revealing a sequence that bears strong homology to albumins (Appendix section 2.1, Figure 3), indicating that albumins in rabbit serum interact with PLGA. To show whether this interaction is specific to rabbit albumins or also occurs in human albumins, human serum was purified analogous to rabbit serum and then analyzed by native gel electrophoresis. The results demonstrated that human serum albumin interacts as well with PLGA (Figure 10).

Considering the results from CLISA, affinity chromatography, native gel and finally mass spectrometry, it is very likely that the color reaction observed in the degradation time assay may also be caused by an unspecific interaction between rabbit albumin immobilized to the implant surface and PLGA NPs. Due to this unspecific interaction, it is recommended for purification of PLGA Abs from immune serum that IgGs should be isolated first and afterwards affinity chromatography can be carried out as described previously. These results show the problems that may arise in principle when PLGA NPs are intended for use as a drug targeting system: Serum albumins will bind to the PLGA surface thus lowering the chance of site specific targeting!
Futhermore, this finding suggests that the surface of PLGA-NPs needs to be coated adequately to prevent binding of serum albumin onto PLGA before injecting the particles into the human body. The coating of PLGA-NPs could be achieved by using biocompatible “non fouling” biodegradable polymers to prevent accumulation of the coating material in the body if frequent injections are necessary and also to allow for reloading. The results presented thus far in this Chapter, as well as the results obtained by ELISA (Chapter 6), demonstratethat the immunisation of rabbit 52 and 53 was not efficient. This led us to change the immunization strategy (see Chapter 6) and search for other targeting systems using biocompatible polymers, such as chitosan (see Chapter 7).

Experimental Methods

Preparation of PLGA-nanoparticles

Nanoparticles (NPs) were produced using the water-in-oil-in-water (w/o/w) solvent–evaporation technique according to Weissenböck et al., 2004. PLGA (Resomer RG 503H, 400 mg, Boehringer Ingelheim) was dissolved in 2 g ethyl acetate under stirring at 4°C. This PLGA solution was emulsified with 400 µl dd. H_2O (or solutions of

drugs, enzymes, etc.) by sonication for 60 sec (Sonifier Bandelin electronic UW 70/HD 70; tip, MS 72/D, Berlin, Germany) under cooling in a water bath. 6 ml of 10% Pluronic F-68 solution was added rapidly and the emulsion was sonicated again for 50 sec under cooling to yield the (w/o)/w emulsion. The (w/o)/w emulsion was rapidly poured into 1% Pluronic F-68 (Sigma Aldrich) solution. After stirring (600 rpm) for 1 hour at room temperature the residual ethyl acetate was removed under reduced pressure (100 mbar) and rotation (170 rpm) for 30 min and finally for further 30 min under high vacuum. The resulting NPs were filtered (Prefilter 1µm glass fiber, Millipore) to eliminate aggregates. The mean particle size and distribution were determined by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Instruments Ltd, U.K.) in triplicate at 20°C. The NPs suspension was stored at -80°C until use. To measure PLGA concentration in a NP suspension and for other applications, as much Pluronic as possible should be removed from the NP suspension using diafiltration as following. Therefore, 20 ml of the PLGA NP suspension was carefully washed (7 times) with 40 ml dd. H_2O on a tangential flow filtration system, using an ultrafiltration membrane (Vivaflow 50; 100000 MWCO PES, Sartorius Vivascience G.m.b.H). The tangential flow was generated by a peristaltic pump (MV-CA 8, Ismatec, Glattbrugg) at a system pressure of 2.5 bar. As the washed NP suspension is less stable when most of Pluronic is removed, it was used immediately. The concentration of PLGA in NPs suspension was determined after lyophilisation of 1 ml washed NPs yielding 2.86 mg/ml.

The average size of the used PLGA-NPs was 130 nm and PdI was 0.162 and the concentration of PLGA used was determined by the weight after lyophilisation and found to be 2.86 mg/ml.

Detection of PLGA antibodies in sera of rabbit 52 and 53

Success of pc PLGA-Ab production was quantified using GCC-labeling as well as ELISA for the sera of rabbits 52 and 53 (see Chapter 6). For this purpose several dilution series of PLGA (RG 503 H) in DMSO were made in a concentration range between 10 to 0.005 mg/ml. In contrast to other organic solvents for PLGA, DMSO doesn’t damage the polystyrene surface material of the microtiterplate. The dotted PLGA samples were incubated with GCC-labeled sera, either serum after immunization (immune-serum) of rabbit no. 52 or 53. Different amounts (from 10 to

0.5 µl) of the sera were added to 10 ml GCC solution. Each dilution of GCC, labeled with immune-serum was incubated in the serial dotted dilutions of either PLGA or Pluronic respectively to test also for the formation of antibodies against Pluronic.

During the synthesis of PLGA nanoparticles, Pluronic is added to stabilize nanoparticles. Therefore, it had to be determined whether the immune sera of rabbits 52 and 53 included Pluronic Abs. Pluronic samples were treated analogous to PLGA samples.

The wells of the microtiterplate (MicroliteTM 2, DYNATECH) were filled with 60 µl sample per well [either PLGA dissolved in DMSO containing 1% (v/v) glycerol, to keep PLGA samples hydrated during drying at room temperature, or Pluronic diluted in dd. H_2O] for one hour at room temperature. The serial dilutions of either PLGA or Pluronic were dotted separately into the wells for incubation in each GCC-labeled solution with either immune-serum of rabbit 52 or rabbit 53. As a negative control, one serial dilution was incubated in 350 µl per well GCC solution including Protein G, GCC-blocking solution and omitting immune sera of rabbit 52 or 53 [10 ml GCC solution containing 10 µl (1mg/ml) Protein G and 1 ml GCC-blocking solution]. The microtiterplate was emptied by shake off and blocking solution [50 mM Tris/HCl pH 7.3 containing 2% (w/v) fish gelatine, 0.1% (v/v) Tween 20] was added to the wells for 20 min then washed 3 times for 5 min in washing solution [50 mM Tris/HCl pH 7.3 containing 0.5% (v/v) Tween 20] under shaking, followed by rinsing 2 times in dd. H_2O. Then eleven GCC-labeled solutions with immune-serum were prepared identically, however each solution included a different amount of immune serum (from 10 µl to 0.5 µl). 10 ml GCC solution was stirred with 10 µl Protein G (1 mg/ml) for 20 min at room temperature. Thereafter, 10 µl of either immune-serum of rabbits 52 or 53 was added respectively under stirring for further 30 min at room temperature, followed by adding of 1ml GCC-blocking solution under continuous stirring for another 30 min. The samples on the microtiterplate were incubated in 350 µl per well of either GCC-labeled immune-serum solution of rabbit 52 or 53, GCC-labeled preimmune serum, or in GCC-labeled LDH solution (see below) separately for 2 hours at room temperature under shaking. The samples on the microtiterplate were washed 3 times in washing solution and then 3 times rinsed in dd. H_2O. The detection of a positive signal (red color of gold clusters) was observed with the naked eye.

Preparation of GCC-labeled LDH (Lactate Dehydrogenase) solution

Three portions of GCC solution (10 ml each) were stirred with 10 µl Protein G (1 mg/ml) for 20 min at room temperature. Thereafter to each solution 5 µl of a solution of 2.5 mg/ml LDH of pork or cow or rabbit respectively diluted in dd. H_2O were added at room temperature under stirring for another 30 min followed by addition of 1ml GCC-blocking solution under continuous stirring for another 30 min.

Preparation of GCC-labeled preimmune serum

10 ml GCC solution was stirred with 10 µl Protein G (1 mg/ml) for 20 min at room temperature. Thereafter, 10 µl of either preimmune serum or immune-serum of rabbits 52 or 53 was added respectively under stirring for further 30 min at room temperature, followed by adding of 1ml GCC-blocking solution under continuous stirring for another 30 min.

Degradation time assay for the degradation of PLGA-nanoparticles and reloading

Antibodies (Abs) in immune sera (of rabbit 52 and 53) were cleaved by means of 2-MEA and then immobilized onto the surfaces of amino-silanized or amino-PEGylated materials (glass, stainless steel 316 L and ceramic). These carriers were then blocked, washed, and incubated in a solution of washed PLGA-NPs. The carriers were washed and then incubated in GCC-labeled immune serum of rabbit 52 or 53.

Procedure:

Organic amino-silanization and amino-PEGylation by means of EDC/Sulfo-NHS of carriers are described in Chapter 1. Abs in immune-sera of rabbit 52 or 53 were cleaved by means of 2-MEA (see below). These cleaved Abs were immobilized onto carriers, activated with sulfo-SMCC (see below). This step was omitted for negative control carriers. PLGA-NPs were preparated (with a size of 151 nm) and washed as described in Chapter 6. The carriers were incubated in PLGA NP suspension (2ml/ carrier) overnight at 4°C on an orbital shaker under gentle shaking. GCC-labeled immune serum of rabbit 52 or 53 was preparated by stirring of 10 ml GCC-stock solution with 10 µl immune-serum of either rabbits 52 or 53 for 30 min at room temperature respectively, then 1 ml of GCC-block solution was added under continuous stirring for another 30 min). The carriers were rinsed in dd. H_2O, then

blocked and washed as mentioned by immobilization of cleaved antibody fragments onto carrier activated with sulfo-SMCC (see below). The carriers were incubated in GCC-labeled immune serum (2 ml/ carrier) for two days at 4°C under gentle shaking on an orbital shaker, then washed as mentioned before. For the degradation time test carriers (amino-silanized glass) were incubated for one year in Ringer's solution (2ml/ carrier) under gentle shaking at 37°C in a water bath. Ringer's solution was replaced repeatedly. These carriers were loaded again with washed PLGA NPs and binding was detected with the GCC labeling method, as described above, after two months incubation in Ringer's solution and again after one year.

Cleavage of disulfide bonds in the hinge region of the antibodies with 2-mercaptoethanolamine

2-mercaptoethanolamine can be used to cleave disulfide in the hinge region of IgG (Yoshitake S. et al. 1979). The most critical aspects are the concentrations of 2-mercaptoethanolamine (2-MEA) and EDTA in the reaction mixture. An optimal reduction of IgG takes place in 50-100 mM 2-MEA and 1-100 mM EDTA. The pH of the reaction may vary from pH 6 to 9, with an optimum of pH 8. The concentration of Ab can vary too. This method was used to cleave IgG in immune sera of rabbits 52 or 53.

Procedure:

6 µl immune serum was diluted in 0.1M PBS with 0.15 M NaCl, pH 7.2 containing 3 µl 10 mM EDTA and 3 µl 1.5 NaCl. 5 mg of 2-MEA was dissolved in 10 µl H_2O, 3 µl was added to immune serum solution and incubated for 90 min at 37°C. The cleaved IgG in serum was separated from the reducing agent by means of gel filtration using Sephadex G-25. Use 0.1M PB, 0.15 M NaCl, pH 7.2 containing 10 mM EDTA as chromatography buffer. 300 µl fractions were collected and selected for free –SH groups of the Fab´ fragments with the Ellman's test. The fractions with positive Ellman's test containing reduced Ab were pooled and immediately used for immobilization onto sulfo-SMCC activated amino-silanized or amino-PEGylated carriers (see below).

Activation of amino-silanized or amino-PEGylated carriers with sulfo-SMCC to immobilize cleaved Abs

The amino-PEGylated (NH_2-PEG-NH_2) or amino-silanized carriers (4x 12 mm glass slides) were washed with buffer (0.1 M PBS with 0.15 M NaCl, pH 7.2). 4 mg sulfo-SMCC was dissolved in 1 ml dd. H_2O and then filled to 11 ml with washing buffer. The carriers were incubated for 30 min in this solution under gentle shaking. The carriers were washed 3 times with buffer for 5 min under gentle shaking. The activated carriers can be stored freeze-dried for at least one year without losing maleimide activity.

Immobilization of cleaved antibody fragments onto carrier activated with sulfo-SMCC

400 µl of cleaved Abs in immune sera of rabbit 52 or 53 using 2-MEA was pipetted onto the carriers activated with sulfo-SMCC. The carriers were incubated for 2 days under gentle shaking at 4°C in a humid chamber. The carriers were washed 3 times for 5 min with washing solution under gentle shaking. Washing solution contained 0.5% (v/v) Tween 20 and 0.1 M PB, 0.15 M NaCl, pH 7.3. The carriers were blocked for 30 min with blocking solution [2% (w/v) gelatine from cold water fish skin dissolved in 0.1 M PB, 0.15 M NaCl, pH 7.4 and 0.1% (v/v) Tween 20] under gentle shaking. The carrier was washed 2 times for 5 min in washing solution under gentle shaking. Now the carriers were for incubation with PLGA-NPs.

Purification of IgGs from rabbit 52 and 53 serum using Dynabeads® M-280 Sheep anti-Rabbit IgG

All IgGs in sera of rabbit no. 52 and 53 were isolated as described in the following.

Washing procedure of Dynabeads® M-280 Sheep anti-Rabbit

The Dynabeads® M-280 Sheep anti-Rabbit were resuspended thoroughly in the vial by vortexing and shaking. 1 ml of resuspended beads was transferred into a 2 ml Eppendorf vial. The vial was placed onto the magnet for 2 min and the supernatant removed by pipetting, carefully avoiding to touch the inside wall of the tube (where the beads attract to the magnet) with the pipette tip. The vial was removed from the magnet and the pellet was resuspended in 1 ml wash buffer (PBS buffer pH 7.4 (washing buffer) containing 0.16 g $NaH_2PO_4.H_2O$, 0.98 g $Na_2HPO_4.2H_2O$, 8.10 g

NaCl was filled with dd. H_2O to 1L). The vial was placed onto the magnet for 2 min and the pellet again resuspended in 1 ml wash buffer.

Separation procedure of target IgG

The pellet of washed beads was resuspended in 1 ml diluted serum of rabbit no. 52 or 53 respectively (300 µl of serum was made up to 1 ml with PBS pH 7.4 and centrifuged briefly at 14.000 rpm) and incubated under slow tilted rotation mixing over night at 4°C. The Eppendorf vial was placed for 2 min on the magnet and the supernatant pipetted off. The Eppendorf vial was removed from the magnet and the pellet resuspended in 1 ml washing buffer. This wash step of the pellet in 1 ml washing buffer was repeated twice. The bound and purified IgGs were now ready to be eluted off the beads.

Elution procedure of isolated IgGs

300 µl 0.1 M citrate buffer pH 2.5 (elution buffer) was added to the beads with immobilized IgGs and mixed well by tilting and rotation for 2 min At pH 3.1 most of the IgGs are expected to be eluted. 3 times consecutively, the Eppendorf vial was placed on the magnet and the supernatant containing purified IgGs transferred to a clean and autoclaved Eppendorf. The pH was immediately neutralised with saturated sodium phosphate (Na_3PO_4) buffer pH 12.7. The pellet was resuspended again in 300 µl 0.1 M citrate buffer pH 2.5 to elute any remaining IgGs and mixed well by tilting and rotation for 2 min.

For a qualitative determination of the protein content Bradford protein assay chemistry was used. 20 µl Bradford reagent (Bio-Rad Protein Assay) diluted 1:5 with dd. H_2O were added to 2 µl of supernatant and the color changed to blue. Then the supernatants of the first and second eluate containing pure IgGs were pooled. The protein concentration was measured using a Qubit Fluorometer (Invitrogen). Subsequently PLGA-Abs were isolated from the pure IgGs via affinity chromatography, using immobilized PLGA onto either Dynabeads® M-450 Epoxy or Gulsenit. The concentration of pure IgGs was determined with the Quant-iT™ Protein Assay Kit (for use with the Qubit™ fluorometer, Invitrogen as described below).

For reuse of the beads their pH was immediately neutralized using a Na-phosphate buffer. For storage, the beads were re-suspended in PBS/BSA buffer (isotonic PBS

buffer pH 7.4 containing 0.1 % BSA and 0.02% sodium azide (NaN_3) as preservative for storage).

Purification of serum from rabbit 52 and 53 using Protein A Antibody Purification Kit

The Protein A Antibody Purification kit provides all reagents necessary for the isolation of IgGs. According to the product information, the Protein A cartridge can bind approximately 5 mg/ml IgG content from 2 ml of human serum.

Serum purification

1 ml binding buffer was added to 500 µl of serum (serum-Binding buffer) and mixed well. A 10 ml syringe was filled with 10 ml HEPES buffer and connected to the top (male connector) of the desalting cartridge. The cartridge was regenerated by passing the buffer through the cartridge at an approximate flow rate of 1 ml/min. A 5 ml syringe was filled with 5 ml Regeneration buffer and connected to the top (male connector) of the Protein A cartridge. The Cartridge was regenerated by passing the buffer though the cartridge at an approximate flow rate of 1 ml/min. A 10 ml syringe was filled with 4 ml Binding buffer. The Protein A cartridge was equilibrated by passing the buffer through the cartridge at an approximate flow rate of 1 ml/min. A 10 ml syringe was filled with serum-Binding buffer mixture. The Protein A cartridge was loaded by passing the serum-Binding buffer mixture through the cartridge at an approximate flow rate of 0.5 ml/min. After the sample was loaded onto the Protein A cartridge, the 10 ml syringe was rinsed with 1 ml H_2O and refilled it with 6 ml Binding buffer and the Protein A cartridge washed by passing the buffer through the cartridge at an approximate flow rate of 1 ml/min. The female end of the Protein A cartridge was attached to the male Luer lock fitting to the Desalting cartridge. The 5 ml syringe was filled with 1.5 ml Elution buffer and attached to the Protein A cartridge. The cartridge was eluted by passing the Elution buffer through the cartridge at an approximate flow rat of 0.5 ml/min. The purified IgGs in the eluate show a neutral pH.
For a qualitative determination of the proteins by means of the Bradford protein assay 20 µl Bradford reagent (Bio-Rad Protein Assay) were diluted 1:5 with dd. H_2O and added to 2 µl of elute and the color changed to blue. The concentration of eluate was determined with the Quant-iT™ Protein Assay Kit (for use with the Qubit™ fluorometer, Invitrogen, as described below). The two cartridges (Protein A cartridge

and Desalting cartridge were detached and regenerated by passing 5 ml Regeneration buffer through the Protein A cartridge and 10 ml HEPES buffer through the Desalting cartridge. Regeneration was proven by a negative Bradford protein assay.

Immobilization of PLGA onto Dynabeads® M-450 Epoxy for affinity chromatography

Washing procedure

The Dynabeads® M-450 Epoxy were resuspended thoroughly by vortexing and shaking. 1 ml of resuspended beads was transferred into a glass tube. The tube was placed on the magnet for 2 min and the supernatant discarded. The vial was removed from the magnet and the pellet resuspended in 1 ml dd. H_2O. Step 3 was repeated and the pellet resuspended again in 1 ml dd. H_2O.

PLGA coupling procedure to D*ynabeads® M-450 Epoxy*

The pellet of washed beads was resuspended in 5 ml of 500 mg PLGA (Resomer RG 502H) dissolved in 2 ml DMSO. The incubation was carried out over night at room temperature under slow rotation. The tube was placed in the magnet and the supernatant was discarded. The pellet was mixed and incubated with 1 ml DMSO for 5 min under rotation to remove uncoupled PLGA. The tube was placed in the magnet and the supernatant was discarded. 3 times consecutively, the pellet was washed again with 1 ml isotonic PBS buffer pH 7.4 and incubated for 5 min under rotation. The tube was placed in the magnet and the supernatant was discarded. The third time the beads were rotated with isotonic PBS buffer pH 7.4 for 10 min.

Affinity chromatography using Dynabeads® M-450 Epoxy with coupled PLGA

Separation procedure of PLGA-Ab

PLGA-Abs were isolated from the pure IgGs of immune sera (rabbit 52, 53) purified with either the Dynabeads® M-280 Sheep anti-Rabbit kit or the Protein A Antibody Purification Kit by means of PLGA coupled to Dynabeads® M-450 Epoxy.

The pellet of washed beads was resuspended in 600 µl of pure IgGs of immune sera (rabbit no. 52 or 53 respectively) and incubated under slow tilt rotation mixing over night at 4°C. The remaining protocol was carried out analogous to the protocol described before in separation procedure of target IgG.

Elution procedure of PLGA-Ab

The elution was carried out analogous to the protocol for the elution of isolated IgGs as described before.

Immobilization of PLGA onto Gulsenit

1 g PLGA (Resomer RG 502H) was dissolved in 20 ml ethyl acetate. NHS (575 µg) was dissolved in 40 ml ethyl acetate. N,N'-Diisopropylcarbodiimide (DIC, 310 µl) was added to the dissolved NHS and this solution immediately pipetted into the dissolved PLGA. Mixing and reaction for one hour was performed at room temperature. The activated PLGA solution was incubated in 20 g amino-silanized Gulsenit (Chapter 1) overnight at room temperature under stirring. The Gulsenit was rinsed 3 times with ethyl acetate to remove unreacted PLGA followed by 3 times with dd. H_2O; using a suction funnel, pore size 4, to remove solvent from carrier after each rinse. The Gulsenit can then be used as a column material for the purification of PLGA-Ab by means of affinity chromatography. The efficiency of immobilization was determined with the Kaiser-test (Chapter 1).

Affinity chromatography using Gulsenit with coupled PLGA

A 2.5 ml chromatography column with a 10 µm pore filter at the bottom (Mo Bi Tec) was filled with PLGA conjugated Gulsenit and washed with 5 column volumes of binding buffer (5 mM PBS, 0.15 M NaCl, pH 7.2). The diluted serum (1:5 with binding buffer) was centrifuged shortly at 14000 rpm and then loaded onto the PLGA conjugated Gulsenit column at a flow rate of 0.15 ml/min. The column was washed with binding buffer until no proteins were detected by Bradford protein assay. 20 µl Bradford reagent (Bio-Rad Protein Assay) diluted 1:5 with dd. H_2O were added to 10 µl of each fraction and blue color indicated the presence of proteins. The PLGA-Abs were eluted with elution buffer (0.1 M acetic acid/ 0.1 M NaOAc, pH 3) at a flow rate of 1.7 ml/min. Every eluted fraction (two drops) was collected in a well of a microtiterplate containing about 10 µl neutralization buffer (0.5 M $NaHCO_3$) to neutralize the purified Abs immediately. The fractions containing protein (detected by Bradford protein assay) were pooled and the pH was adjusted to a physiological pH. For a qualitative determination of the proteins by means of the Bradford protein assay, 20 µl Bradford reagent (Bio-Rad Protein Assay) diluted 1:5 with dd. H_2O were added to 10 µl of each fraction and the color changed to blue. These fractions were

pooled. The sample was concentrated for 5 min by 4000 rpm at 4°C using an Amicon ultra centrifuge filter. The concentration of the eluate was determined using the Quant-iT™ Protein Assay Kit (for use with the Qubit™ fluorometer, Invitrogen as described below).

Quant-iT™ Protein Assay Kit (Invitrogen)

Three BSA standards (0 ng/µl, 200 ng/µl and 400 ng/µl in TE buffer with 2 mM sodium azide) were used for calibration of the Qubit™ Fluorometer. 10 µl of each standard was diluted in 190 µl Quant-iT™ working solution, prepared by diluting the Quant-iT™ protein reagent 1:200 in Quant-iT™ protein buffer and measured after 15 min incubation at room temperature.1-20 µl (dependent on the concentration of the sample) of sample were diluted in Quant-iT™ protein buffer to obtain finally 200 µl solution. The diluted samples were mixed well (avoiding air bubbles) and the protein concentration measured after 15 min incubation at room temperature with the Qubit™ Fluorometer. The concentration of the sample was calculated using the following equation:

$$Concentration\ of\ \ your\ sample = QF\ value \times \frac{200}{x}$$

QF value = the value given by the Qubit™ fluorometer and

x = microlitres of sample added to the assay tube.

Native gel and Western blot

Buffers and solutions

Stacking gel

The stacking gel solution contained 1 ml 30% acrylamide/ 0.8% Bisacrylamide ; 4.8 ml dd. H_2O; 2 ml 0.5 M Tris/ HCl pH 6.8; 80 µl 10% (w/v) APS and 8 µl TEMED.

Separating gel

The separating gel solution contained 6 ml 30% acrylamide/ 0.8% Bisacrylamide; 11.8 ml dd. H_2O; 6 ml 1.5 M Tris/ HCl pH 8.8 ; 200 µl 10% (w/v) APS and 20 µl TEMED.

Running buffer (pH 8.4)

The running buffer contained 3.0 g Tris; 14.4 g glycine and was filled to 1 L with dd. H_2O.

Sample preparation and electrophoresis

1 µl serum was diluted 1:40 in 50 mM Tris-HCl (pH 7.4) or running buffer and then 40 µl sample buffer pH 6.7 [2.5 ml 125 mM Tris/ HCl pH 8.4; 20% (v/v) glycerin (2 ml)]; 1 mg bromophenol blue and was made up to 10 ml with dd. H_2O was added. The gel slots were loaded with 20 µl of sample.

The separation of proteins was performed at 150 V and 60 mA for approximately 1 to 1.5 hours. After separation of the proteins by electrophoresis, they have to be either fixed in the gel with methanol/ acetic acid / water-solution resulting in denaturation and precipitation of the proteins or they can be blotted onto a nitrocellulose membrane (NC) by Western Blotting. Gel, NC-membrane and Whatman paper for blotting were treated with transfer buffer [Tris (5.8 g); glycine (2.9 g); dd. H_2O to 1 L, pH 6.5]. By semidry-blotting (NovaBlot, Pharmacia, Multiphor II SemiDry) separated proteins can be transferred from the gel to the NC-membrane at 10 V and 150 mA within 1.5 hours. The proteins adhere to the nitrocellulose membrane by hydrophobic interactions and can be detected on the membrane by AP (alkaline phosphatase) according to manufacturer's instructions.

Visualization of proteins after gel electrophoresis: CBB-staining

The separated proteins can be detected directly in the gel after PAGE by staining with Coomassie-Brilliant-Blue-R250 (CBB-R250). In acidic solution, CBB binds unspecifically to basic amino acids, primarily arginines, showing an absorbance shift from 465 nm to 595 nm. Gels were incubated in staining solution (1.25 g CBB-R250; 5 ml acetic acid; 15 ml methanol and 30 ml dd. H_2O) under shaking for 30 min and then in de-staining solution (25 ml acetic acid; 60 ml methanol filled to 200 ml with dd. H_2O) over night.

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Chapter 6

Production of polyclonal antibodies against the biocompatible polymer PLGA using silica amino microspheres as a carrier

One of the specific aims in this thesis was to **search and develop a biorecognitive reloadable binding system for drug delivery system e.g. nanoparticles.**

The first chosen drug delivery system was based on functionalized PLGA nanoparticles. These particles should be capable of binding to antibodies as molecular receptors embedded on the applied biocompatible coated implant material. In this Chapter the production of polyclonal antibodies against the biocompatible and biodegradable PLGA polymer under very stringent immunisation protocol is described.

This manuscript will be submitted for publication in Biotechnology Letters

Production of polyclonal antibodies against the biocompatible polymer PLGA using silica amino microspheres as a carrier

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Abstract

A very stringent immunisation protocol was developed in rabbits for the production of polyclonal antibodies against a biocompatible and biodegradable polymer, such as PLGA. To achieve this goal a number of technical problems regarding the purification of the antibodies and titer determination had to be solved. By direct purification of PLGA antibodies from sera of immunized rabbits, using affinity chromatography, a protein which interacts with PLGA was isolated. Analysis of this protein by means of native gel electrophoresis and mass spectrometry revealed that this protein has a homology to albumins. Therefore, we investigated the effect of human serum albumin on PLGA, and could prove its binding to PLGA nanoparticles. Together our results suggest that coating PLGA-NPs as a drug targeting system with biocompatible and biodegradable polymer before injecting them into the human body eliminates unspecific coating with serum albumin, which could lowering the chance of site specific targeting in our design.

Keywords

Biocompatible; Biodegradable; Antibody-titer; PLGA-antibody; PLGA-nanoparticle; Silica-amino- microspheres

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Introduction

PLGA (poly (D,L-lactide-co-glycolide) is a random copolymer of glycolic acid and lactic acid and is widely used for bioabsorbable sutures or drug delivery systems. The ratio of glycolic acid to lactic acid determines the rate of degradation, mostly by hydrolysis. Degradation of PLGA yields lactic acid and glycolic acid, both also products of the endogenous metabolism. A large number of *in vivo* and *in vitro* studies have shown the full biocompatibility and biodegradability of PLGA nanoparticles and their degradation product (Athanasiou K. et al. 1996; Anderson J. et al. 1997; Tracy MA. et al. 1999).

PLGA's versatile degradation kinetics, established safety and biocompatibility, makes it an ideal material for drug delivery applications. Moreover, PLGA nanoparticles not only modify the pharmacokinetics of encapsulated drugs, but they also protect the occluded drugs from enzymatic attack.

Currently, a number of FDA-approved products on the market utilize PLGA as excipients to achieve sustained release of the active ingredient, such as e.g. Neutropin Depot®, Sandostatin LAR® or Trelstar Depot®. PLGA nanoparticles are used also as a carrier for AI-700 that is used for stress echo cardiological evaluation.

The efficiency of PLGA nanoparticles as a local drug delivery system (for example dexamethasone) to decrease neointimal proliferation, without having significant systemic effects was already shown in the rat carotid injury model after ballon injury (Anderson J. et al. 1997).

Production of PLGA antibodies can have a benefit in clinical and research imaging. For example targeting of drugs or drug-delivery system to the site of interest drug containing nano-particles can be conjugated with specific antibodies against certain characteristic components of the tissue of interest. An example of such a passive targeting is the preferential accumulation of chemotherapeutic agents in solid tumours as a result of the enhanced vascular permeability of tumour tissues, compared with healthy tissue. For various therapeutic and diagnostic targeting purposes to PLGA-nanoparticles PLGA-antibodies are needed: A spacer molecule might be coupled on one end with ligands that are selectively recognized by receptors on the surface of the cells of interest and the other end of the spacer molecule then bound to PLGA antibodies which interact specifically with PLGA nanoparticles containing drugs for therapeutical purposes or visualisation dyes for

diagnostic purposes or, e.g. gadolinium for MRI (magnetic resonance imaging) or PET (positron emission tomography) live imaging. This could allow for a more precise targeting to the site of interest (for example in solid tumors) since ligand–receptor interactions can be highly selective.

The production of polyclonal antibodies (pAb) against a biocompatible and biodegradable polymer, such as PLGA (poly (D, L-lactide-co-glycolide), is still a challenge. Here, an approach using PLGA (a short chain polymer Resomer RG 502H) coupled to amino group containing SAM (silica-amino- microspheres, diameter 0.50-0.99 µm) as an antigen carrier for immunization is taken. These 100% solid microspheres do not contain latex, which has been shown to induce allergic reactions (Nutter AF. et al. 1979; Gelfand DW. et al. 1991).

Results and Discussion

Conjugation of PLGA to SAM

Conjugation of PLGA to amino group containing SAM was achieved using DIC chemistry. Prior to the conjugation reaction, the terminal carboxylate groups of PLGA were chemically activated by the addition of DIC (diisopropylcarbodiimide), a non-allergenic, N-substituted carbodiimide cross-linker to PLGA in ethyl acetate (Figure 1). N-substituted carbodiimides react with carboxylate groups to form highly reactive O-acylisourea intermediates. To enhance the coupling efficiency to the attacking amine, N-hydroxysuccinimide (NHS) was added (Williams A. and Ibrahim I.A. 1981) and the efficiency of PLGA immobilization to SAM was assessed by determining the amount of unreacted free amines using the Kaiser-Test (1970).

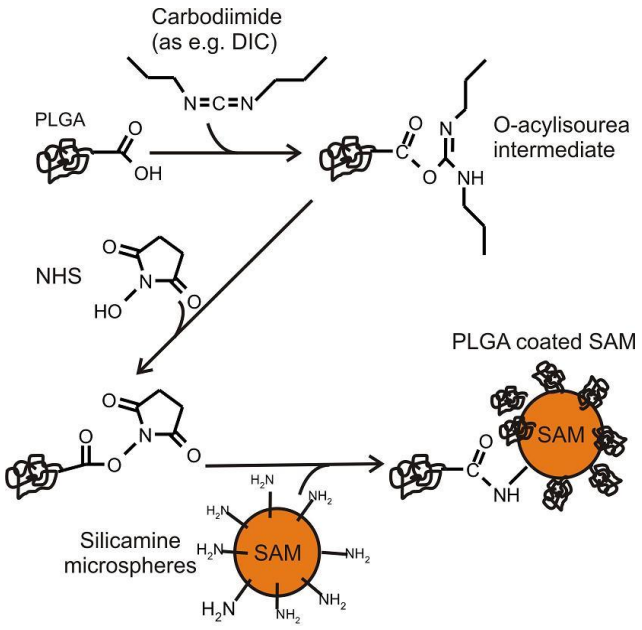


Figure 1. PLGA (poly (D, L-lactide-co-glycolide) binding to the amino groups of silica microbeads (SAM). The conjugated complex of PLGA and SAM is used as an antigen for immunization.

The injection sample contained SAM conjugated with PLGA polymer. The efficiency of PLGA immobilization to SAM was assessed by the Kaiser-Test (Chapter 1). Figure 2 shows the color reaction of free amino groups in sample that contains SAM before PLGA conjugation with ninhydrin inducing a pink color in contrast to SAM after PLGA conjugation.

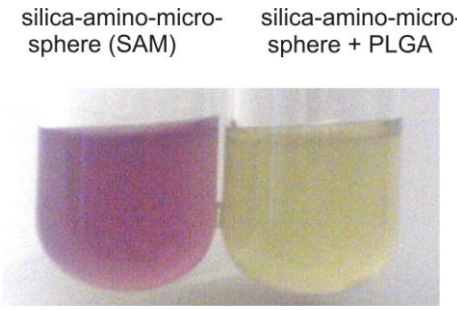


Figure 2. The Kaiser-Test shows a purple color after reaction with the free amino groups of SAM. After binding of PLGA onto SAM, no color reaction is observed.

Immunization and titer determination

Two rabbits (numbers 95 and 96) were immunized. The injection sample contained SAM conjugated with PLGA polymer. The conjugated complex of PLGA and SAM is used as an antigen for immunization. To enhance production of specific PLGA-pAbs, the amount of PLGA was increased with each immunization (Table 1 in experimental

methods). The injection of conjugated silica microspheres into New Zealand white rabbits (age 3 weeks) was carried out according to Mihee K. et al, 2007. Eight successive, sub-cutaneous immunization injections were carried out in total, with the exception of the 7th injection that was administered intravenously (Table 1 see experimental methods). Anti-PLGA antibody titer was determined by ELISA for each stage after the third immunization. The highest titer found was observed after the fourth immunization (Figure 3). No signal was detected with pre-immune serum (before immunization). Only injections with immune serum yielded a positive signal. Both rabbits no. 95 and 96 were immunized identically; however rabbit no. 96 showed higher PLGA-antibody titers than rabbit no. 95 (Figure 3, 4). This indicates successful immunization compared to the previous immunizations of rabbits 52 and 53 (see Appendix, section 3.1, Table 1 and Figure 5 F, G).

By determination of Anti-PLGA antibody titer using ELISA the concentrations of 25 and 12.5 µg /ml PLGA were too high for the small well size of the microtiterplate, because of multilayer formation. These layers bind the specific PLGA-antibodies, but each washing step peels off one layer. The signal intensity of positive interaction between antigen and captured antibody decreased dramatically after several washing steps. Therefore a concentration of 5 µg/ml was chosen to show specificity of the binding between antigen and PLGA antibody. The dotted 100 µl of sample contain 500 ng of PLGA completely covering the surface of a single well in the microtiterplate with a monolayer. The absorbance values and curves of serial diluted sera as a relative measure for the amount of captured antibodies are presented in Figure 4. The titer of the fourth immunization of rabbit 96 showed an endpoint titer at a dilution of 1:8000 of serum and an absorbance (ABS) of 0.07 (Figure 4 B). The dilutions of sera after the fifth immunization showed an ABS of 0.05 at 1:8000, and after the seventh and eighth immunization at 1:5000 (Appendix, section 3.1, Table 1, 2). Rabbit 95 produced a low titer after the fourth immunization with an ABS value of 0.01 at a 1:8000 dilution (Figure 4 G, Appendix, section 3.1, Table 3) and no titer at subsequent immunizations (Appendix, section 3.1, Table 1 and 3, Figure 5 B, C).

These results show that four immunizations are sufficient for the production of PLGA-Ab. We observed that the addition of PLGA powder to immobilized silica amino microspheres (SAM) and PLGA-NPs during the third and fourth immunization was essential for PLGA-Ab production, despite the difficulties that occurred when injecting the sample (see experimental methods for more details). To minimize these

problems during immunization in the future, the amount of PLGA powder can be reduced. However, intravenous injection of PLGA-NPs alone during the seventh immunization, and also the subcutaneous injection of PLGA conjugated to SAM only, did not increase the production of PLGA-Ab.

The analysis of pre-immune serum and immune serum of rabbit 96 are shown on silver stained native gels in the next section.

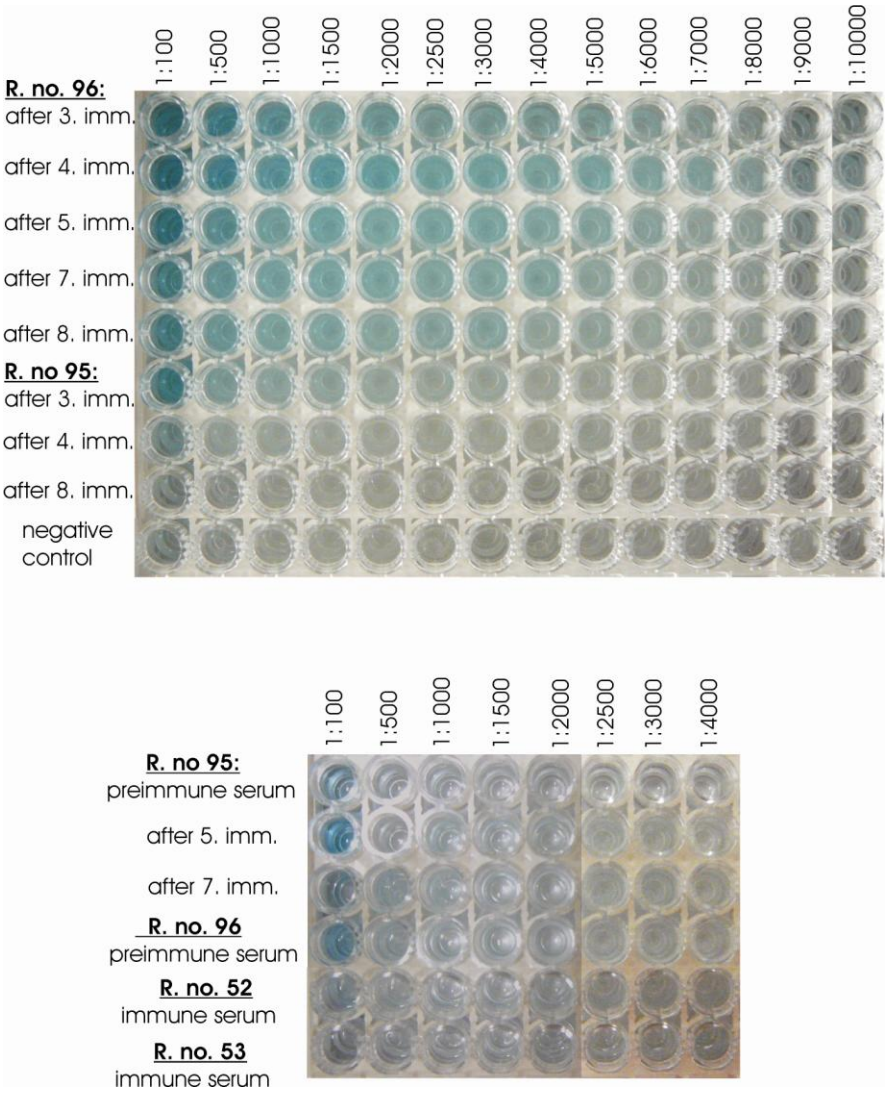


Figure 3. Titer determination of rabbit (R.) number (no.) 95, no. 96, no. 52 and no. 53 by ELISA. The highest titer is observed after the fourth immunization (imm.) of rabbit 96. Rabbit 96 showed higher PLGA-antibody titers after immunizations than rabbit 95 with a negative signal except after the 3rd and 4th Immunization, when a low positive signal was detected. Pre-immune sera of rabbit 95 and 96, immune sera of rabbit 52 and 53 and the negative control show no signal.

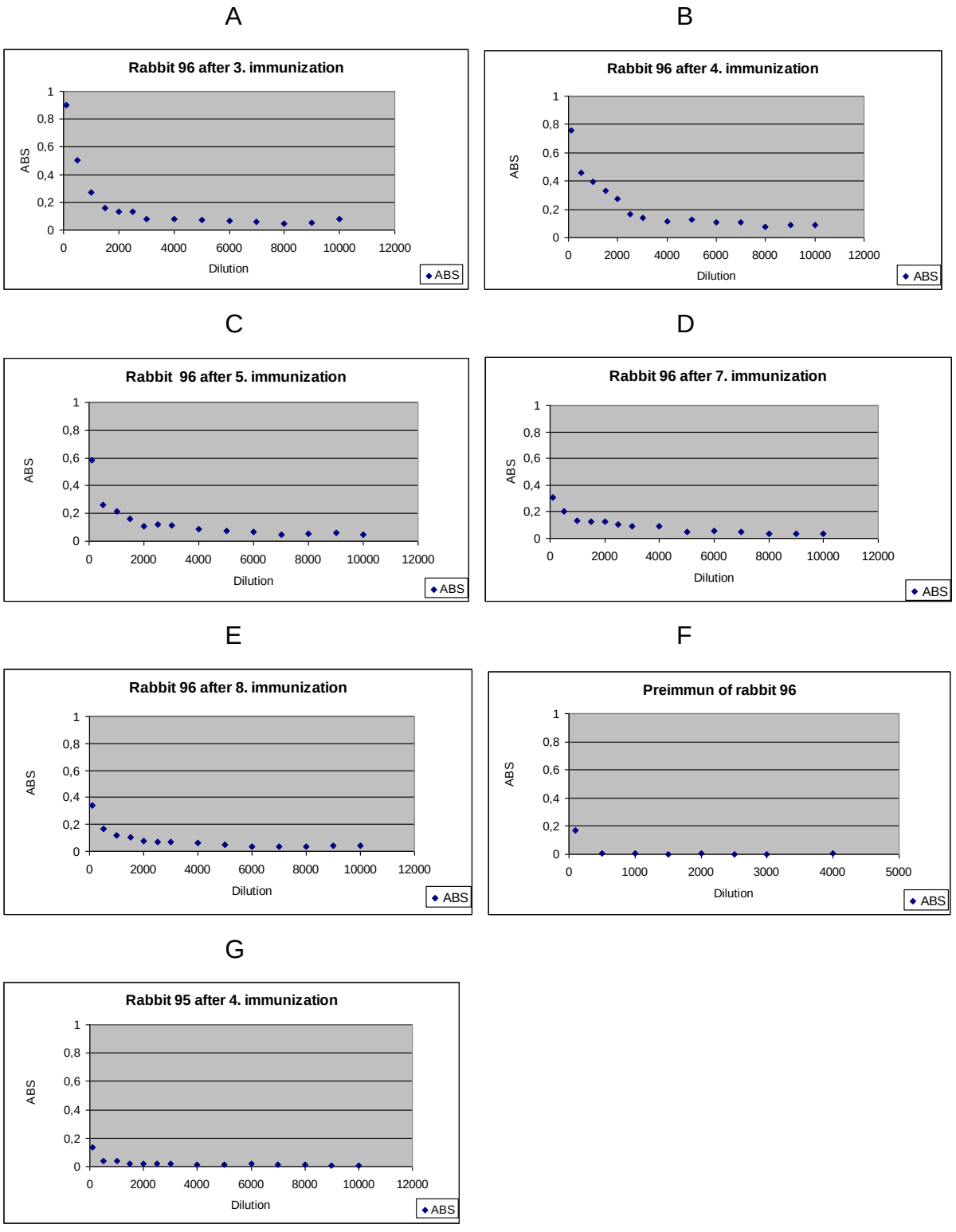
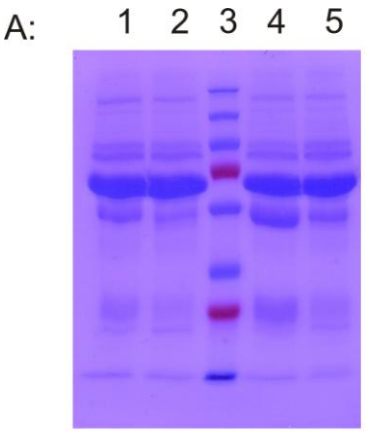


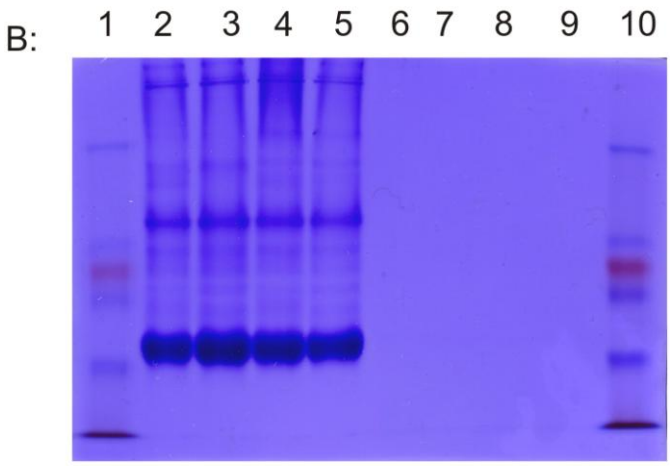
Figure 4. Absorbance curves of different dilutions of immune and pre-immune serum from rabbit no. 96. (Appendix section 3.1, Tables 1 and 2). The 4th immunization shows the highest endpoint titer at 1:8000 dilution and an ABS value of 0.07. The 5th immunization shows an ABS value of 0.05 at 1:8000 dilution and the same ABS value after the 7th and 8th immunization at a dilution of 1:5000 This summarizes that four immunization steps are sufficient for PLGA-Ab production.

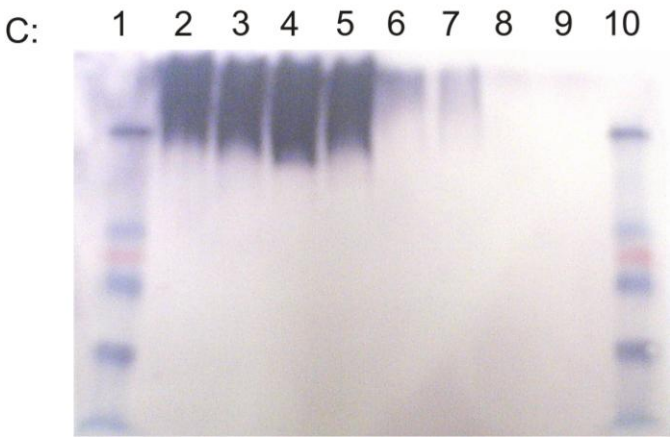
Analysis of gel electrophoresis, western blot and AP detection

To show PLGA-Ab production in sera after the third immunization SDS- and native-PAGE were performed. The gels were stained with a Coomassie Brilliant Blue (CBB) stain. Neither SDS- nor native- gels showed a difference between immune sera (after immunization) and preimmune sera of both Rabbits (no. 95, 96, Figure 5 A). To determine the zone of IgGs in the native gel a Western blot and ELISA-AP (alkaline phosphates) was carried out. The NC-membrane after blotting was incubated with anti rabbit IgG conjugated with AP and stained with AP substrate. The purified IgGs (using Dynabeads M-208 sheep anti-rabbit IgG see Chapter 5) from immune serum after the third immunization of rabbit 95 & 96 did not show any bands in the native gel with CBB-staining (Figure 5 B no. 6, 7) but a positive signal was observed using AP staining (Figure 5 C no. 6,7). However, the PLGA-Abs isolated with Epoxy beads coupled with PLGA (see Chapter 5) from the previously purified IgGs did not show a signal due to low titer concentration after the third immunization (Figure 5 C no. 8, 9).



1	*Preimmune serum R. no. 95*
2	*Preimmune serum R. no. 96*
3	*Protein-ladder*
4	*Immune serum R. no. 95 after 3rd immunization*
5	*Immune serum R. no. 96 after 3rd immunization*





1	*Protein-ladder*
2	*Preimmune serum R. no. 95*
3	*Preimmune serum R. no. 96*
4	*Immune serum R. no. 95 after 3rd immunization*
5	*Immune serum R. no. 96 after 3rd immunization*
6	*Purified IgGs from immune serum of R. no. 95 after 3rd immunization*
7	*Purified IgGs from immune serum of R. no. 96 after 3rd immunization*
8	*Isolated PLGA-Ab from purified IgGs of R. no. 95 after 3rd immunization*
9	*Isolated PLGA-Ab from purified IgGs of R. no. 96 after 3rd immunization*
10	*Protein-ladder*

Figure 5. Preimmune serum and immune serum after third immunization of both rabbits 95 and 96 (R=rabbit) seem similar in the SDS gel (A) and native-gel (B) after CBB-stain. Rabbit IgGs are isolated from immune serum using Dynabead purification. PLGA-antibodies are isolated from the whole IgGs via Epoxy beads. No bands are observed with isolated IgGs and purified PLGA-Ab after the third immunization. The isolated IgGs show a positive signal when detected using AP-staining (C: 6, 7) and no signal of purified PLGA-Ab after third immunization (C: 8, 9).

The visualization of proteins in native gels is enhanced by silver stain, which is generally accepted to be between 20 and 200 times more sensitive than CBB-stain and thus can detect approximately 0.1 ng protein/ band (Merril C. et al. 1987; Rabilloud T. et al. 1990).

As is illustrated in Figure 6, additional bands can be detected in the native gel with silver staining after the fifth immunization, in contrast to the preimmune serum (as a negative control). These bands may be specific for the production of PLGA-Ab after immunization. However, titer determinations using ELISA have shown that the highest titer of PLGA-Ab production is observed after the fourth immunization. These additional bands were cut and analyzed in mass spectrometry using a protein database containing Mammalian proteins (Appendix, section 3.3 Table 1). The bands show sequence similarity to components of different proteins (Appendix, section 3.3, Tables 2, 3) such as Apolipoprotein A-I, Inter-alpha inhibitor proteins and proteins of the complement cascade. Apolipoprotein A-I is the major protein component of HDL (high density lipoprotein). HDL carries many protein and lipid species, many of which have very low concentrations but are biologically very active. For example, HDL and their protein and lipid constituents help to inhibit inflammation, activation of the endothelium, coagulation, and platelet aggregation. It has been postulated that the concentration of large HDL particles more accurately reflects protective action, as opposed to the concentration of total HDL particles (Kwiterovich P.O. et al. 2000). Inter-alpha inhibitor proteins (IaIp) are a family of structurally related serine protease inhibitors. Recent studies have implicated a role for IaIp in sepsis, and have demonstrated their potential as biomarkers in sepsis and cancer (Josic D. et al. 2006). This would be consistent with a stimulation of the immune system to produce biologically active fragments that can either directly attack foreign antigens or enhance the functions of certain types of inflammatory leukocytes (Goldman AS. et al. 1996).

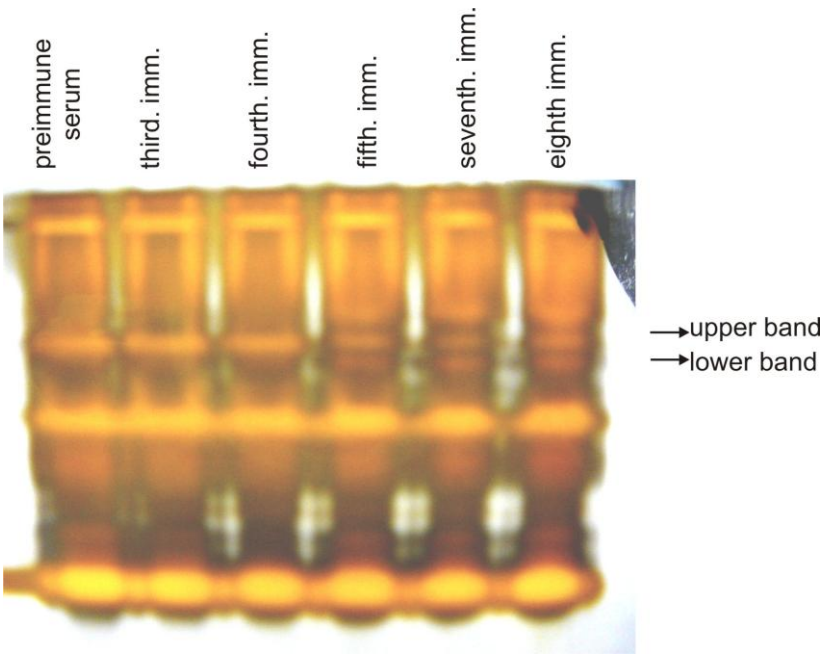


Figure 6. Native gel of preimmune serum und immune serum of rabbit 96 visualized by silver stain. Two bands (higher and lower band) appeared after the fifth immunization and were analyzed by mass spectrometry.

Experimental Methods

Injection sample preparation and pre-treatment of SAM

The PLGA-SAM coupling reaction was carried out immediately before each immunization. SAM (100% solid silica amine microspheres, 100 mg) were suspended in ethanol (2 ml) and vortexed. The SAM suspension was sonicated in a sonic water bath first for 10 min to disperse clumps of unsuspended microspheres, and thereafter for another 10 min, until the microspheres were completely dispersed. Aggregates have to be avoided as they cause problems after subcutaneous injection. The sonicated silica suspension was viewed under a light microscope to confirm complete dispersal of microspheres (Figure 7). The microsphere suspension was centrifuged at 14000 rpm for 5 min. The supernatant was removed and the microsphere pellet was re-suspended in the PLGA-conjugate solution.

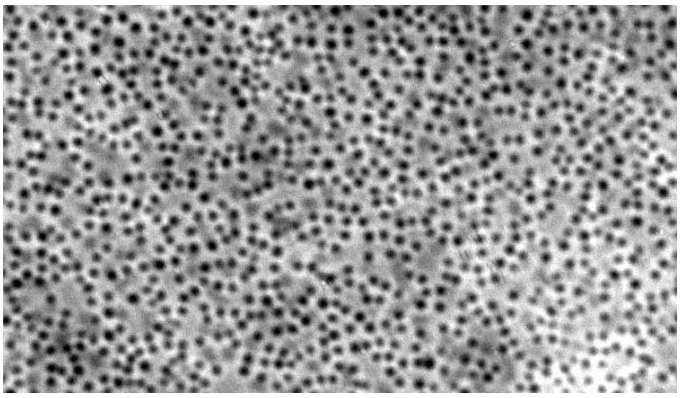


Figure 7. Individual SAMs (without PLGA) under a transmitted light microscope at 100x magnification.

Preparation of PLGA-conjugate and injection sample

See Table 1 for the amount of used components for the different immunization steps.

PLGA (Resomer RG 502H, 68 mg) was dissolved in 1 ml ethyl acetate. NHS (5 mg) was dissolved in 20 µl methanol. DIC (22 µl) was added to the dissolved NHS and immediately added to the dissolved PLGA. The reaction mixture was stirred for 30 minutes at room temperature. Subsequently the activated PLGA solution was added to the prepared SAM suspension for conjugation (see above). The reaction mixture was kept for 2 days at room temperature. The reaction mixture was centrifuged for 1 min at 4000 rpm at room temperature. The pellet was washed with ethyl acetate to dissolve any unconjugated PLGA. *Due to problems (described below) that might appear in this step from the fourth immunization onwards the centrifugation time was reduced to a short run and washing with ethyl acetate was omitted.* After another centrifugation step (conditions as above) the pellet was washed with methanol to remove any isourea that has formed as a by-product of the cross-linking reaction. For separation the suspension was again centrifuged (1 min, 4000 rpm, room temperature) and the pellet was used to prepare the injection sample. *In this step after centrifugation the pellet of the SAM conjugated with PLGA had a gel-like consistency and could not be re-suspended completely in ethyl acetate to remove unconjugated PLGA. Using shorter centrifugation times also was not successful. Even sonication of the conjugate for 5 min did not lead to re-suspension of the gel-like conjugate in methanol or in washed PLGA-NPs.*

Due to this problem this step was also omitted from the fourth immunization onwards.

Then the injection sample contained a mixture of the pellet prepared in previous step and 1 ml washed PLGA-NPs. For the third and fourth immunization PLGA-powder

(Table 1) was added to injection sample. *The same problem described before was encountered in this step too.* Therefore these centrifugation/re-suspension and addition of very finely ground PLGA powder to the injection sample steps were omitted after the third immunization and the injection samples of the fifth, sixth and eighth immunization contained only the PLGA conjugated SAM.

Immunization

The cumulative injection volume per immunization per rabbit was 0.5 ml and was administered subcutaneously into the back skin in five subsequent injections of 100µl each, using a 32-gauge syringe. Two Rabbits (No. 95, 96) were immunized. The interval for booster injections was two weeks.

Immuni-zation	SAM (mg)	PLGA (mg)	EtAc (ml)	DIC (µl)	NHS (mg/µl MeOH)	PLGA-NPs 2.86mg/ml	PLGA-powder	Injection type
1	100	68	1	22	5 /20 µl	1ml	None	subcutaneous
2	100	136	2	44	10 /40 µl	1ml	None	subcutaneous
3	100	204	3	66	15 /60 µl	1ml	7 mg	subcutaneous
4	200	408	6	132	30 /120 µl	1ml	30 mg	subcutaneous
5	250	816	12	264	60 /240 µl	none	none	subcutaneous
6	250	816	12	264	60 /240 µl	none	none	subcutaneous
7	none	none	none	none	none	1ml	none	intravenous
8	250	816	12	264	60 /240 µl	none	none	subcutaneous

Table 1. Amounts of used components and conditions for the respective immunization steps.
(SAM = silica-amino- microspheres, PLGA = poly (D,L-lactide-co-glycolide, EtAC = ethyl acetate, DIC = diisopropylcarbodiimide, N-hydroxysuccinimide =NHS, PLGA-NPs= PLGA-nanoparticles,)

Determination of antibody titer using ELISA

For Abs titer determination blood samples were taken from the ear vein after the third immunization. The blood was kept for at least 30 min at room temperature and then centrifuged at 4000 rpm, for 10 min at 4°C. The supernatant (serum) was aliquoted and stored at -20°C.PLGA-pAb production was tested by ELISA. The sample concentration of PLGA dotted onto the well of the sample reader micro-titerplate is important for the assay efficiency. Therefore different PLGA concentrations, 25, 5,

2.5µg/ml were tested. 5µg/ml gave the best assay readings and therefore, this concentration of PLGA was used.

A 5 µg/ml PLGA (RG 502 H) dilution was prepared in DMSO (Merk). Then 100 µl of PLGA dilution was added onto the surface of each well of a microtiterplate (Nunc-Immuno Plate MaxiSorp, 96-well). For each immunization 14 wells were needed. As a negative control 100 µl dilution buffer was used. The uncovered microtiterplates were incubated for 1 hour at room temperature, then emptied and the wells were filled with blocking solution [2% (w/v) gelatine from cold water fish skin (Sigma Aldrich) was dissolved in 50 mM Tris/HCl buffer (50 mM Tris/HCl, 0.15 M NaCl pH 7.4, and 0.1% (v/v) Tween 20, Sigma Aldrich)] for 30 min under gentle shaking. The microtiterplates were washed 2 times with washing buffer [0.5% (v/v) Tween 20 was added to 50 mM Tris/HCl buffer] for 5 min under gentle shaking. The sera after immunizations were diluted in dilution buffer [50 mM PB, pH 7.2, 30 mM NaCl and 0.5% (w/v) PEG (polyethylenglycol, Sigma Aldrich)] at 1:100, 1:500, 1:1000, 1:1500, 1:2000, 1:2500, 1:3000, 1:4000, 1:5000, 1:6000, 1:7000, 1:8000, 1:9000 and 1:10.000. Then 100 µl of each dilution was added per well of washed microtiterplates. The microtiterplates were covered and incubated overnight at 4°C under gentle shaking in a humid camber, then washed 3 times with washing solution for 5 min. The microtiterplates were incubated for 1 hour with 100µl of anti-rabbit IgG, in dilution buffer, conjugated with HRP (Sigma Aldrich) at room temperature under shaking then washed 3 times with washing solution for 5 min. For detection, 100 µl TMB-substrate (Sciotec) was added to each well. The microtiterplates were incubated for 20 min at room temperature and the reaction was stopped with 50 µl 1M H_2SO_4. The microtiterplates were then read out at 450 nm in a Microplate Reader, Model 450, Bio-Rad).

Gel Electrophoresis

The formation of antibodies was examined by SDS and native gel electrophoresis.

SDS-PAGE

Gel composition and preparation

For SDS- and native-PAGE the Mini-Protean II Electrophoresis system from Bio-Rad was used. According to the molecular weight of the examined proteins, separating gels with 7.5% of acrylamide/bisacrylamide were prepared.

Buffers and solutions

Stacking gel (4%)

The stacking gel solution contained 1.3 ml of 30% acrylamide/ 0.8% bisacrylamide; 6.1 ml dd. H_2O; 2.5 ml 0.5 M Tris/HCl, pH 6.8; 100 µl 10% (w/v) SDS; 50 µl 10% (w/v) APS and 10 µl TEMED.

Separating gel (7.5%)

The separating gel contained 5 ml of 30% acrylamide/ 0.8% bisacrylamide; 9.7 ml dd. H_2O; 5 ml 1.5 M Tris/HCl, pH 8.8; 200 µl 10% (w/v) SDS; 100 µl 10% (w/v) APS and 10 µl TEMED.

Running buffer

The running buffer contained 3.03 g Tris; 14.6 g glycine; 1 g SDS and was filled to 1L with dd. H_2O.

Sample preparation and electrophoresis

For SDS PAGE 1 µl serum was diluted 1:40 in 50 mM Tris-HCl (pH 7.4) and then 40 µl sample buffer [2.5 ml 0.5 M Tris/ HCl pH 6.8; 20% (v/v) glycerol (2 ml); 0.4 g SDS; 0.31 g dithiothreitol; 1 mg bromophenol blue and made up to 20 ml with dd. H_2O] was added. The sample was heated to 95°C for 5m 95°C. The gel slots were loaded with 20 µl of sample. A molecular mass standard mixture (PageRuler Fermentas, Protein Ladder, 2 µl) was used for comparison of molecular mass.

The separation of proteins was performed at 150 V and 60 mA for approximately 1 to 1.5 hour.

Native PAGE and Western blot

The protocol is described in Chapter 5.

Visualization of proteins after gel electrophoresis: CBB-staining

The protocol is described in Chapter 5.

Visualization of protein gels after electrophoresis: silver staining

After running the gel, the separated proteins were visualized with silver staining according to Blum (Blum H., 1987). The staining process relies on the reduction of Ag^+-ions to metallic silver, resulting in staining of the protein bands.

All steps were carried out on a shaker and all solutions were prepared freshly before staining!

Step	Reagent	Duration
Fixing	40% ethanol / 10% acetic acid in dd. H_2O	≥ 1 h
Washing	30% ethanol in dd. H_2O	2 x 20 min
Washing	dd. H_2O	1 x 20 min
Sensitizer	0.02% Sodium thiosulfate in dd. H_2O	1 min
Washing	dd. H_2O	3 x 20 sec.
Silver	0.1% Silver nitrate in dd. H_2O	20 min at 4 °C
Washing	dd. H_2O	3 x 20 sec
The gel chamber was changed at this step to avoid free silver on the surface of the chamber		
Development	3% sodium carbonate 0.05% formaldehyde (37%) in dd. H_2O	3-5 min
Washing	dd. H_2O	1 x 20 sec
Stopping	5 % acetic acid in dd. H_2O	5 min
Washing	dd. H_2O	3 x 10 min
Storing	1 % acetic acid in dd. H_2O	-

Preparation and washing of PLGA-nanoparticles

The preparation of PLGA-nanoparticles is described in details in Chapter 5 according to Weissenböck et al., 2004.

Organic amino-silanization of Gulsenit:

Gulsenit (MAGINDAG, Austria) is an active magnesium silicate mineral having a particle size less than 10 µm and a density of about 3.2 kg/l. Gulsenit was covered

with freshly prepared 5% (v/v) (3-aminopropyl) triethoxysilane solution (APTS, Sigma Aldrich) in 95% EtOH and reacted for one hour under gentle shaking at room temperature. Gulsenit was washed with 95% (v/v) EtOH 3 times for 5 min under gentle shaking and cured at 110°C over night (Pittner F. 2002).

Immobilization of PLGA onto Gulsenit

1g PLGA (Resomer RG 502H) was dissolved in 20 ml ethyl acetate. 575 µg NHS (Sigma Aldrich) was dissolved in 40 ml ethyl acetate. 310 µl N,N'-Diisopropylcarbodiimide (DIC) (Sigma Aldrich) was added to the dissolved NHS and this solution was immediately pipetted into the dissolved PLGA. The activation reaction was carried out for one hour at room temperature. The activated PLGA solution was incubated with 20 g amino-silanized Gulsenit overnight at room temperature under stirring. Then the Gulsenit was rinsed 3 times with ethyl acetate to remove unreacted PLGA followed by 3 washes with dd. H_2O; using a suction funnel, pore size 4, to remove solvent from carrier after each rinse. The Gulsenit can then be used as a column material for the purification of PLGA Ab by means of affinity chromatography. The efficiency of immobilization was determined using the Kaiser-test.

Affinity chromatography using Gulsenit with coupled PLGA

A 2.5 ml chromatography column with a 10 µm pore filter at the bottom (Mo Bi Tec) was filled with PLGA conjugated Gulsenit and washed with 5 column volumes of binding buffer (5 mM PBS, 0.15 M NaCl, pH 7.2). The diluted serum (1:5 with binding buffer) was centrifuged shortly at 14000 rpm and then loaded onto the PLGA conjugated Gulsenit column at a flow rate of 0.15 ml/min. The column was washed with binding buffer until no proteins were detected by Bradford protein assay. Briefly, 20 µl Bradford reagent (Bio-Rad Protein Assay) diluted 1:5 with dd. H_2O were added to 10 µl of each fraction and changes in the color of the solution to blue indicated the presence of proteins. The PLGA-Abs were eluted with elution buffer (0.1 M acetic acid/ 0.1 M NaOAc, pH 3) at a flow rate of 1.7 ml/min. Every eluted fraction (two drops) was collected in a well of a microtiterplate containing about 10 µl neutralization buffer (0.5 M $NaHCO_3$) to neutralize the purified Abs immediately. The fractions containing protein (detected by Bradford protein assay) were pooled and the pH was adjusted to a physiological pH. For a qualitative determination of the proteins

the Bradford protein assay was used. Fractions which showed a positive Bradford test were pooled and concentrated for 5 min by 4000 rpm at 4°C using an Amicon ultra centrifuge filter. The concentration of the eluate was determined using the Quant-iT™ Protein Assay Kit (for use with the Qubit™ fluorometer, Invitrogen). The isolated protein was examined by native gel electrophoresis. The gel was stained with a Coomassie Brilliant Blue (CBB) stain and the protein bands were then subjected to digestion and sequence analysis by mass spectrometry.

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Chapter 7

Development of biocompatible carrier coating with binding sites for biodegradable drug delivery system allowing reloadability

According to the results illustrated in Chapter 5 concerning deposition of human serum albumin onto PLGA another drug delivery system had to be found to reach the afforded aims for this thesis:

To introduce binding sites for degradable, drug loaded nanoparticles into the biocompatible coating and

To provide a proof of principle testing of the interaction between the nanoparticles and their in biocompatible coating embedded binding site

To reach this goal another type of nanoparticles had to be looked for, biodegradable by enzymes circulating in the bloodstream. Chitosan nanoparticles, degradable by lysozyme are known for its biocompatibility, biodegradability, bio-and mucoadhesivity, and hydrophilic character. This increases the bioavailability of poorly absorbable drugs across various epithelial barriers (Alonso M. J. et al. 2007).

This Chapter illustrates the successful of introduction of Abs as binding sites for chitosan NPs on the amino-silanized, amino-PEGylated and SH-PEGylated implant material. Furthermore the binding of CS-NPS labeled with the fluorescent dye BODIPY 493/503 to the biocompatibly coated implant surface could be shown. This novel developed targeting set up can be used for drug delivery system.

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Development of biocompatible carrier coating with binding sites for biodegradable drug delivery system allowing reloadability

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Abstract

Drug delivering implants are becoming increasingly important as tools in the treatment of cardiovascular diseases and other diseases. Here we describe the development of novel, bio-compatible coatings for medical implant materials with antibodies as receptors for bio-degradable and biocompatible chitosan nanoparticles. These may serve as drug carriers to mediate local pharmacological activity. After biodegradation of the drug containing nanoparticles by lysozyme, an enzyme circulating in the bloodstream, the binding sites become free again and can be reloaded with fresh drug delivering particles. The design of the receptor is of great importance as any bio- or chemorecognitive interaction with other components circulating in the blood has to be avoided. Furthermore, the binding between receptor and the particles has to be strong enough to keep them tightly bound during their lifetime, but on the other hand allow reloading after final degradation of the particles. The nanoparticles suggested as a drug delivery system for implant can be loaded with different pharmaceuticals such as antibiotics, growth factors or immuno-suppressives. This concept may enable for changing medication even after implantatation of the medical device if afforded by patients needs.

Keywords

Antibody immobilization; Biocompatible coating; Chitosan nanoparticles; Drug targeting; Medical device.

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Introduction

Drug delivering implants such as drug eluting stents (DESs) play an important role in the treatment of cardiovascular diseases. These stents have been introduced as a new and promising technique to reduce restenosis after stent implantation. Several substances with immunosuppressive, antiproliferative and cytostatic activities have been tested as active stent coatings, and were shown to reduce restenosis in humans (Chieffo A. et al. 2000; Grube E. et al. 2004). Despite these advantages, restenosis rates still remain substantial in high-risk patients, such as patients with long lesions and complex lesion morphologies in bifurcations. The DESs currently in use contain only a certain amount of drugs, and restenosis may take place if this reservoir is used up. These limitations can be overcome by reloading the stent (or other implant material) with drugs. To address this limitation, we and others have sought to develop biocompatible drug delivery systems that allow continous administration of drugs to meet the clinical need of the patients. The general design of our drug delivery systems (Figure 1) was on the basis of the following: 1) use of carrier materials coated with a biocompatible layer covalently bound to the carrier backbone. This forms a passive protection layer preventing rejection of the implant material; 2) anchoring of antibodies in this protective layer to act as specific bio-recognitive sites. These were subsequently used to load biodegradable, drug containing nanoparticles onto the implant. After implantation of the medical device into the patient, such a structure will serve as a re-loadable drug delivery system. Biodegradable nanoparticles containing pharmaceutical drugs are injected and while circulating in the bloodstream will bind to these specific binding sites. The particles are then slowly degraded by enzymes in the surrounding body fluids, thus gradually releasing the drugs (Figure 1). At the end of this process, when the polymer of the nanoparticle is degraded, the bio-recognitive sites should be free again to be reloaded with new drug containing particles.

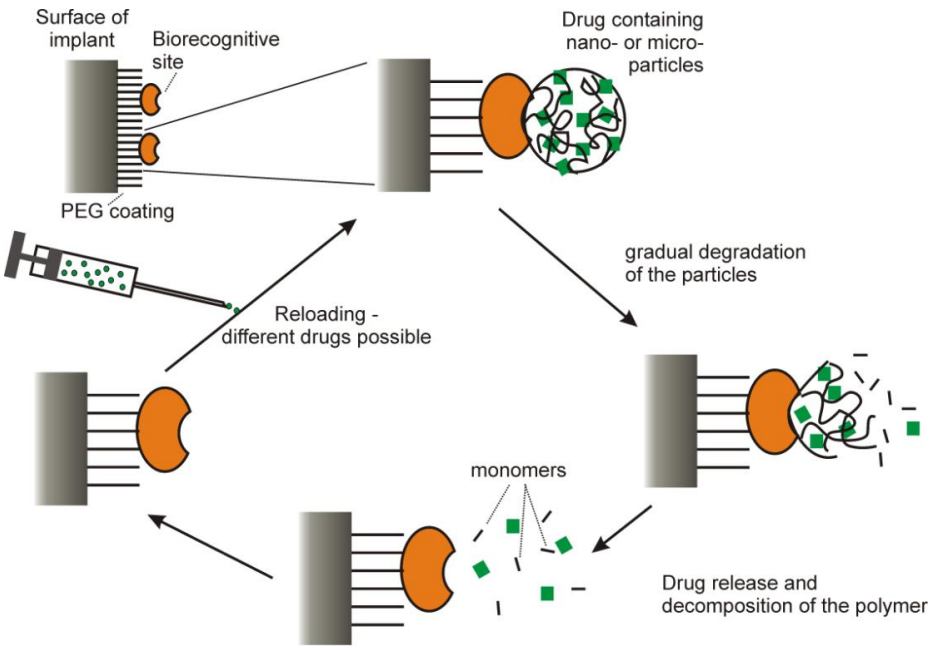


Figure 1. Principle of nanoparticle binding, drug release and reloading.

The possibility of re-loading allows not only patient-adequate dosage of drugs but also change of medication after implantation, using different drugs at different stages of the treatment process. In case of bone implants nanoparticles can be loaded with growth factors that promote replication, differentiation, protein synthesis and/or migration of respective cell types. The successful development of this technology will provide a better means for controlled drug release from the implant due to the site directed binding of the drug carrier nanoparticles to implant surfaces. The more efficient and directed drug release at the target site should improve the clinical outcome after implantation thus improving the healing process.

The biocompatible coating in this study is based on polyethyleneglycol (PEG) derivatives which are covalently bound to the backbone carrier serving as a passive coating. PEG is employed extensively in pharmaceutical and biomedical applications. It can be chemically modified for attachment to other molecules and surfaces (Ratner B.D. et al. 2004). The drug delivery system in this study is based on chitosan nanoparticles. The low toxicity (Aspden T.J. et al. 1995), chemical properties and excellent capacity for the entrapment of proteins of chitosan make it a suitable candidate for use in gene- and drug-delivery systems (Calvo P. et al. 1997b, De la Fuente M. et al. 2008)**.** Chitosan has been a focus of increasing attention in the design and engineering of novel nanoparticulate drug delivery systems, due to their

desirable properties such as biocompatibility, biodegradability, bio-and mucoadhesivity, and hydrophilic character that facilitate the administration and increase the bioavailability of poorly absorbable drugs across various epithelial barriers, such as corneal, nasal and intestinal mucosa (Alonso M.J. et al. 2007). An additional advantage of chitosan nanoparticles is their production under aqueous and fairly mild conditions, thus making them especially suitable to preserve the bioactive conformation of delicate macromolecules (e.g. proteins, hormones, antigens, pDNA, siRNA, growth factors, heparin etc.) that otherwise would be prone to enzymatic degradation and hydrolysis (Alonso M. J. et al. 2007). The physicochemical, biomedical and pharmaceutical properties of chitosan have been described in detail in several articles (Janes K. A. et al. 2001; Csaba N. et al. 2006). Drug delivery systems including chitosan-beta-cyclodextrin nanoparticles and nanocore-coated type capsules were the focus of a review by Alonso et al. (Alonso M. J. et al. 2007). Chitosan has thoroughly been utilized in the development of potentially innovative drug delivery, tissue engineering and wound dressing systems over the last decade. Over 50% of the total number of filed patents in 2006 (Alonso M.J. and Goycolea F.M 2008) claim the use of this biopolymer as a substantial part of the invention are related to drug delivery, tissue engineering and wound healing (Skaugrud O. et al. 1999; Agnihotri A. et al. 2004; Ravi Kumar M.N.V. et al. 2004; George M. et al. 2006; Rinaudeo M. 2006, 2008). Chitosan is a linear amino polysaccharide composed of approximately 20% 1, 4-linked N-acetyl-D-glucosamine (GlcNAc) and approximately 80% β1, 4-linked D-glucosamine (GlcN) produced by partial deacetylation of chitin in hot alkali. CS is degradable by lysozyme circulating also in the bloodstream, thus making it a welcome material for the production of drug containing nanoparticles being slowly degraded and thus releasing their content. The interaction between the chitosan-fluorescence labeled nanoparticles (containing BODIPY 493/503 as a fluorescent tag) and their receptor (antibodies) immobilized onto the carrier surface is shown in this study using fluorescence microscopy.

Results and Discussion

As examples for medical implant materials stainless steel L316 as well as zirconia were used. The successful set up procedure of the biocompatible coating was already shown for these materials as well as for silicate glass (Al-Dubai H. et al.

2010). Because of the uneven surface of zirconia and quenching of optical signals in case of metals silicate glass coated under the same conditions was used as a model substance when using fluorescence labeling as an analytical tool.

For immobilization of the biocompatible coating using either the bifunctional bis-amino PEG (NH_2-PEG-NH_2; amino-PEGylation) or monofunctional mercapto-PEG (SH-PEG; SH-PEGylation) the surface of used carriers needs to be previously activated via chemical etching followed by amino-silanization (Pittner F. 2002). Bi-functional PEGs or SH-PEG were bound covalently to the previously amino-silanized carrier surface to enhance biocompatibility. The choice of PEG-coupling procedure depends on the functional groups of the PEG derivative. For amino-PEGylation in a first step, succinic anhydride was coupled to the amino-silane followed by EDC/sulfo-NHS [(1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/ N-hydroxysulfosuccinimide] activation (Pittner F. 2002; Hoare D. and Koshland D. 1966). Bis-amino-PEG can also be coupled directly to the amino-silanized carrier using p-chloro-anil as a crosslinker (Figure 2). p-Chloro-anil linkers reacts with amino groups and thus can be coupled to the silane moiety under mild conditions (Pittner F. 2002). In a second reaction step the amino groups of a polymer as e.g. NH_2-PEG-NH_2 can then be coupled in para position.

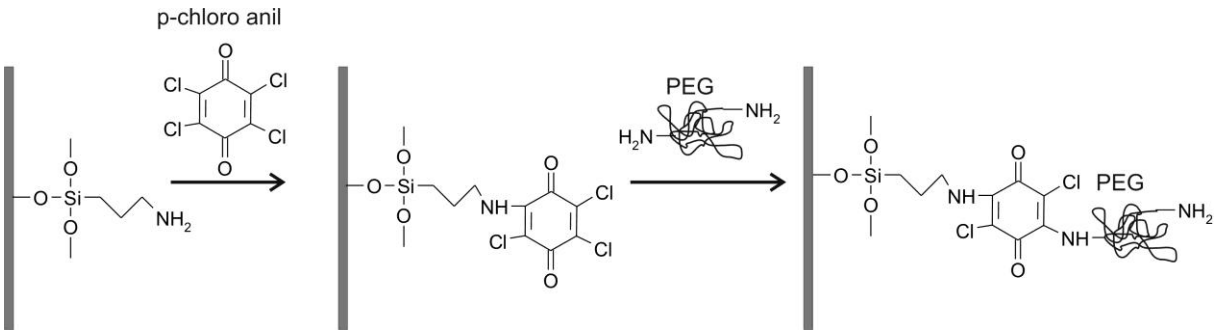


Figure 2. p-Chloro-anil reacts with amino groups and thus can be coupled to the silane moiety under mild conditions. In a second reaction step the amino groups of a polymer such as NH_2-PEG-NH_2 or protein antibodies (e.g. chitosan-Abs) can then be coupled in p-position.

The immobilization of the biorecognitive binding site (antibodies, Abs) for drug delivery (chitosan nanoparticles, CS-NPs) onto the PEGylated or amino-silanized carrier was carried out by using either the sulfo-SMCC [sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylat] cross linking method or the p-chloro-anil

method (Hashida S. and Ishikawa E. 1985; Pittner F. 2002). In the here presented system the used Abs for capture of the CS-NPs was a mouse monoclonal anti-ß-O-linked acetylglucosamine (O-GlcNAc) IgM isotype that recognizes O-GlcNAc. Throughout this paper it is referred to as chitosan-antibody (CS-Ab). CS-Ab molecules are immobilized onto the terminal amino groups of the amino-PEGylated carrier or directly onto an amino-silanized carrier using either the sulfo-SMCC (Figure 3) or the p-chloro-anil crosslinking method.

The SH-PEGylation using monofunctional SH-PEG is carried out on amino-silanized slides already containing CS-Ab molecules. The coupling of CS-Ab using sulfo-SMCC crosslinker is followed by SH-PEGylation. The free maleimide end of sulfo-SMCC not bound to CS-Abs couples specifically to the sulfhydryl groups of SH-PEG (Figure 3).

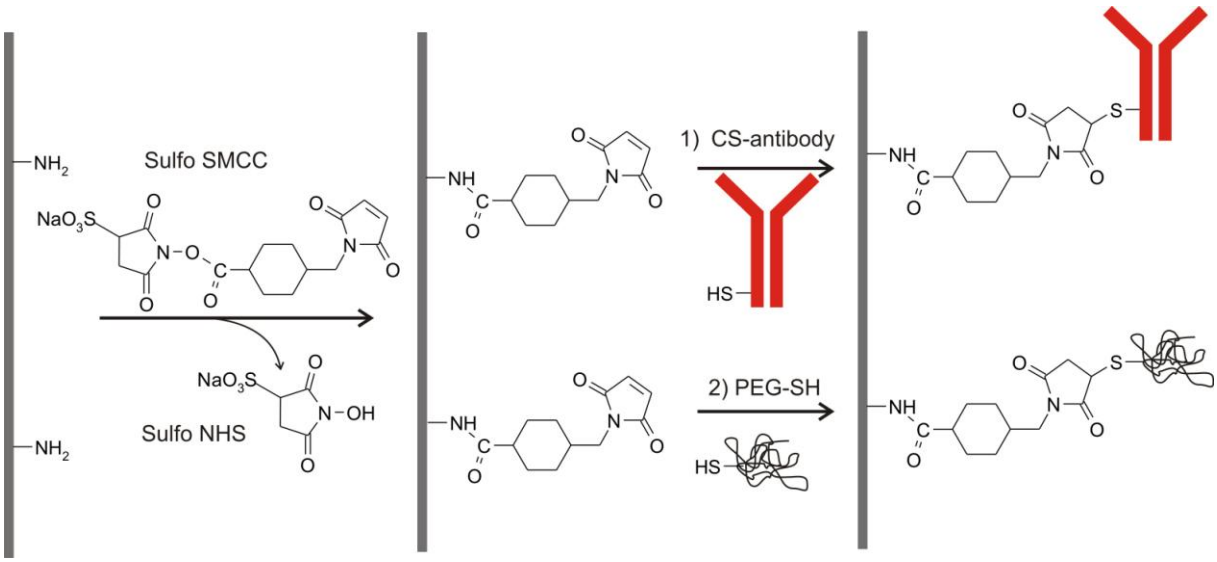


Figure 3. Binding of whole Chitosan antibodies onto the carrier via sulfo SMCC followed by reaction with PEG-SH for enhancing biocompatibility. Any unreacted sites were finally blocked with ß-mercaptoethanol (ßME) – not shown here.

The interaction between CS-NPs and their antibodies immobilized onto a carrier was demonstrated using CS-fluorescence labeled particles (Figure 4) and fluorescence microscopy.

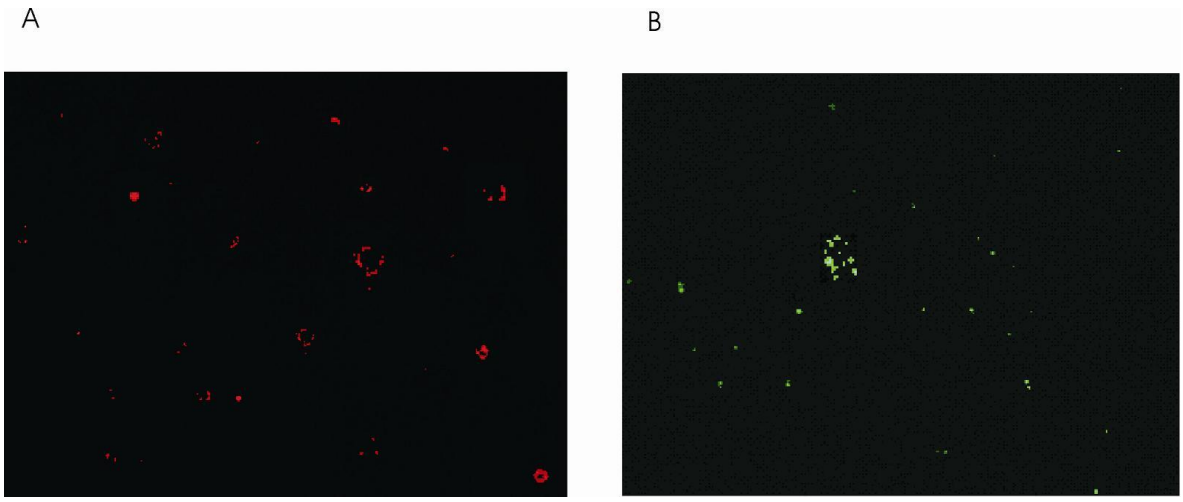
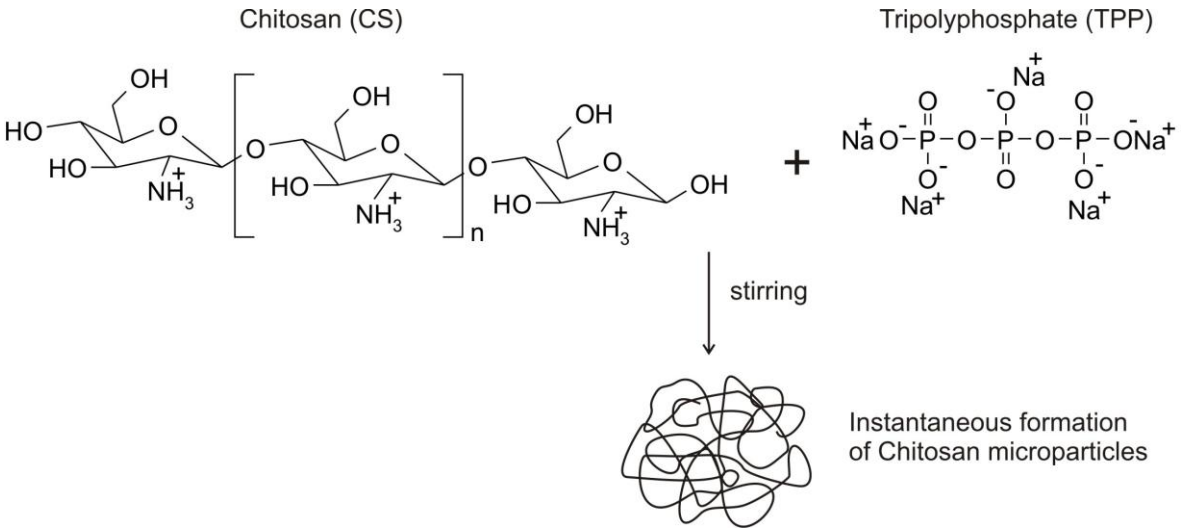


Figure 4. Fluorescence microscopy images of CS-NPs including fluorescent marker: A; Nile red, B; BODIPY 493/503).

Formation of Chitosan nanoparticles

The formation of CS-NPs occurs spontaneously under mild gelation conditions with no need for sonication, high temperatures, or organic solvents. It has been shown earlier that the inter- and intra-molecular linkages created between the positively charged amino groups of CS and the negatively charged TPP are responsible for the initiation of the gelation process showed in Figure 5A (Bodmeie R. et al. 1989; Shiraishi S. et al. 1993; Rajaonarivony M. et at. 1993). The gelation conditions were optimised using different dilutions of TPP. A concentration of 1.5 mg TPP/ml dd. H_2O was found to be the best condition for the formation NPs with a size of 253 nm and a PLI of 0.280 (Figure 5B). CS-NPs generated under these conditions were tagged with fluorescent marker molecules (BODIPY 493/503). The size of CS-NPs conjugated to BODIPY 493/503 was 275.1 nm and its PLI was 0.239.

A)



B)

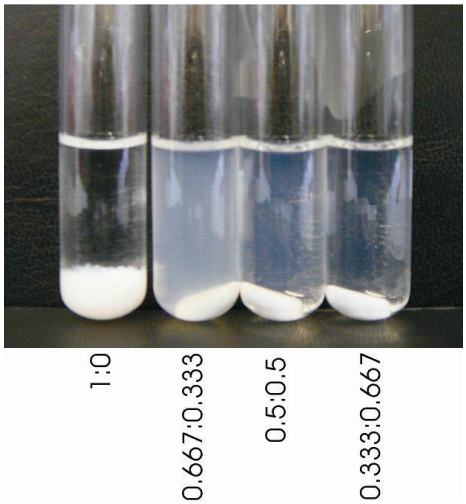


Figure 5. A: Schematic depiction of the strategy for preparation of CS-NPs by inducing the gelation of a CS solution with TPP. B: Visualisation of opalescence of the CS-NPs preparation after addition of different concentrations of TPP to CS solution 1; TPP-dilution (1:0), 2; TPP dilution (0.667:0.333), 3; TPP-dilution (0.5:0.5), 4; TPP- dilution (0.333:0.667). The optimal composition of CS-NPs is observed at 0.5:0.5 TPP-dilution.

Immobilization of recognitive binding sites for CS-NPs onto amino-silanized carriers

One of the aims of this study was to develop a process that allows coating of carriers with a biocompatible layer that provides free binding sites suitable to load drug molecules onto the implant. This was achieved by immobilizing CS-Ab molecules

onto a carrier as binding sites for CS-NPs. This approach was demonstrated successfully first on amino-silanized carriers (Figure 6). CS-NPs were labeled with fluorophores and binding to their binding site (CS-Abs) immobilized on carrier using either sulfo-SMCC (Figure 6A) or p-chloro-anil (Figure 6C) was shown by fluorescence microscopy. In negative controls where CS-Abs were omitted, no fluorescent CS-NPs could be detected in sulfo-SMCC carriers (Figure 6B) and only a low background is observed with p-chloro-anil carriers (Figure 6D).

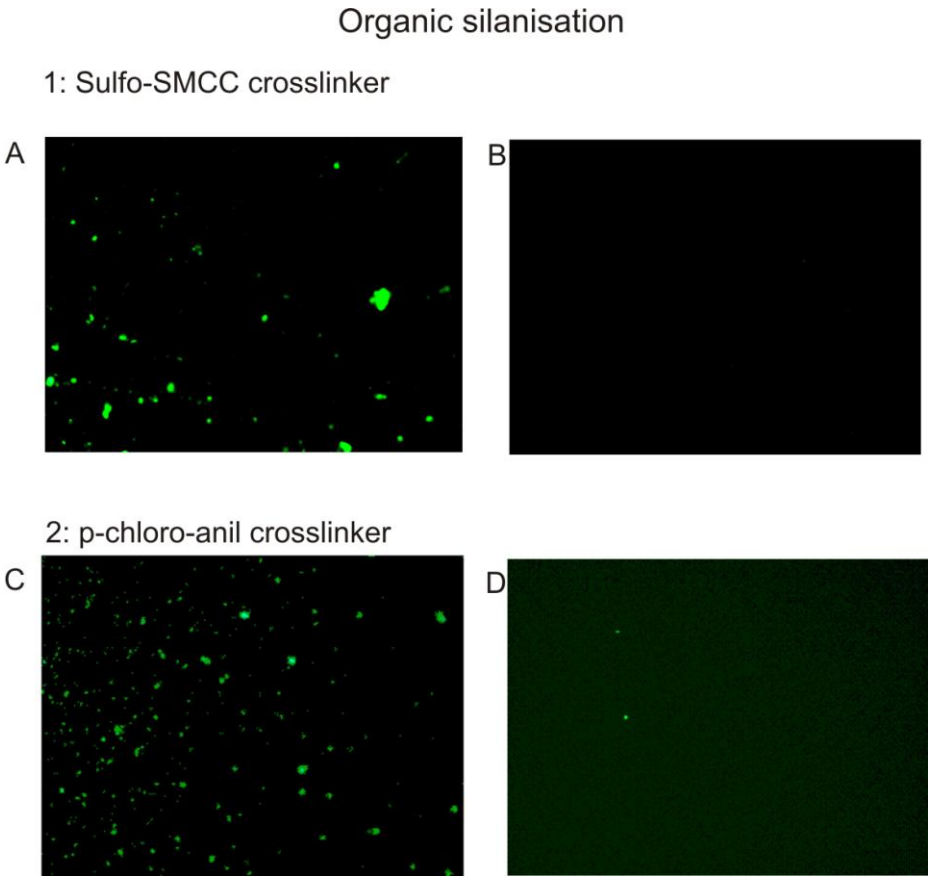


Figure 6. Fluorescence microscopy images illustrating the binding efficiency of CS-NPS labeled with BODIPY 493/503. The immobilization of CS-Ab was carried out by means of sulfo-SMCC (A) or p-chloro-anil (C) respectively. No signal was observed for the negative control (without immobilized CS-Ab) using sulfo-SMCC crosslinker (B) and only a low background is observed using p-chloro-anil crosslinker (D).

SH-PEGylation of carriers containing CS-Abs

The SH-PEGylation at pH 7.3 was carried out on the amino-silanized carrier after the coupling of CS-Ab by means of a sulfo-SMCC cross linker as shown in Figure 7. The interaction between immobilized CS-Ab and the labeled CS-NPs is presented in Figure 7A. Figure 7B shows that no signal was observed in a negative control, i.e., in the absence of immobilized CS-Ab**.**

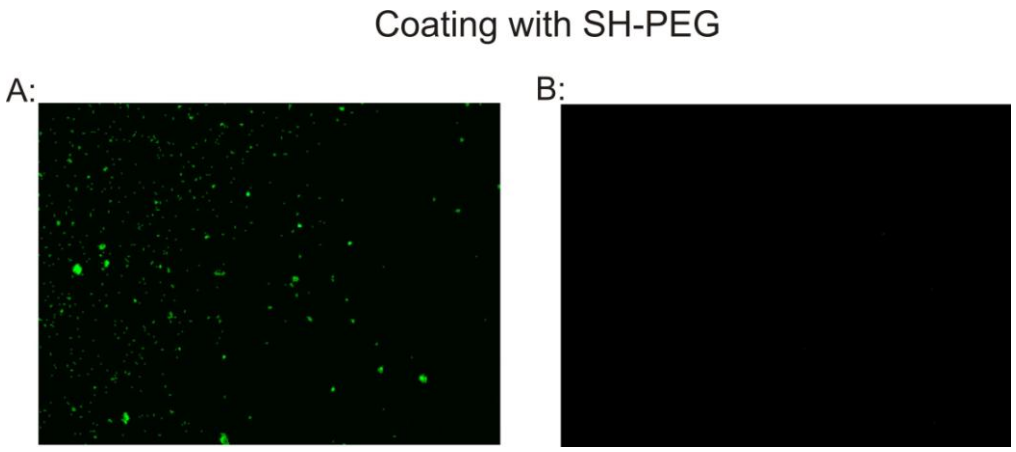


Figure 7. Fluorescence microscopy images of coated silicate glass carriers containing immobilized CS-Abs followed by SH-PEGylation (A). No signal was observed for the negative control (B) omitting the immobilization of CS-Ab.

Immobilization of recognitive binding site of CS-NPs onto amino-PEGylated carrier

The amino-PEGylation of carriers was carried out by means of EDC/sulfo-NHS or p-chloro-anil at different pH (5 or 10). Amino-PEGylation at pH 5 results in a higher binding affinity of CS-NPs (Figure 8A, C) than at pH 10 (see supplementary materials Figure 9). That was consistent with the hypothesis that under acidic conditions amino groups on PEG tend to the surface of the molecule because of stronger interactions with the hydrophilic surrounding and thus become available for binding of the crosslinkers (EDC/sulfo-NHS, Figure 8A, B or p-chloro-anil, Figure 8C, D) necessary for further immobilization of CS-Abs using sulfo-SMCC. Figure 8 demonstrates also the different crosslinking efficiency of EDC/sulfo-NHS and p-chloro-anil used for amino-PEGylation. Coating of NH_2-PEG-NH_2 using EDC/sulfo-NHS is more time consuming than the p-chloro-anil method, but p-chloro-anil gives some low

background (Figure 8D) signals, possibly as a result of an incomplete cross-linking reaction.

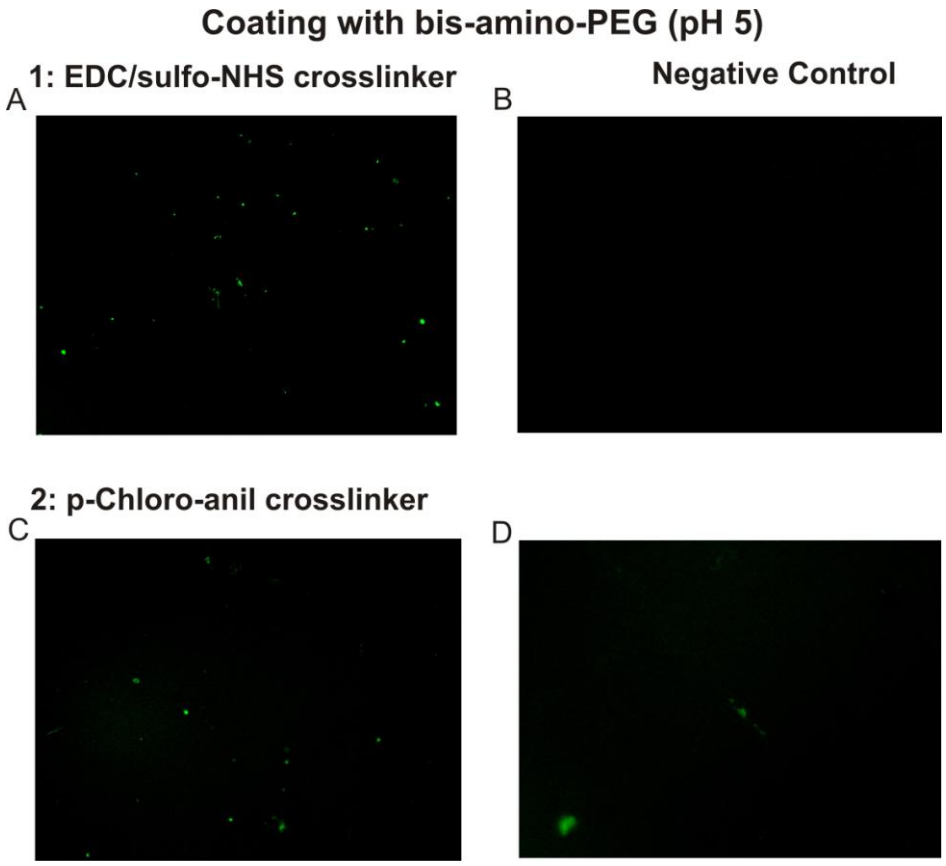


Figure 8. Fluorescence microscopy images of carriers coated with NH_2-PEG-NH_2 at pH 5. The crosslinkers either EDC/sulfo-NHS (A, B) or p-chloro-anil (C, D) were used to immobilize NH_2-PEG-NH_2 onto organic silanized carriers. The immobilization of CS-Ab was applied by means of sulfo-SMCC. EDC/sulfo-NHS crosslinker shows no signal in the negative control (B) and more captured fluorescent CS-NPs than when using p-chloro-anil crosslinker (D).

EDC/sulfo-NHS may be the method of choice for polymer coating of an implant surface as the resulting amide bond linkage does not elicit any immunogenic response.

Experimental Methods

Organic silanizaton of inorganic carriers

Inorganic carriers used here were silicate glass slides (Ø12 mm, Assistant, Austria). Silicate glass slides are etched with 1M NaOH for 30 min. The carriers were washed several times with dd. H_2O and then soaked for at least two days in dd. H_2O under

gentle shaking. Replacement of dd. H_2O is highly recommended several times to enhance hydroxyl group formation. The surface of carriers is then modified by amino-silanization with 3-aminopropyl triethoxysilane in order to immobilize NH_2-PEG-NH_2 or SH-PEG. The used carriers were covered with freshly prepared 5% (v/v) (3-aminopropyl) triethoxysilane solution (APTS, Sigma, Austria) in 95% EtOH and reacted for one hour under gentle shaking at room temperature. The carriers were washed with 95% (v/v) EtOH 3 times for 5 min under gentle shaking and incubated at 110°C over night.

Binding of succinic anhydride to the amino-silanized surface

Succinic anhydride is applied to introduce carboxylate groups onto the surface of amino-silanized material: 1 g of succinic anhydride (Merck, Austria) was suspended in 25 ml PBS buffer pH 6. The pH was monitored and adjusted to 6 with 1M NaOH to prevent severe acidification of the reaction solution, which might damage the molecule to be coupled in the further step. This solution was added to the amino-silanized carrier and reacted at room temperature over night to assure complete blocking of all amino groups (control the pH over the first few hours of the reaction). The carrier was washed 3 times for 5 min with buffer followed 3 times with dd. H_2O.

EDC combined with sulfo-NHS

0.1mM EDC (Sigma, Austria) and Sulfo-NHS (Pierce, Switzerland) in a final concentration of 5 mM were dissolved in 50 ml dd. H_2O and the pH adjusted to 10 with 1M NaOH. The carboxylated carrier prepared with succinic anhydride was incubated in this solution for about 1 to 2 h (maximum) at room temperature under gentle shaking. The activated carrier was washed several times with dd. H_2O.

Coupling of p-chloro-anil as a linker

As p-Chloro-anil is sensitive to light all reaction steps have to be carried out under light protection until NH_2-PEG-NH_2 or Ab is immobilized. The amino-silanized carrier was washed 2 times with toluene (J.T. Baker, Austria) for 5 min under shaking then incubated with a 1% (w/v) solution of p-chloro-anil (Fluka, Austria) in toluene for 1 h at room temperature under gentle shaking. The carrier was washed 2 times with toluene, acetone (J.T. Baker, Austria) and dd. H_2O respectively.

Amino-PEGylation

1 g O,O′-bis (3-aminopropyl) polyethylene glycol (NH_2-PEG-NH_2, MW 1500, Fluka, Austria) was dissolved in 50 ml of 0.1M citric acid-sodium citrate buffer pH 5 or 50 ml of 0.1M carbonate-bicarbonate buffer pH 10 respectively. The carrier, activated with either EDC/Sulfo-NHS or p-chloro-anil, was incubated in the above prepared solutions separately at least overnight. Each amino-PEGylated carrier was washed 3 times for 5 min under shaking with the buffer to remove unreacted PEG and then washed several times with dd. H_2O.

SH-PEGylation

SH-PEG (O-[2-(3-Mercaptopropionylamino) ethyl]-O´-methyl-polyethylene glycol, MW 5000) was provided from Sigma (Austria). Amino-silanized carriers already activated with sulfo-SMCC and then coupled with CS-Ab were incubated in 500 µl of a solution of 121mg/ ml SH-PEG (dissolved in 50 mM sodium phosphate buffer pH 7.3) per carrier (Ø12 mm silicate glass cover slides) under argon in a humid camber over night at 4°C under shaking. The carriers were washed 3 times for 5 min with Tween 20 washing solution [washing solution contains 0.5% (v/v) Tween 20 (Sigma) and 0.1 M phosphate buffer, 0.15 M NaCl pH 7.3] on an orbital shaker. The washed carriers were blocked in 2-mercaptoethanol (ßME, Fluka) blocking solution (35 mM ßME in 0.1 M sodium phosphate buffer pH 7.3) for 30 min under shaking. The blocked carriers were washed as before and incubated with fluorescent chitosan-nanoparticles (CS-NPs).

Immobilization of antibodies onto the carrier

The amino-PEGylated or amino-silanized carriers (4x Ø12 mm silicate glass cover slides) were washed with buffer (0.1 M PBS with 0.15 M NaCl, pH 7.2). 4 mg sulfo-SMCC [Sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate), Pierce] was dissolved in 1 ml dd. H_2O and than filled it to 11 ml with the above mentioned 0.1 M PBS buffer. The carriers were incubated for 30 min in this solution under gentle shaking. The carriers were washed 3 times with buffer for 5 min under gentle shaking. Uncleaved CS-Ab (2mg/ ml diluted 1:55 in 50 mM phosphate buffer pH 7.5) was pipetted onto the carrier activated with either sulfo-SMCC or p-chloro-anil and incubated for 1h at 37°C. The carrier was washed 3 times for 5 min with Tween 20 washing solution under gentle shaking. Silicate glass carriers with Abs

immobilized by means of sulfo-SMCC crosslinker were blocked with 35 mM ßME in 0.1 M sodium phosphate pH 7.3 for 30 min under shaking. Silicate glass carriers containing Abs immobilized via p-chloro-anil crosslinker were blocked using ethanolamine (1:10, v/v) in dd. H_2O for 30 min on an orbital shaker. Silicate glass carriers for negative control were silanized, activated with p-chloro-anil and activated with sulfo-SMCC omitting immobilization of CS-Abs. After the blocking step the silicate glass surfaces were washed 3 times for 5 min under shaking in Tween 20 washing solution and subsequently rinsed with dd. H_2O. Fluorescent CS-NPs were diluted 1:10 with dd. H_2O. 100 µl of diluted fluorescent was pipetted onto washed carriers. The carriers were then incubated over night at 4°C in a humid chamber. The carriers were washed 3 times for 5 min with Tween 20 washing solution under shaking. Binding of fluorescent CS-NPs to the silicate glass carriers was visualized by fluorescent microscopy (Olympus BX41) using excitation/emission wavelengths of 493/503 nm for BODIPY 493/503. Images were taken in Color View software (Soft Imaging System) at 40x magnification and edited in Cell^D life science documentation software (Olympus Soft Imaging Solutions, Münster, Germany).

Preparation of Chitosan-nanoparticles

A CS solution (2 mg/ml) in 5% stoichiometric excess of acetic acid (HAc) was prepared. In our case theoretically 0.063% HAc was needed for CS (Sigma) with a deacetylation degree of 75-85% and a MW between 50 000 to 190 000. The resulting pH of the solution was adjusted to 4.3 with 5% HAc to obtain a clear solution of CS. A TPP stock solution in a concentration of 3mg/ml was prepared in dd. H_2O. 5 ml of the following dilutions of the TPP stock were prepared (TPP stock: water) 1: stock solution, 2: dilution 2:1, 3: dilution 1:1, 4: dilution 1:2. An aliquot of 3 ml CS was pipetted into a silicate glass test tube that was placed upright into a 50 ml Erlenmeyer flask. 1 ml of the TPP solution (prepared as detailed before) was added under stirring into the center of the vortex formed in the CS solution in the test tube. After adding TPP the opalescence of the solution increased with TPP concentration. After stirring for 10 min the mixture was kept standing for 10 min. Then the solution was visually inspected for aggregates. Formation of visible particles is a sign of unwanted aggregation. The final pH of the NP suspension is shown in Table 1. With stock solution precipitation was observed. Particle size was measured for the formulations

that showed no aggregation or precipitation. A 1:1 dilution of TPP was found to give best results (Table 1).

TPP-dilution (3mg/ml TPP stock: water)	pH of NP suspension	Size of NPs (nm)	PLI (polydispersity index)
2:1	5.05	352	0.499
1:1	4.97	253	0.280
1:2	4.87	293	0.293

Table 1. The mean CS-NPs size and distribution were determined by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Instruments Ltd, U.K). All measurements were carried out in triplicate at 20°C after 5 min of equilibration time.

After NP formation they were recovered by centrifugation: 1 ml of the NP suspension was transferred into 1.5 ml Eppendorf vials with a 20 µl glycerol bed at the bottom of the vial (glycerol is used to avoid aggregation and facilitate resuspension of the isolated NPs). The vials were centrifuged at 10000 x g for 40 min at 25°C and subsequently the supernatant was removed. 100 µl of water was added and the MP-pellet resuspended with a 100 µl micropipette by vigorous, repeated mixing. The preparation of fluorescent CS-nanoparticles tagged with BODIPY 493/503 *(*4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene, Invitrogen) is carried out as described above. In this case, 100 µl of BODIPY 493/503 solution (dissolved in ethylacetate at a concentration of 1mg/ ml) was added to 3 ml CS solution (2 mg/ ml) by stirring vigorously at room temperature for 20 min. The solution was filtered through a 5 µm membrane filter (MILLIPORE, sterile MILLEX-SV) and 1 ml of TPP solution (1:1) was added analogous to above. The size of NPs tagged with BODIPY 493/503 was 275.1 nm and their PLI was 0.239. Labeled NPs were centrifuged, resuspended in 100 µl dd. H_2O as described earlier, and used for detection of immobilized CS-Ab on either amino-silanized silicate glass surfaces, or amino-PEGylated or SH-PEGylated silicate glass surfaces.

Supplementary Materials

We tested the influence of pH during the amino-PEGylation coating with bi-functional NH_2-PEG-NH_2. CS-NPs show a better binding affinity to carriers with NH_2-PEG-NH_2 when bound at pH 5 (Figure 8) compared to pH10 (Figure 9).

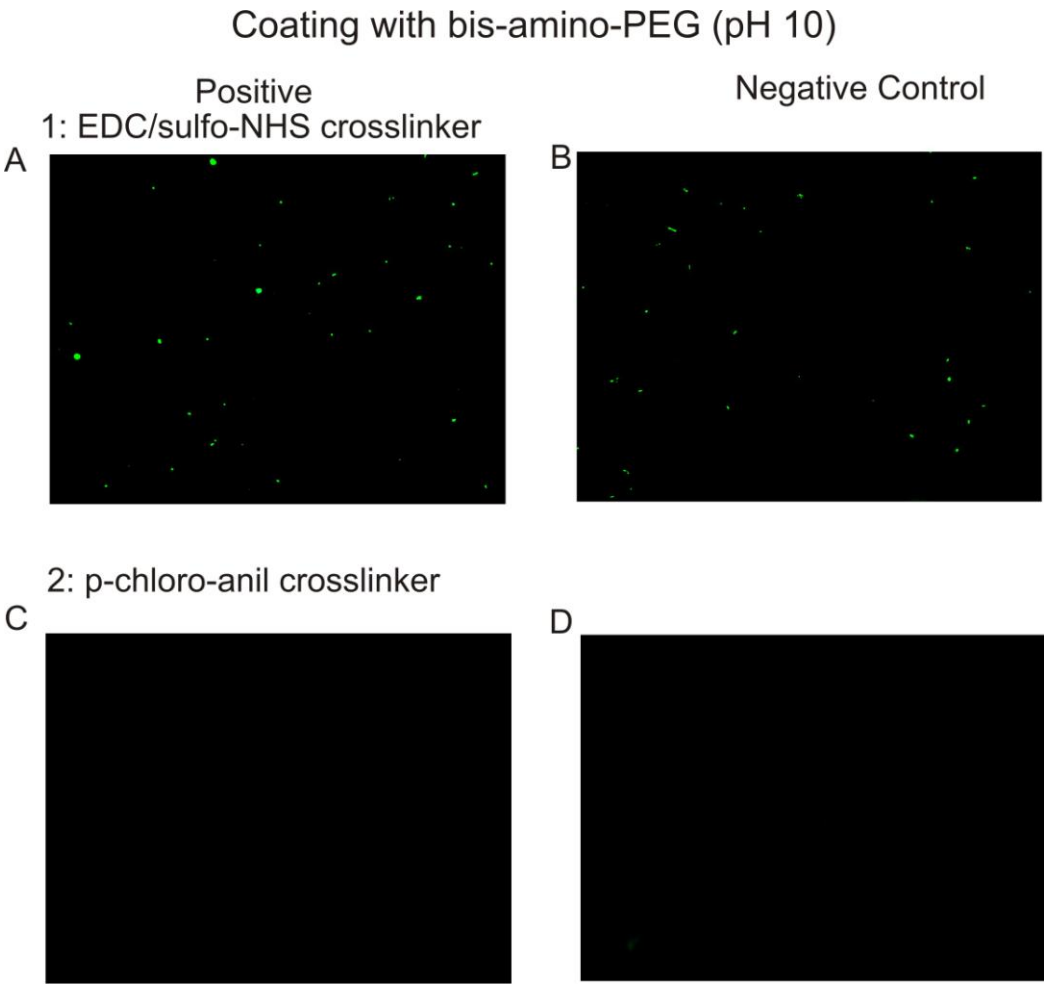


Figure 9. Fluorescence microscopy images of carriers coated with NH_2-PEG-NH_2 at pH 10. The crosslinkers either EDC/sulfo-NHS (A, B) or p-chloro-anil (C, D) were used to immobilize NH_2-PEG-NH_2 onto organic silanized carriers. The immobilization of CS-Ab was applied by means of sulfo-SMCC. EDC/sulfo-NHS crosslinker shows signal even in the negative control (B). No captured fluorescent CS-NPs than when using p-chloro-anil crosslinker (C, D).

In case of EDC/sulfo-NHS crosslinker the positive carrier and the negative control carrier show a signal although no CS-Ab is immobilized. The coating layer of bis-amino-PEG prepared at pH 10 might have been inhomogeneous and possibly didn't cover the entire carrier surface. As a consequence the EDC activated surface could be still free and active and serve as a binding site for CS-NPs.

In case of p-chloro-anil crosslinker in the negative control no signal was observed. The blocking with ethanolamine is efficient and protects the free binding site for CS-NPs. The positive carrier shows no signal. The immobilized bis amino PEG at pH10 using p- chloro-anil did not provide free amino groups as a binding site for Ab.

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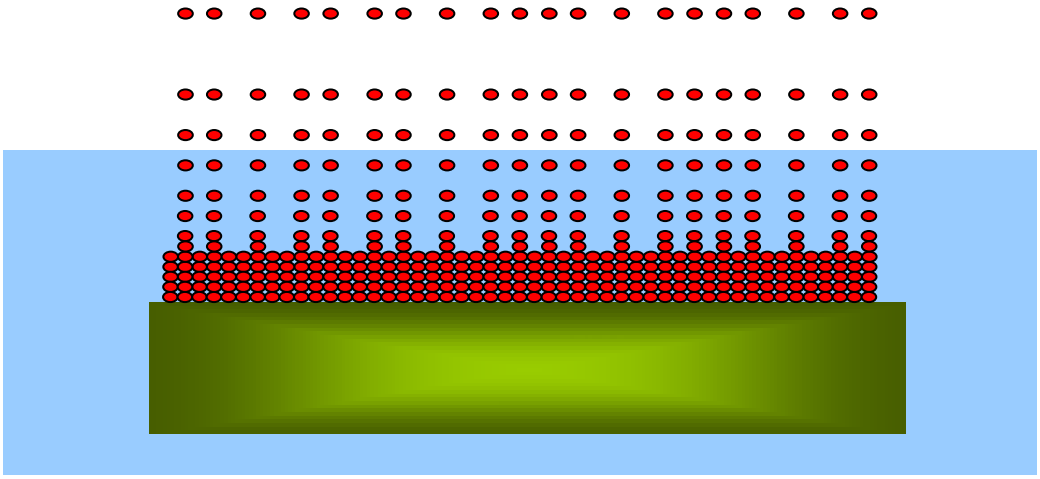
Summary and further work



Summary

Although a number of methods were developed earlier to increase biocompatibility of implant materials, the design of polymer and implant materials poses still challenges for modern biopharmaceutical engineering. Since the introduction of drug eluting stents (DES) for clinical cardiology, several studies have shown the superiority of drug eluting coronary stent implantation over conventional angioplasty. In addition, drug eluting implants that can reduce the local immune response at the implant site have proven beneficial in several applications. However, currently existing drug eluting materials contain drug volumes sufficient only for several weeks after stent implantation and restenosis still remains a major post operative risk, even after implanting a DES. Any improved implant material should not only have excellent mechanical properties, durability, resistance to corrosion and good biocompatibility, but should also allow to increase the time period of drug delivery, as well the option to release different drugs.

In order to develop novel implant materials with such desired characteristics we have used stainless-steel and zirconia (ZrO_2-TZP-A BiO-HIP) with active and passive coating in this study. The passive PEG polymer coating was covalently bound to the implant backbone to serve as a barrier with good biocompatibility on the surface of the implant materials.

The active coating, that was designed to release drugs, such as anti-proliferative agents, antibiotics, or growth factors, as long as required by the patient. To allow for re-loading as well as change of drugs, a drug delivery system was developed based on nanoparticles as vehicles which bind to their embedded receptors (antibodies) on the passive polymer-coated (PEG) implant materials. For proof of principle studies, dyes were successfully embedded into the nanoparticles, which allowed visual tracing of the successful setup of the implant coating. While the nanoparticles are degraded either by hydrolysis or by enzymes in the surrounding body fluids, the drugs are released gradually. At the end of this process the biorecognitive sites become free again and able to be reloaded with fresh drug containing nanoparticles. Nanoparticles used in this study were made of the well described, biocompatible and biodegradable polyester PLGA (Poly (D, L-lactide-co-glycolide)) that is degradable by mere hydrolysis, or of chitosan that is degradable by lysozyme circulating in human blood. Several studies have already shown the possibility to load and release different drugs from PLGA- or chitosan-nanoparticles.

In contrast to the currently available drug eluting implant materials, the novel implant materials developed in this study allow drug delivery via nanoparticles throughout the entire implant life if necessary, to considerably improve the recovery of the patient after implantation. As nanoparticles can be loaded with different drugs, this technology allows easy adjustment to patient-specific medication needs as well as the administration of compounds that would either degrade quickly, or that are difficult to dose, when administered orally or intravenously (e.g. growth factors, immuno-suppressants, thrombolytic enzymes made biocompatible by chemical engineering). More over, this concept also allows the change of medication after implantation by using nanoparticles containing various drugs at different stages of the healing process when reloading the implant.

Bi-functional PEGs or SH-PEG were bound covalently to the previously amino-silanized implant surface to enhance biocompatibility. The choice of PEG-coupling procedure depends on the functional groups of the PEG derivative. For immobilization of bi-functional NH_2-PEG-NH_2, in a first step, succinic anhydride was coupled to the amino-silane followed by EDC/sulfo-NHS activation. For the monofunctional SH-PEG sulfo-SMCC was used as a linker. The immobilization of antibodies (Abs) or their Fab´ fragments onto the PEGylated or amino-silanized carrier was carried out by using the sulfo-SMCC cross linking method or the p-chloro-anil method. The Ab molecules were selectively cleaved with a reducing agent such as 2-MEA (2-mercaptoethanolamine), or the newly optimized method developed during this thesis using dihydrolipoamide immobilized onto amino-silanized Gulsenit to create two Fab` fragments. The determination of Fab´ fragments immobilized onto amino-silanized or amino-PEGylated glass carriers was shown by OPA–Test (o-phthaldialdehyde). The OPA-Test was used among other methods, such as the Kaiser assay, alkaline phosphatase assay, and finally sulfo-SDTB assay, to determine amino groups after amino-silanization and amino-PEGylation of the carriers.

The active coating developed in this study is based on the effect of drugs that were embedded in nanoparticles. These nanoparticles bind specifically to their immobilized recognitive site (antibodies) on the surface of the carrier (implant material). In case of PLGA-nanoparticles PLGA antibodies were raised for the first time in this study. The

raising of polyclonal antibodies against a biocompatible and biodegradable polymer, such as PLGA, requires special efforts under normal conditions. Therefore, a very stringent immunisation protocol has been developed for antibody production in rabbits. By direct purification of PLGA antibodies from immune rabbit sera, using affinity chromatography, a protein was isolated which interacts with PLGA. Analysis of this protein by means of native gel electrophoresis and mass spectrometry showed that this protein has a homology to albumins. Therefore, we investigated also the effect of human serum albumin on PLGA, and could prove its binding to PLGA nanoparticles.

According to these results, it is recommended to coat PLGA-NPs before injecting them into the human body to avoid unspecific coating with serum albumin. The design of such coating of PLGA-NPs has to be carried out carefully, as it needs to be biocompatible, rejecting proteins circulating in the body fluids to avoid fouling, and also be biodegradable, preferably by the same mechanisms as PLGA. In this case, production of antibodies against these biocompatible and biodegradable polymers that serve as binding sites for polymer coated PLGA-NPs might be a good solution. The raising of antibodies against these polymers can be carried out in analogy to the production of PLGA antibodies.
This deposition of albumins onto PLGA was demonstrated using CLISA (Cluster Linked Immunosorbent Assay). This method was utilized for the detection of several antigen/ antibody interactions was adapted in this thesis for proving interactions between implant coating and nanoparticles in a reloading test. It was further applied for other analytical purposes, such as the detection of allergens in patient sera.

Moreover, a drug delivery system based on chitosan nanoparticles was also studied in this thesis to show proof of principle for the novel developed implant material. The interaction between chitosan nanoparticles and their antibodies immobilized onto a carrier was demonstrated on silicate glass material employing CS-fluorescence labeled particles using fluorescence microscopy. However, this method is not finally applicable to metals, because of quenching of optical signals, nor to ceramics due to its uneven surface characteristics. Therefore, the chemical coating of implant materials with PEGs and antibodies as well as antibody fragments was shown with

the above mentioned implant materials. To give the final visual proof by fluorescence microscopy, the same methods were then carried out using glass cover slips.

Future work

The principle of implant coating demonstrated in this thesis can be rextended using other implant materials such as titanium or glassy carbons. Other biocompatible and biodegradable coatings should be also tested for binding of the nanoparticle - recognition system. Coatings with patient specific surfaces as e.g. endothelial cells of the respective patient should be a goal for future developments to achieve even safer stents. For coronary artery stents nanoparticles should be loaded with Prostacyclin (PGI_2) which inhibits platelet aggregation and induces vasodilatation.

The compatibility of new developed implant materials should then be tested *in-vitro* with endothelial cells e.g. the HUVEC cell line (human venous endothelial cells) and finally in animal and clinical studies.

Appendix

Synthesis of amino-methoxy-PEG and carboxy-methoxy-PEG for biocompatible coating of implant materials

Introduction

PEG itself possesses no reactive site moieties due to the repeating ethylene oxide units. For binding to other compounds terminal functional groups have to be inserted through a series of reactions to more active functional groups (Jing Li et al. 2003) e.g. amino groups. To find an efficient way of preparing a hydrophilic polymer bearing a positively charged group at one end with economical cost led us to synthesize amino-methoxy-PEG. Two different reasonable procedures were tried to synthesize mono amino PEG, the first according to Colombo R. (1981) and the second via carboxy-methoxy-PEG.

In this study the implant material has to be coated with biocompatible polymer (PEG). A mixture of bis-amino-PEG and amino-methoxy-PEG can be used if the distance between the biorecognitive receptors bound to the implant surface turn out to be too small and the drug loaded particles too big for adequate fit. This mixture would help to regulate the number of immobilized biorecognitive capture sites (Ab) on the surface of implant material if necessary, since only the free amino group of bis-amino-PEG can serve as a binding site for the biorecognition system (Figure 1).

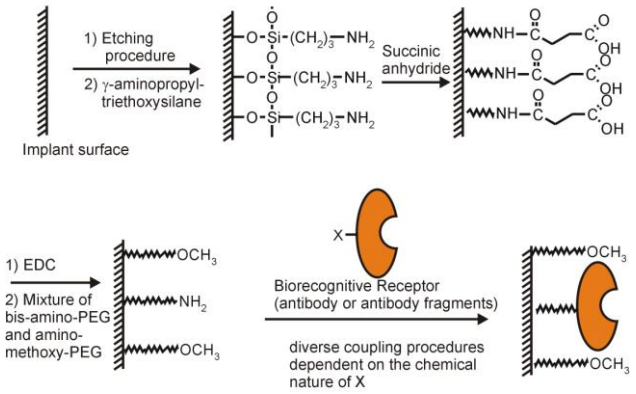
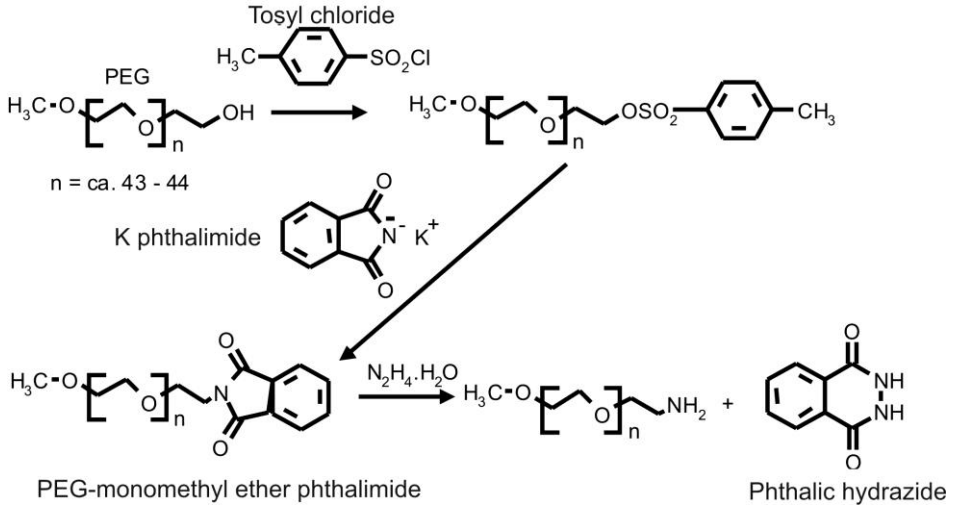


Figure 1. Reaction scheme for binding of biorecognitive receptors using a mixture of bis-amino-PEG and amino-methoxy-PEG.

Synthesis of Amino-methoxy-PEG 2000

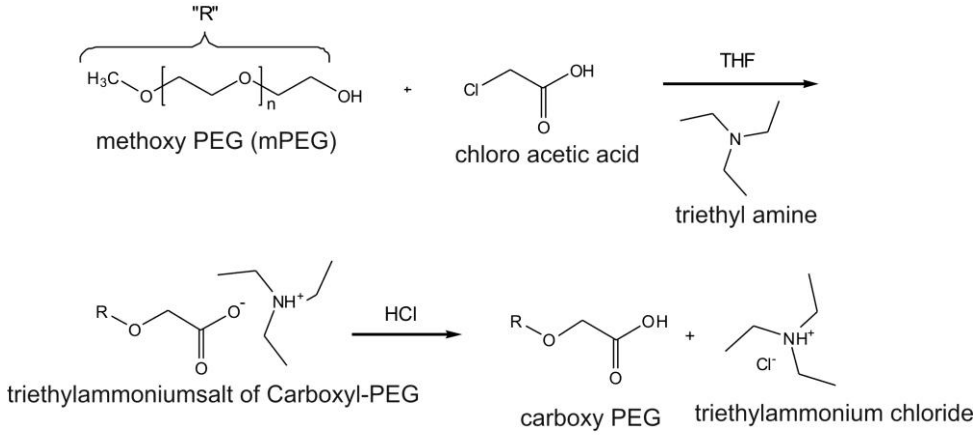
The synthesis of amino-methoxy-PEG was carried out analgous to Colombo R. et al 1981 as shown in Scheme 1.



Scheme 1

Synthesis of Carboxy-methoxy-PEG

The procedure was carried out analogous to a method developed and described in Rumplayr K. 2006.



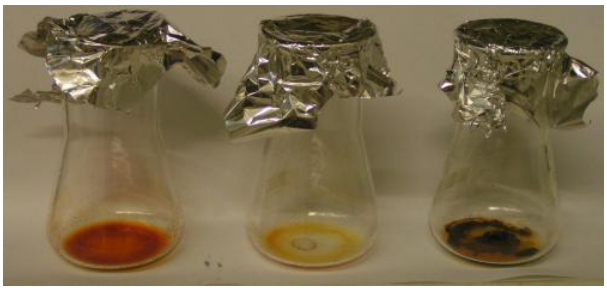
Scheme 2

Results and Discussion

Detection of the carboxyl group

The alkali salt of the hydroxamic acid which was obtained under alkaline conditions was reacted with $FeCl_3$ after acidification. The Carboxy-methoxy-PEG (c-m-PEG) preparation resulted in a red-brownish colouring. As a control the same reaction was

performed with methoxy-PEG (m-PEG) where only the color of $FeCl_3$ could be obtained. Amino-methoxy-PEG (NH_2-m-PEG) reacted immediately and strongly with thionyl chloride resulting in a burnt, black substance (Figure 2).



c-m-PEG *m-PEG* *NH_2-m-PEG*

Figure 2. Test for carboxyl groups in the product of c-m-PEG synthesis.

IR Spectroscopy

IR spectra of synthesized carboxy-methoxy-PEG are compared to spectra of methoxy-PEG (Figure 3 A and B) showing the characteristic absorbance for CO-groups in the region of 1750 cm^{-1}, absent in methoxy-PEG.

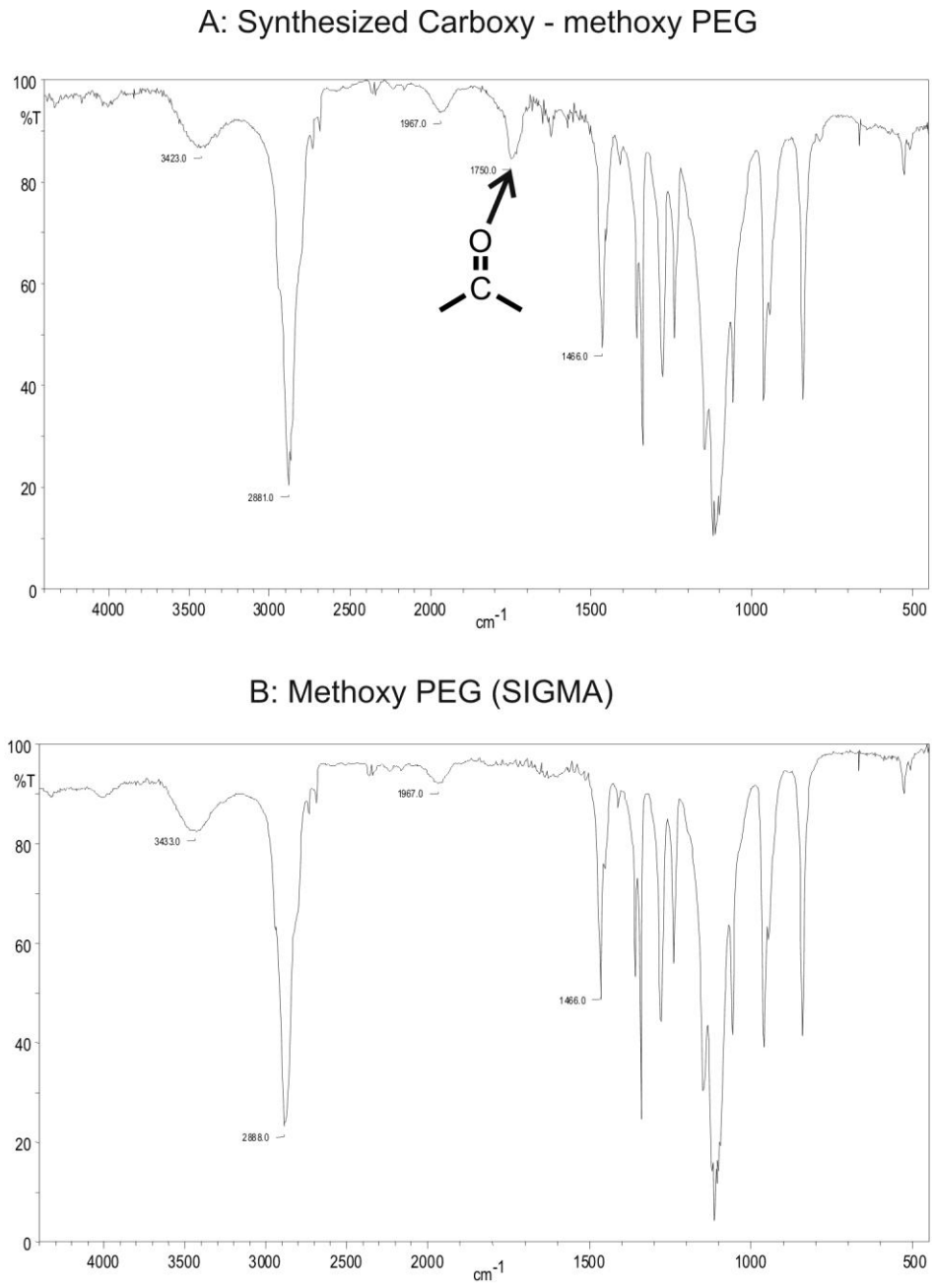


Figure 3. IR spectra of synthesized Carboxy – methoxy PEG compared to the starting material Methoxy PEG (SIGMA). The absorbance in the region of 1750 cm-1 is characteristic for CO-groups in carboxy-methoxy-PEG.

Experimental Methods

Synthesis of amino-methoxy-PEG 2000

PEG 2000 (10 g, 5.0 mmol) was dried in vacuum at 60°C over night, dissolved in 150 ml dry methylene chloride containing 10 ml pyridine. Then 4-toluene sulfonylchloride (27 g, 143 mmol) was added to the solution and the mixture stirred under Argon at room temperature for 24 hours and then under reflux for 4 hours. The reaction

mixture was concentrated to about 50% of the original volume and the polymer precipitated from this solution by dropwise addition of 400 ml anhydrous diethylether under vigorous stirring. After 30 min the precipitate was filtered and thoroughly washed with cold diethylether. The thus obtained tosylate was twice crystallized from absolute ethanol, filtered, washed with diethylether and dried under vacuum. The yiel was 9.36 g.

2.8 g of the polymer (3.6 mmol) and 5 g K-phthalimide (26.9 mmol) were dissolved in 60 ml dry DMF and the mixture stirred over night under Argon and then refluxed for 3 hours. The precipitate was filtered off, and the product precipitated from the clear filtrate by dropwise addition of 300 ml dry anhydrous diethyl ether with stirring, kept for 1 hour at – 10°C, the precipitate then filtered and washed several times with diethyl ether. The precipitate was dissolved again in the minimal amount of chloroform ($CHCl_3$), filtered, concentrated to half the volume and then the polymer precipitated again by addition of diethylether, filtered, washed with diethylether and dried in vacuum to give the the phthalimide of PEG-monomethlyether. The yield was 4.11 g

Phthalimide-PEG-monomethylether (6 g) was dissolved in 80 ml absolute ethanol and 6 ml hydrazine-hydrate (6 ml) added and refluxed under Argon for 18 hours. The mixture was cooled to 10°C, the product precipitated by dropwise addition of 80 ml anhydrous diethyl ether under stirring. The precipitate was filtered, washed with ether and dissolved in the minimum amount of dichloromethane (CH_2Cl_2). After filtering again the clear filtrate was concentrated and the polymer precipitated by dropwise addition of diethylether. The thus obtained amino PEG monomethylether was filtered, washed several times with diethyl ether and dried under vacuum. The yield was 2 g.

Synthesis of carboxy-methoxy-PEG

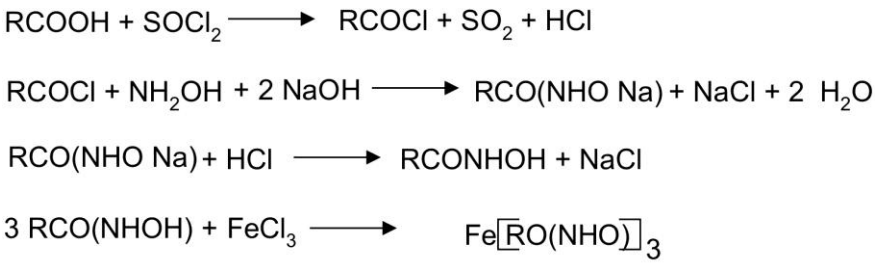
Methoxy-PEG 2000 (20 g, 10 mmol) was dissolved in 50 ml dry tetrahydrofurane (THF), 2.86 g (30 mmol) chloroacetic acid and 7 ml (50 mmol) triethyl amine were then added and the mixture stirred at 40°C for 4 hours. The product was then precipitated with 100 ml hexane, filtered, washed with hexane and dried in vacuum. The yield was 22.5 g of a white powder.

This white powder was then dissolved in 120 ml H_2O, 10 ml HCl (conc.) added, stirred for a few minutes, and then extracted with 3 x 20 ml $CHCl_3$, the extracts

combined, dried over Na_2SO_4 and the solvent then evaporated. The yield was 17.66 g.

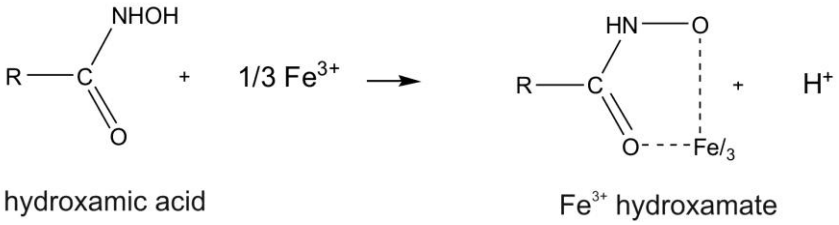
Detection of the carboxyl group in carboxy-methoxy-PEG

Proof of carboxy content was carried out according to the procedure by Feigl F. (1960). Carboxylic acids can be first reacted with thionyl chloride forming the acd chloride and it then readily gives the alkali salt of hydroxamic acid on treatment with hydroxylamine and alkali (Scheme 3).



Scheme 3

All hydroxamic acids give a red or violet color with ferric chloride by formation of water soluble inner complex salts (Scheme 4).



Scheme 4

The conversion into hydroxamic acid and the formation of hydroxamate is used as a test for carboxylic acids and their derivates.

Procedure:

Five drops of thionylchloride were added to 10 mg of synthesized carboxy-methoxy-PEG. The mixture was then evaporated to dryness on a heating plate to convert the carboxylic acid into its chloride. Five drops of saturated alcoholic solution of hydroxylamine chloride were then added and a few drops of alcoholic alkali until the

liquid reacted basic. The mixture was then acidified with a few drops of 0.5 M HCl. Reaction took place during reheating by formation of hydroxamic acid.

The mixture was treated with five drops of 1% aqueous solution of ferric chloride ($FeCl_3$). In presence of carboxyl groups the color changes from brown-red to dark violet.

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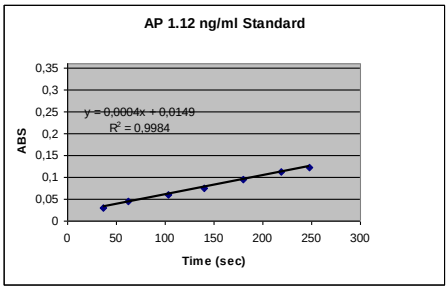
Appendix of Chapter 1

1.1 Calculation of calibration curve for amino group detection using AP enzyme assay

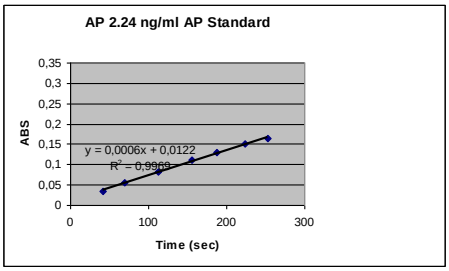
A. 1.12 ng/ml AP-standard

Time (sec)	ABS
37	0,03
62	0,044
103	0,06
140	0,076
180	0,096
219	0,113
248	0,123



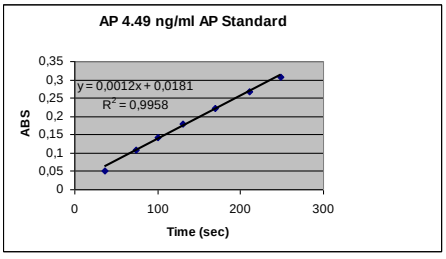
B. 2.24 ng/ml AP-standard

Time (sec)	ABS
0,035	41
0,055	70
0,082	112
0,111	156
0,131	187
0,15	223
0,164	253



C. 4.49 ng/ml AP-standard

Time (sec)	ABS
37	0,052
74	0,109
101	0,143
131	0,179
170	0,222
211	0,267
248	0,307



D. Calibration curve

AP concentration	Slope
1,12	0,0004
2,24	0,0006
4,49	0,0012

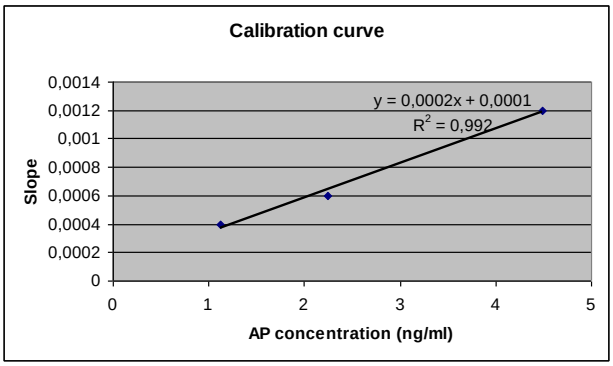
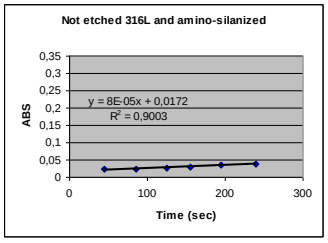


Figure 1. The calibration curve was calculated by measuring the extinction of several concentrations of AP over the time separately. The definite concentration of each AP and its slopes (A, B, C) are used to give the equation for standard function (y = 0.0002x + 0.0001) of calibration curve (D). The amount of immobilized enzymes onto carriers (Figure 2 and 3) was calculated using this standard calibration function.

1.2 Quantification of amino groups with glutaraldehyde immobilized AP onto amino-silanized carriers

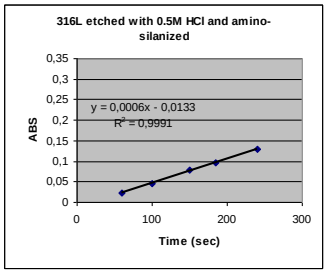
A. Not etched 316L and amino-silanized 316L stainless steel

Time (sec)	ABS
45	0,023
85	0,023
125	0,026
155	0,028
195	0,036
240	0,037



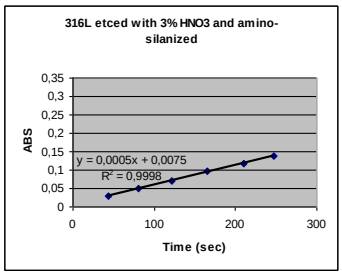
B. 316L stainless steel etched with 0.5 M HCl and Amino-silanized

Time (sec)	ABS
60	0,023
100	0,045
150	0,078
185	0,096
240	0,13



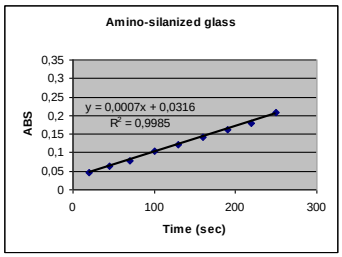
C. 316L stainless steel etched with 3% HNO_3 and amino-silanized

Time (sec)	ABS
43	0,03
80	0,05
121	0,072
165	0,096
210	0,118
247	0,139



D. Amino-silanized glass

Time (sec)	ABS
20	0,045
45	0,064
70	0,078
100	0,103
130	0,122
160	0,143
190	0,163
220	0,18
250	0,208



F. Amino-silanized ceramics

Time (sec)	ABS
28	0,02
64	0,029
94	0,034
142	0,044
183	0,053
216	0,061
246	0,067

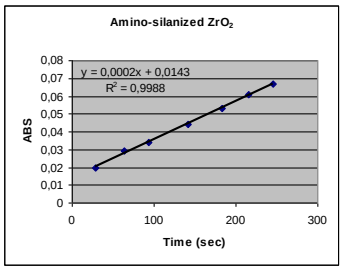
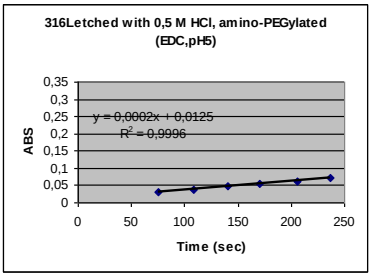


Figure 2. Measurement of AP activation on amino-silanized carriers. The slope of each sample is proportional to the concentration of the enzyme, which should be somewhat equal to the number of amino groups on the surface of the carrier. Using the standard equation (Figure 1 D) and the slope of each sample the amount of immobilized amino groups can be calculated. The highest number of detected amino groups was observed for ceramics (F) than glass (D) material. After etching of 316 L stainless steel a relative equal AP activation was observed (B, C), however by the silanized non etched 316L stainless steel no AP activation was measured (A).

1.3 Quantification of amino groups with glutaraldehyde immobilized AP onto amino-PEGylated carriers

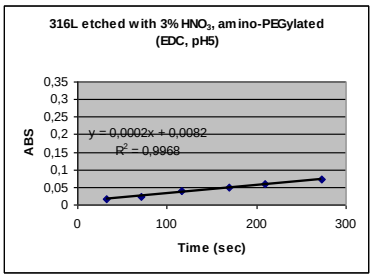
A. 316L stainless steel etched with 0.5 M HCl, amino-PEGylated (EDC, pH5

Time (sec)	ABS
75	0,031
108	0,039
140	0,047
170	0,055
206	0,063
237	0,071



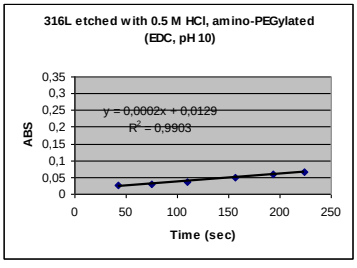
B. 316L stainless steel etched with 3% HNO_3, amino-PEGylated (EDC, pH5)

Time (sec)	ABS
32	0,015
71	0,024
117	0,038
169	0,049
209	0,058
273	0,072



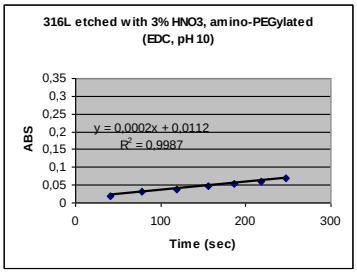
C. 316L stainless steel etched with 0.5 M HCl, amino-PEGylated (EDC, pH10)

Time (sec)	ABS
42	0,025
75	0,03
110	0,037
156	0,048
193	0,059
224	0,067



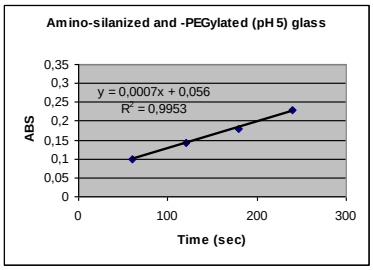
D. 316L stainless steel etched with 3% HNO_3, amino-PEGylated (EDC, pH10)

Time (sec)	ABS
41	0,02
78	0,03
119	0,039
156	0,048
186	0,054
218	0,061
247	0,069



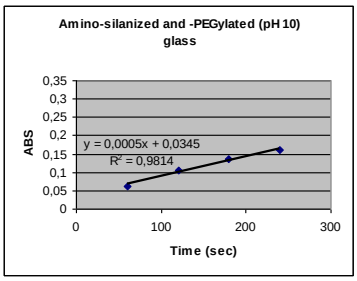
E. Amino-silanized and amino-PEGylated (pH 5) glass

Time (sec)	ABS
60	0,099
120	0,144
180	0,179
240	0,23



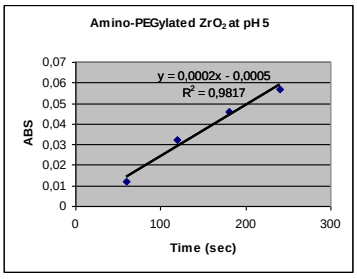
F. Amino-silanized and amino-PEGylated (pH 10) glass

Time (sec)	ABS
60	0,062
120	0,106
180	0,137
240	0,161



G. Amino-silanized and amino-PEGylated (pH 5) ceramics

Time (sec)	ABS
60	0,012
120	0,032
180	0,046
240	0,057



H. Amino-silanized and amino-PEGylated (pH 10) ceramics

Time (sec)	ABS
60	0,013
120	0,021
180	0,034
240	0,046

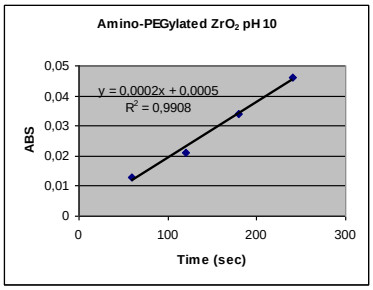


Figure 3. Measurement of AP activation on amino-PEGylated carriers. Using the standard equation (Figure 1 D.) and the slope of each sample the amount of immobilized amino groups can be calculated. The highest detected amount of amino groups was observed for amino-PEGylated ceramics (F, G) than glass at pH 5 (E). The amino-PEGylated 316L stainless steel samples (A, B, C, D) show relatively similar amount of amino groups.

Appendix of Chapter 5

2.1 Mass spectrometry analysis of purified immune serum of rabbit 52 using Gulsenit conjugated with PLGA

Rabitt 52

gi|126723746 Mass: 70861 Queries matched: 1021

serum albumin precursor [Oryctolagus cuniculus]

```
   1 MKWVTFISLL FLFSSAYSRG VFRREAHKSE IAHRFNDVGE EHFIGLVLIT
  51 FSQYLQKCPY EEHAKLVKEV TDLAKACVAD ESAANCDKSL HDIFGDKICA
 101 LPSLRDTYGD VADCCEKKEP ERNECFLHHK DDKPDLPPFA RPEADVLCKA
 151 FHDDEKAFFG HYLYEVARRH PYFYAPELLY YAQKYKAILT ECCEAADKGA
 201 CLTPKLDALE GKSLISAAQE RLRCASIQKF GDRAYKAWAL VRLSQRFPKA
 251 DFTDISKIVT DLTKVHKECC HGDLLECADD RADLAKYMCE HQETISSHLK
 301 ECCDKPILEK AHCIYGLHND ETPAGLPAVA EEFVEDKDVC KNYEEAKDLF
 351 LGKFLYEYSR RHPDYSVVLL LRLGKAYEAT LKKCCATDDP HACYAKVLDE
 401 FQPLVDEPKN LVKQNCELYE QLGDYNFQNA LLVRYTKKVP QVSTPTLVEI
 451 SRSLGKVGSK CCKHPEAERL PCVEDYLSVV LNRLCVLHEK TPVSEKVTKC
 501 CSESLVDRRP CFSALGPDET YVPKEFNAET FTFHADICTL PETERKIKKQ
 551 TALVELVKHK PHATNDQLKT VVGEFTALLD KCCSAEDKEA CFAVEGPKLV
 601
ESSKATLG
```

gi|30794280 Mass: 71274 Score: 786 Queries matched: 82 emPAI: 2.21

Albumin[Bostaurus]

```
  1 MKWVTFISLL LLFSSAYSRG VFRRDTHKSE IAHRFKDLGE EHFKGLVLIA
 51 FSYLQQCPF DEHVKLVNEL TEFAKTCVAD ESHAGCEKSL HTLFGDELCK
101 VASLRETYGD MADCCEKQEP ERNECFLSHK DDSPDLPKLK PDPNTLCDEF
151 KADEKKFWGK YLYEIARRHP YFYAPELLYY ANKYNGVFQE CCQAEDKGAC
201 LLPKIETMRE KVLTSSARQR LRCASIQKFG ERALKAWSVA RLSQKFPKAE
251 FVEVTKLVTD LTKVHKECCH GDLLECADDR ADLAKYICDN QDTISSKLKE
301 CCDKPLLEKS HCIAEVEKDA IPENLPPLTA DFAEDKDVCK NYQEAKDAFL
351 GSFLYEYSRR HPEYAVSVLL RLAKEYEATL EECCAKDDPH ACYSTVFDKL
401 KHLVDEPQNL IKQNCDQFEK LGEYGFQNAL IVRYTRKVPQ VSTPTLVEVS
451 RSLGKVGTRC CTKPESERMP CTEDYLSLIL NRLCVLHEKT PVSEKVTKCC
501 TESLVNRRPC FSALTPDETY VPKAFDEKLF TFHADICTLP DTEKQIKKQT
551 ALVELLKHKP KATEEQLKTV MENFVAFVDK CCAADDKEAC FAVEGPKLVV
601 STQTALA
```

Figure 4. Mass spectroscopy analyses of purified immune serum of rabbit 52 using Gulsenit conjugated with PLGA showing a homology to albumins.

More Details

Type of mass spectrometry: Thermo Finnigan LTQ

Proteins found:

- Serum albumin precursor [Oryctolagus cuniculus], Queries matched: 1021 (red letters)
- Albumin [Bos taurus], Queries matched: 82 (red letters)

Used scoring system: Search algorithm: Mascot: Mascot incorporates a probability based implementation of the Mowse algorithm

Appendix of Chapter 6

3.1 Absorbance of different dilutions of immune and preimmune serum

Table 1.

Dilution	ABS of preimmune serum from R.96	ABS of preimmune serum from R. 95	ABS of immune serum from R. 95 after 5th immunization	ABS of immune serum from R. 95 after 7th immunization	ABS of immune serum from R. 52	ABS of immune serum from R. 53
100	0,173	0,116	0,333	0,058	0,05	0,028
500	0,008	0,015	0,044	0,042	0,004	0,026
1000	0,007	0,013	0,031	0,026	0,003	0,025
1500	0,002	0,006	0,025	0,016	0,001	0,021
2000	0,004	0,007	0,018	0,013	0,003	0,019
2500	0,003	0,007	0,012	0,015	0,002	0,016
3000	0,001	0,003	0,01	0,011	0,001	0,012
4000	0,004	0,006	0,007	0,006	0,002	0,012

Table 2.

Dilution	ABS of R.96 after 3rd immunization	ABS of R. 96 after 4th immunization	ABS of R. 96 after 5th immunization	ABS of R. 96 after 7th immunization	ABS of R. 96 after 8th immunization	Negative control
100	0,898	0,76	0,581	0,309	0,341	0,061
500	0,503	0,461	0,264	0,204	0,17	0,061
1000	0,27	0,398	0,212	0,134	0,117	0,062
1500	0,161	0,334	0,159	0,124	0,105	0,05
2000	0,134	0,276	0,11	0,124	0,073	0,051
2500	0,133	0,163	0,119	0,102	0,072	0,05
3000	0,081	0,139	0,111	0,091	0,069	0,049
4000	0,081	0,114	0,089	0,088	0,065	0,052
5000	0,073	0,125	0,077	0,05	0,051	0,054
6000	0,063	0,107	0,07	0,055	0,038	0,053
7000	0,06	0,107	0,048	0,047	0,037	0,058
8000	0,045	0,076	0,052	0,038	0,038	0,058
9000	0,055	0,087	0,06	0,035	0,043	0,055
10000	0,081	0,09	0,048	0,034	0,043	0,055

Table 3.

Dilution	ABS of R.95 after 3rd immunization	ABS of R. 95 after 4th immunization
100	0,465	0,134
500	0,092	0,039
1000	0,058	0,036
1500	0,047	0,02
2000	0,041	0,02
2500	0,027	0,018
3000	0,027	0,017
4000	0,013	0,015
5000	0,029	0,013
6000	0,012	0,019
7000	0,021	0,015
8000	0,023	0,013
9000	0,014	0,008
10000	0,026	0,008

Table 1, 2, and 3. ABS values of preimmune- and immune- serum of R. 95 and 96 from after the 3rd to after the 8th immunization. The negative control is tested with preimmune serum of rabbit 96 after 8th immunization and an average value is taken (from all ABS values of the negative control). This average value is 0.05 (=blank). All presented ABS values in table 1, 2 and 3 are after subtraction of blank.

3.2 Absorbance curves of different dilutions from preimmune serum and immune serum

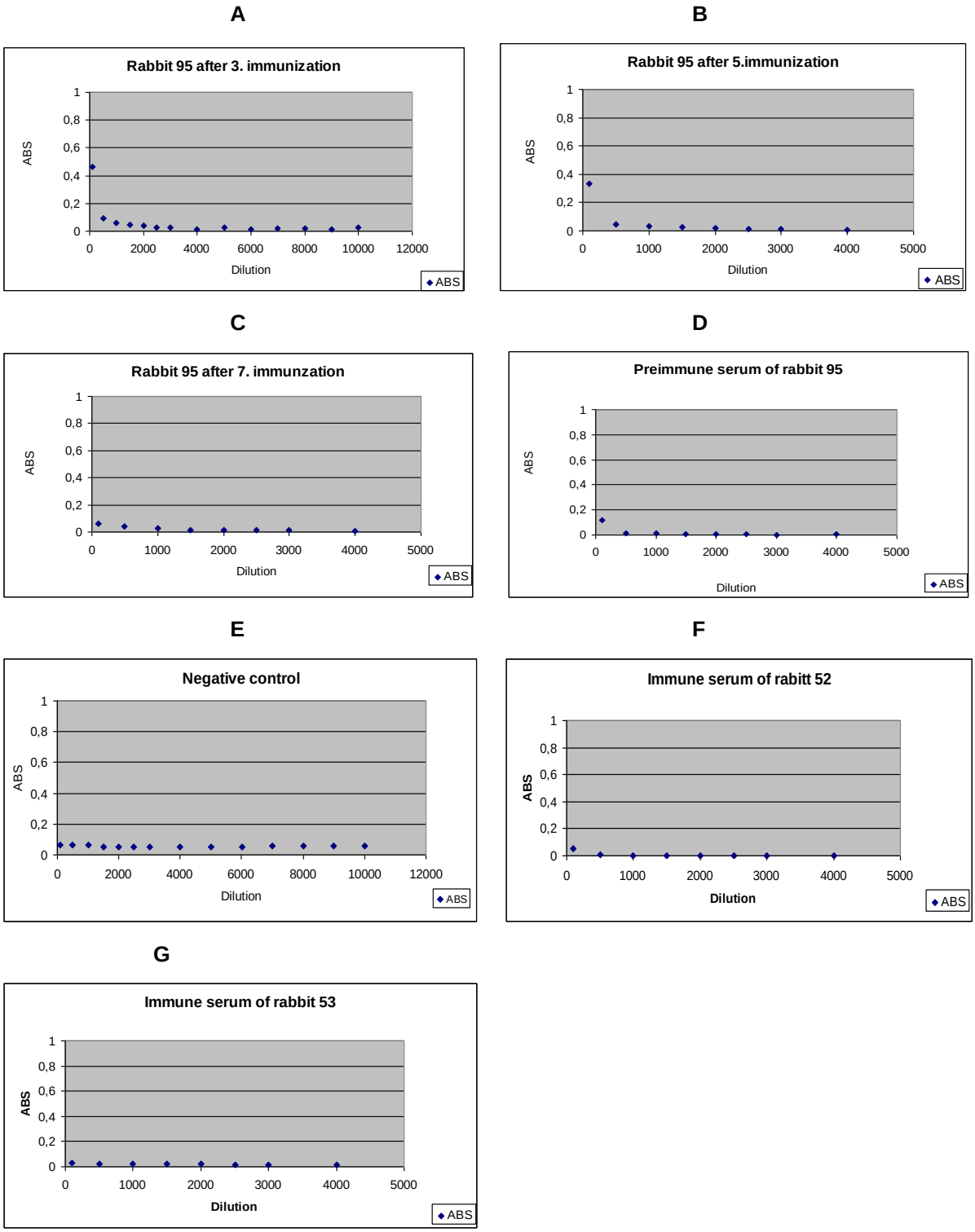


Figure 5. Absorbance curves of different dilutions from preimmune serum and immune serum of Rabbit 95, 52 and 53 and negative control. Preimmune serum of rabbit 95 (D) shows no signal. A very low titer was found in rabbit 95 after 3rd, 5th, 7th and 8th (A, B, C) immunization. A negative titer was observed in rabbit 52 (F) and rabbit 53 (G). Rabbit 52 and 53 were immunized only with PLGA-NPs.

3.4 Mass spectroscopy results for the upper and lower band

Table 1, 2 and 3 summarize results of mass spectrometry for the and lower band of immune sample after the fifth immunization on a native gel:

Table 1

Version: unknown
Charge States Calculated: unknown
Deisotoped: unknown
Textual Annotation: unknown
Database Name: the nr_2009-130.fa database
Version: unknown
Taxonomy: Mammalia
Number of Proteins: 732230
Search Engine: Mascot
Version: Mascot
Samples: All Samples
Fragment Tolerance: 0,80 Da (Monoisotopic)
Parent Tolerance: 2,0 Da (Monoisotopic)
Fixed Modifications: +57 on C (Carbamidomethyl)
Variable Modifications: +16 on M (Oxidation)
Database: the nr_2009-10-30.fa database (selected for Mammalia, unknown version, 732230 entries)
Digestion Enzyme: Trypsin
Max Missed Cleavages: 2
Peptide Thresholds: Spectrum Mill: score greater than 11, SPI% greater than 0, Fwd-Rev greater than -2147483647, Rank 1-2 greater than -2147483647, and norm Fwd-Rev greater than 0, Phenyx: z-Scores of greater than 8,0,OMSSA: -Log (E-Value) scores of greater than 2,0, X! Tandem: -Log(Expect Scores) scores of greater than 2,0, Mascot: ion scores must be greater than both the associated identity scores and 35, 30, 30 and 40 for singly,doubly, triply and quadruply charged peptides, Sequest: deltaCn scores of greater than 0,10 and XCorr scores of greater than 1,8, 2,5, 3,5 and 3,5 for singly, doubly, triply and quadruply charged peptides
Protein Thresholds: 2 peptides minimum

Table 2. Identified proteins and accession number of upper and low band of immune serum after fifth immunization.

Number	Identified Proteins	Accession Number
1	apolipoprotein A-I (predicted) [Oryctolagus cuniculus]	gi|217418328
2	inter-alpha (globulin) inhibitor H1 [Oryctolagus cuniculus], gi|6579183|dbj|BAA88322.1| inter-alpha-trypsin inhibitor heavy chain H1 [Oryctolagus cuniculus]	gi|130491439
3	inter-alpha-trypsin inhibitor heavy chain2 [Oryctolagus cuniculus], gi|11041696|dbj|BAB17301.1| inter-alpha-trypsin inhibitor heavy chain2 [Oryctolagus cuniculus]	gi|130498817
4	complement component 3 [Rattus norvegicus], gi|149028133|gb|EDL83571.1| rCG45082 [Rattus norvegicus]	gi|158138561
5	serum albumin precursor precursor [Oryctolagus cuniculus], gi|44889024|sp|P49065.2|ALBU_RABIT RecName: Full=Serum albumin; Flags: Precursor, gi|29653363|gb|AAB58347.2| serum albumin precursor [Oryctolagus cuniculus]	gi|126723746
6	liver transferrin [Oryctolagus cuniculus], gi|1751|emb|CAA41424.1| liver transferrin [Oryctolagus cuniculus]	gi|156119356 (+2)
7	Chain A, Complex Of Bdellastasin With Porcine Trypsin	gi|257472074
8	paraoxonase 1 [Oryctolagus cuniculus], gi|130676|sp|P27170.2|PON1_RABIT RecName: Full=Serum paraoxonase/arylesterase 1; Short=PON 1; AltName: Full=Serum aryldialkylphosphatase 1; AltName: Full=A-esterase 1; AltName: Full=Aromatic esterase 1, gi|165660|gb|AAA31452.1| serum paraoxonase, gi|7579911|gb|AAB27713.2| paraoxonase; PON [Oryctolagus cuniculus]	gi|126722853 (+2)
9	complement component 3 [Oryctolagus cuniculus], gi|116596|sp|P12247.1|CO3_RABIT RecName: Full=Complement C3 alpha chain, gi|164863|gb|AAA31190.1| complement component C3 alpha-chain	gi|126723309
10	hemopexin precursor [Oryctolagus cuniculus], gi|1708184|sp|P20058.2|HEMO_RABIT RecName: Full=Hemopexin; Flags: Precursor, gi|6137344|pdb|1QHU|A Chain A, Mammalian Blood	gi|130500366

	Serum Haemopexin Deglycosylated And In Complex With Its Ligand Haem, gi|7245995|pdb|1QJS|A Chain A, Mammalian Blood Serum Haemopexin Glycosylated-Native Protein And In Complex With Its Ligand Haem, gi|7245996|pdb|1QJS|B Chain B, Mammalian Blood Serum Haemopexin Glycosylated-Native Protein And In Complex With Its Ligand Haem, gi|433687|emb|CAA34452.1| hemopexin precursor [Oryctolagus cuniculus]	
11	PREDICTED: similar to complement component C5 [Equus caballus]	gi|194225695
12	keratin 1 [Homo sapiens], gi|39794653|gb|AAH63697.1| Keratin 1 [Homo sapiens]	gi|11935049 (+2)
13	Chain A, Crystal Structure Of The Rabbit Igg Fc Fragment, gi|203282278|pdb|2VUO|B Chain B, Crystal Structure Of The Rabbit Igg Fc Fragment	gi|203282277 (+2)
14	cytokeratin 9 [Homo sapiens]	gi|435476 (+1)
15	PREDICTED: similar to complement component 5 [Macaca mulatta]	gi|109110418
16	PREDICTED: similar to keratin 10 isoform 2 [Macaca mulatta]	gi|109115260 (+11)
17	complement component 3 [Mus musculus], gi|28175786|gb|AAH43338.1| Complement component 3 [Mus musculus]	gi|126518317 (+3)
18	immunoglobulin heavy chain VDJC-alpha transrecombinant region [Oryctolagus cuniculus]	gi|4063643
19	Ig kappa chain b4 [Oryctolagus cuniculus]	gi|165371
20	RecName: Full=Haptoglobin; Contains: RecName: Full=Haptoglobin alpha chain; Contains: RecName: Full=Haptoglobin beta chain	gi|123511 (+3)

Table 3. Molecular weight, number of unique peptides and percent coverage of Identified Proteins in upper and lower band of immune serum after fifth immunization

Number	Upper band			Low band	
	molecular weight	number of unique peptides	percent coverage	number of unique peptides	percent coverage
1	31 kDa	12	44%	10	36%
2	100 kDa	11	12%	12	16%
3	106 kDa	7	7,60%	9	10%
4	186 kDa	6	3,80%	4	2,60%
5	69 kDa	7	14%	6	12%
6	77 kDa	4	6,80%	12	16%
7	23 kDa	4	24%	3	20%
8	40 kDa	2	5,00%	2	5,30%
9	82 kDa	4	7,40%	2	3,90%
10	52 kDa	0	0	2	5,20%
11	192 kDa	5	3,30%	2	1,50%
12	66 kDa	7	14%	2	3,40%
13	25 kDa	2	12%	0	0
14	62 kDa	5	11%	0	0
15	189 kDa	2	1,40%	0	0
16	62 kDa	3	5,90%	0	0
17	186 kDa	2	1,70%	2	1,70%
18	30 kDa	2	8,60%	0	0
19	23 kDa	2	11%	0	0
20	36 kDa	0	0	2	7,00%

Materials

Acetic acid	J.T. Baker
Bovine serum albumin (BSA)	Sigma
2-Mercaptoethanol	Fluka
3-Aminopropyltriethoxysilane	Sigma
4-Toluene sulfonylchloride	J.T. Baker
Acetone	J.T. Baker
Acrylamide	Sigma
$AgNO_3$	Sigma
Amicon ultra centrifuge filter	Amicon
Amino-methoxy-PEG	Fluka
Anti rabbit IgG, HRP conjugated	Sigma
Anti-mouse IgG for HSA	Sigma
APS	Serva
Bisacrylamide	Sigma
BODIPY 493/503 (4,4-difluoro-1,3,5,7,8 pentamethyl, 4-bora-3a,4a-diaza-5-indacene), cat.no. D-3922	Invitrogen
Bradford reagent (Protein assay)	Bio-Rad
Bromophenol blue	Fluka
Carboxy-methoxy-PEG	Fluka
CBB-R250	Serva
Chitosan (MW 50 000-190 000Dalton,75-85% deacetylation degree)	Sigma
Chloroacetic acid	Sigma
Chloroform	J.T. Baker
Column (2.5 ml)	Mo Bi Tec
DIC	Sigma
Dichloromethane	J.T. Baker
Diethyl ether	J.T. Baker

DMF	Pierce
DMSO	Merck
Dynabeads® M-280 Sheep anti-Rabbit	Invitrogen
Dynabeads® M-450 Epoxy	Invitrogen
Dynal® MPC™, Magnetic Particle Concentrator	Invitrogen
DYNATECH Microtiter™ 2 plates	DYNATECHLABORATORIES, INC (U.S.A)
EDC (N-3-Dimethylaminopropyl-N'-ethylcarbodiimide)	Pierce
Ellman's reagent (DTNB), MW 396.35	Pierce
Ethanol	J.T.Baker
Ethyl acetate	Merk
Ethylene diamine	Sigma
$FeCl_3$	Sigma
Filter (10 µm pore)	Mo Bi Tec
Fish gelatine	Sigma
Formaldehyde	J.T.Baker
Gelatine from cold water fish skin	Sigma
Glass powder	Sartorius
Glass slides (Ø12 mm)	Assectent
Glassfiber Prefilter (1µm)	Millipore Filter
Glycerol	Fluka
Glycine	AppliChem
Gulsenit	MAGINDAG, FEUERFEST, Leoben, Austria
H_2SO_4	J.T.Baker
$HAuCl_4 \cdot 3H_2O$	Sigma-Aldrich
HCl	J.T.Baker
HNO_3	J.T.Baker
Human serum albumin (HSA)	Sigma
Hydrazine-hydrate	Sigma

Isopropanol	Fluka
KCN	Sigma
K-Phthalimide	Fluka
LDH (cow)	Sigma
LDH (pork)	Roche
LDH (rabbit)	Sigma
Lipoic acid	Sigma
Maxisorp microtiterplate	NUNC-Immuno
Methanol	J.T.Baker
Methylene chloride	J.T.Baker
$MgCl_2$	Sigma
Mk anti Human HSA -Ab (7.8 mg/ml)	Sigma
Mk anti ß-galactosidase -Ab (2.5 mg/ml)	Promega
Na_2CO_3	Sigma
Na_2HPO_4	Sigma
Na_2SO_4	Riedel-de Haen
Na_3PO_4	Fluka
N-Acetyl-L-cysteine	Sigma
NaCl	Riedel-de Haen
NaH_2PO_4	Merck
$NaHCO_3$	Riedel-de Haen
NaOAC	Fluka
NaOH	Riedel-de Haen
Natrium azide	Sigma
NC membrane (pore size 0.45 µm)	Protran BA 85, Schleicher & Schuell)
NHS (N-Hydroxysuccinimide)	Sigma
Nile red	Sigma
OPA (O-phtaldialdehyde)	Fluka
p-Chloro-anil	Fluka
Perchloric acid	Sigma
Peroxidase (502 U/ mg)	Fluka
Peroxidase antibody (41mg /ml)	Sigma

Phenol	Fluka
Pk anti BSA-Ab (3.6 mg/ml)	Sigma
Pk anti Peroxidase Ab (41 mg/ml)	Sigma
PLGA, 50:50 lactide/glycolide: Resomer RG503H (acid number min 3 mg KOH/g), Resomer RG 502 H (acid number min 6 mg KOH/g)	Boehringer Ingelheim
Pluronic F-68	Sigma
p-NPP	Fluka
Prick-/Scratch testing solutions (3 ml)	Allergopharma Vertriebsges
Protein A, Antibody Purification kit	Sigma stock no. PURE-1A
Protein G	Fluka
Protein Molecular Weight Marker MBI (PageRuler Protein Ladder, #SM0661)	Fermentas
Pyridine	Sigma
Quant-iT™ Protein Assay Kits	Invitrogen
Rabbit anti bovine lactoglobulin Ab	Bethyl
Ringer' solution	Baxter
SDS	Serva
SH-PEG, MW 5000	Sigma
Silica amine microspheres	Bangs Laboratories, Inc.
Silica gel plates gel 60 with fluorescence indicator	MERCK
Sodium hydrosulfite ($Na_2S_2O_4$)	Sigma
ß-Lactoglobulin from bovine milk	Sigma
Stainless-steel L 316 (foil)	Goodfellow
Succinic anhydride	Merk
Sulfo-NHS	Pierce
Sulfo-SDTB	Pierce
Sulfo-SMCC	Pierce

TEMED	Bio-Rad
THF	J.T. Baker
Thionyl chloride ($SOCl_2$)	Sigma
TMB	Sciotec
TNBS	Sigma
Toluene	J.T. Baker
TPP	Sigma
Triethyl amine	Sigma
Tris Base Ultrapure	US Biological
Trisodium citrate	Riedel-de Haen
Tween 20	Sigma
Vivaflow 50; 100000 MWCO PES	Sartorius Vivascience G.m.b.H
Zirconia (ZrO2-TZP-A BiO-HIP**)**	Z-Systems AG

Curriculum Vitae

Curriculum Vitae- Mag. Haifa Al-Dubai

PERSONAL DETAILS

Date of birth	December 20th, 1968
Place of birth	Taiz - Yemen
Citizenship	Austrian
Social state	Married, two daughters
Languages	Arabic (mother tongue)
	German (fluent in speaking and writing)
	English (good in speaking and writing)
Address	Simmeringer Hauptstraße16/2/11, Vienna, Austria
E-mail	haifa.al-dubai@unicvie.ac.at

EDUCATION

2005-2009 PhD in Biochemistry: University of Vienna, Department of Biochemistry, ***Advisor:*** Univ. Prof. Dr. Fritz Pittner Department of Biochemistry University of Vienna and MFPL Dr. Bohr Gasse 9 A-1030 Vienna, Austria, ***Thesis title:*** Feasibility Studies for Development of Specific Re-loadable Binding Sites for Drug Delivery Systems Immobilized onto Implant Surfaces

2004 - 2005 Education for agent to drugs sale, at Pharma Akademie Österreich (PAÖ) and the courses took place in Liechtensteinstraße 127/1/9, A-1090 Vienna (Austria), The first section of the courses included medical issues of the following human tracts or systems and their diseases (histology, locomotor system, cardiovascular system, respiratory tract, gastrointestinal tract, urogenital tract, blood system, immunology, hormone system, neurology, biochemistry, dermatology, pathology, ENT, ophthalmology, psychiatry, microbiology, active ingredient) and the second section of the courses

included pharmacological issues (chemistry/physic, pharmaceuticals law and technology)

Dec.1998 Masters in Microbiology and Biochemistry, University of Vienna, Department of medical Biochemistry (Austria)

Advisor: Univ. Prof. Dr. Georg Weitzer

Thesis title: Culture and in Vitro Differentiation of Embryonic Stem Cells: Analysis of Intermediate Filament Expression in Transgenic Cells with different Desmin Alleles

RESEARCH EXPERIENCE

2005-2009 **PhD Candidate:** Department of Biochemistry University of Vienna and MFPL (Austria), The fundamental goal of my thesis research is to realize the development of the reloadable drug delivery system for biocompatible coated implant materials. To achieve this goal, my thesis project focused on achieving three fundament objectives. The first objective focused on the production of covalent binding of a biocompatible coating and development of analytical tests for proof of a successful set-up. The second objective focused on the development of new analytical tools for demonstration of protein binding to solid surfaces and binding of PLGA-nanoparticles as a drug delivery system to the immobilized receptor on the implant surface. The third objective focused on providing a proof of concept of binding of nanoparticles serving as drug carriers to immobilized receptors onto the biocompatible-coated implant surfaces and long term test showing degradation of nanoparticles and reloadability.

2005 **Research Associate *(6 months)*:** Department of Biochemistry University of Vienna and MFPL (Austria), in the lab of Prof. Pittner Fritz. As a research associated, I worked on the adaptation of gold colloid nanoparticles synthesis in different sizes and engineering of lateral-flow test for rapid diagnostic application.

1996-1998 **Master student:** University of Vienna, Department of medical Biochemistry (Austria), The overall objective of my master thesis project focused on the development regulation of gene expression patterns of the intermediate filament proteins desmin, vimentin and cytokeratin 8 during cardiomyocyte differentiation in embryoid bodies. A system to optimize the in vitro

differentiation system of cardiomycten in early emboryonic differentiation events in vitro was adapted using embryoid bodies. Moreover the effect of leukaemia inhibitory factor on cardiomyogensis and life duration of cardiomyocytes in embryoid bodies was tested in this system. In addition the gene expression pattern of previously mentioned intermediate filament proteins was compared in emboryoid bodies generated from different embroyonic stem cells lines [AB2.2 (wild type), 1B44 (desmin ΔN48 amino acid) and knock out ($des^{-/-}$] .

TEACHING EXPERIENCE

2006-2009 **Instructor and advisor of Masters and Trainees Students,** during my tenure as a PhD candidate at the MFPL, I supervised several Trainees Students (6) and master students (4). Responsibilities included training the students on standard laboratory techniques, organic synthesis, protein measurement methods and purification methods, gel electrophoresis, Western blot, analytical chemistry (CLISA), ELISA, immobilization of polymers onto solid surfaces, design, plan and participate in the execution of their experiments.

2009 **Teaching assistant,** in protein chemistry practical I at the Department of Biochemistry supervised experiments to second and third year bachelor students in chemistry, molecular biology, genetic and microbiology. Responsibilities and taught a class of 8-10 students on the theory and practice of protein determination, isolation from eukaryotic cells, purification and analysis, technique for homogenisation of plant cells to study photosynthetic pigments, enzyme kinetic and regulation, included, exam questions and grading of exams.

2006- 2009 **Tutor in protein chemistry** practical I and II at the Department of Biochemistry, Taught and supervised bachelor and master level students in chemistry, molecular biology, genetic and microbiology practical courses.

FIELDS OF EXPERTISE

Scientific areas and techniques in which I have extensive knowledge experience are listed under each category.

Immobilization

- Silanization
- Immobization of polymers and antibodies molecules or fragments onto solid surfaces
- Determination of immobilized molecules or polymers onto solid surfaces
- Modification of functional groups on solid surfaces

Immunization

- Preparation of sample injection
- Production of antibodies against biocompatible and biodegradable polymer
- Titerdetermination
- Antibodies purification from immune sera

Drug delivery system

- PLGA nano- or micro-particle preparation including enzyme, fluorescent dye or drug
- Chitosan nanopatricle preparation including fluorescent dye
- Degradation of PLGA-nanoparticles

Protein biochemistry

- Splitting of antibodies into fragments
- Protein purification techniques, including all chromatographic techniques
- SDS-PAGE and western blot
- Enzyme kinetics

Analytic chemistry

- Dot blot
- Immunoprecipitation
- ELISA
- CLISA

Organic Chemistry

- Organic synthesis
- Purification methods (thin layer chromatography, distillation, extraction…)
- Spectroscopic a analysis of small molecules using IR, HPLC

Cell biology

- Cell culture
- Embryonic stem cell culture adherent and in suspension: AB2.1, AB2.2, D3 and fibroblast cells (STO, SNL76/7)
- Embryoid bodies formation and in vitro differentiation
- Immuncytochemistry and Immunohistochemistry

Molecular biology

- Recombinant DNA techniques (Cloning, PCR)
- Gel electrophoresis, Western blotting, Northern blotting
- Isolation and analysis of RNA

Computer skills

- Microsoft (Word, Excel, Powerpoint, Corel Draw)

SCIENTIFIC TALKS AND POSTER PRESENTATIONS

TALKS in English

Feb. 1998 Department of medical Biochemistry, University of Vienna (Austria), take about differentiation of embryonic stem cells in vitro and expression of intermediate filament proteins in transgenic cells.

May 2008 Department for Biomolecular Structural Chemistry, University of Vienna (Austria) and MFPL, take at the Symposium of the Computational and Structural Biology Programme about Biocompatible coatings of medical implants allowing reloadable drug targeting and drug release.

POSTER PRESENTATIONS

2009 Live Imaging of Nano Gold Particles. Lichtscheidl I., Al-Dubai H., Maier I., Reipert S., Pittner F., Super and High Resolution Imaging Conference, Lipica – Slovenia

2008 Gold Cluster Labeling as an Alternative to ELISA. Al-Dubai H., Pittner G., Pittner F. 2nd International AllergoOncology Symposium, Vienna – Austria, (oral presentation in English)

2008 A novel dot-blotting method for immunoassays. Al-Dubai H., Hinterwirth H., Pittner F., Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGFI), Vienna – Austria, (oral presentation in English)

2005 Resonance Enhanced Fluorescence Biochips – Homogeneity and Corrosion Stability of Interlayer Systems in REA Biosensor Devices. Hagelmüller J., Al-Dubai H., Koelber M., Dragosits M., Gauglhofer C., Mayer C., Bauer G., Genov S., Pittner F., Schalkhammer T. Surface Plasmon Photonics 2 (SPP“), Graz- Austria

2005 Resonance Enhanced Cluster Absorption (REA) in Sensoric Applications. Hagelmüller J., Dragosits M., Gauglhofer C., Koelber M., Al-Dubai H., Maier

I., Pittner F., Schalkhammer T. Nanotechnology in Biodiagnostics and Analytics (NBDA) Conference, Grenoble - France

1997 Development regulation of intermediate filament protein expression in embryonic stem cell derived cardiomyocytes. Al-Dubai H., Weizer G. Annual meeting of the ÖBG (Österreichische Biochemische Gesellschaft) & ÖGGGT (Österreichische Gesellschaft für Genetic und Gentechnik), Vienna – Austria

P U B L I C A T I O N S (* CORRESPONDING AUTHOR)

1. Hinterwirth H. H., Strobl M., **Al-Dubai H.***, Analytical methods for detection of small amounts of amino groups on solid surfaces – a survey. Chemical Monthly (2009), ***In press,*** *Manuscript Number: MOCHEM-D-09-00441R2.*

2. **Al-Dubai H.***, Oberhofer G., Kerleta V., Hinterwirth H. H., Strobl M., Gabor F. ;Cleavage of Antibodies using Dihydrolipoamide and Anchoring of Antibody Fragments onto Biocompatible Coated Carriers; Chemical Monthly (2010), ***In press,*** *DOI: 10.1007/s00706-010-0284-6.*

3. **Al-Dubai H.***, Pittner G., Pittner F. A dot-blot test using gold colloid cluster technology as a miniaturizable alternative to ELISA and hapten inhibition tests; Chemical Monthly (2008) 139: 1531-1536.

4. **Al-Dubai H. ***, Strobl M., Lichtscheidl I., Pittner G., Pittner F. Immunosorbent assay using gold colloid cluster technology for determination of IgEs in patient sera. Journal of Biotechnology (2010), ***to be submitted soon***

5. **Al-Dubai H. ***, Hermann M., Pittner G., Pittner F., Gabor F. Production of Polyclonal Antibodies against the Biocompatible Polymer PLGA using Silica Amino Microspheres as a Carrier, Biotechnology Letters (2010), ***to be submitted soon.***

6. **Al-Dubai H.*,** Pittner G., Pittner F., Gabor F. Carrier with Binding Sites for Biodegradable Drug Containing Nanoparticles Allowing Reloadability; Open Biomedical Engineering Journal (2010) ***to be submitted soon.***

7. Hölligl A., Puz S., **Al-Dubai H**, Kim JU, Capetanaki Y, Weitzer G. Amino-terminally truncated desmin rescues fusion of des$^{(-/-)}$ myoblasts but negatively

affects cardiomyogenesis and smooth muscle development; Differentiation(2002) 523 (1-3): 229-33.

8. Bader A., Gruss A., Höllrigl A., **Al-Dubai H., C**apetanaki Y., Weitzer G. Paracrine promotion of cardiomyogensis in embryoid bodies by LIF modulated endoderm; Differentiation (2001) 68:31-43.

9. Bader A, **Al-Dubai H.**, Weitzer G. Leukemia Inhibitory Factor Modulates Cardiogenesis in Embryoid Bodies in Opposite Fashions; Circulation Research (2000) 86:787-794

PATENT

Prof. Pittner F., **Al-Dubai Haifa**, Hinterwirth H., Strobl M., Gold-cluster labelling as a simple test system for detection of specific proteins in microtiterplates, U 7117/DB, submitted to "Forschungsservice und internationale Beziehungen", Dipl. Ing. Claudia Doubek.

PROFESSIONAL EXPERIENCE

2006	Delivery of health lectures for Arabic women living in Vienna in cooperation with M-17 and FEM-Süd (Health Center) to encourage integration of Arabic women into Austrian society
1999- 2000	Sales representative, Karl Ohrfandl Company/Sigma-Laborzentrifugen

R E F E R E NCES

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Univ. Prof. Dr. Marcela Hermann: Max F. Perutz Laboratories, Department of Molecular Genetics, Medical University of Vienna, Dr. Bohrgasse 9, A-1030 Vienna, Austria, marcela.hermann@univie.ac.at

Univ. Prof. Dr. Irene Lichtscheidl: Institute of Cell Imaging and Ultrastructure Research, Althanstrasse 14 A-1091 Vienna, Austria, irene.lichtscheidl@univie.ac.at