DIPLOMARBEIT

Contributions to the Development of Novel Biocompatible Coatings of Medically Relevant Materials with Regard to "Drug-Targeting"-Efficiency

Beiträge zur Entwicklung neuartiger biokompatibler Oberflächenbeschichtungen medizinisch relevanter Materialien in Hinblick auf "Drug-Targeting"-Tauglichkeit

zur Erlangung des Akademischen Grades

Magister der Naturwissenschaften (Mag. rer. nat.)

an der Universität Wien

Verfasser:	Hinterwirth Helmut Hubert
Matrikel-Nummer:	0205847
Studienkennzahl:	A419
Studienrichtung:	Chemie
Betreuung durch:	Ao. Univ. Prof. Dr. Fritz Pittner

Wien, am 08.06.2009

Danksagung

An allererster Stelle möchte ich meiner Mutter Maria Hinterwirth danken, die mich das ganze Studium hindurch unterstützt hat. Auf diesem Wege möchte ich ihr für alles sagen:
DANKE!

Mein besonderer Dank gilt meinem Betreuer Professor Fritz Pittner, der stets ein offenes Ohr für mich hatte und von dem ich bei zahlreichen Gesprächen, Diskussionen und Brainstormings sehr viel lernen durfte.
Ein Dank gilt auch seiner lieben Frau Gisi, deren Tür fürs uns immer offen stand und die uns immer mit Rat und Tat zur Seite stand.
Danke euch beiden!

Für die kollegiale Zusammenarbeit möchte ich vor allem Mag. Haifa Al-Dubai danken, aber auch allen meinen Laborkollegen Martina, Georg, Nadira, Mirca, Margit und Ullrich. Ich werde die Kaffeepausen mit euch vermissen!
Danke für die schöne Zeit im Labor

Ein Dank gilt auch Frau Professor Irene Lichtscheidl für die freundliche Bereitstellung vom Fluoreszenzmikroskop, Professor Franz Gabor und seiner Arbeitsgruppe für die Zusammenarbeit im Bereich Nanopartikel sowie Dr. Albert. Missbichler und Professor Marcela Hermann für Zusammenarbeit bei der Immunisierung.
Herzlichen Dank für die Unterstützung

Last but not least gilt mein Dank allen meinen Freunden, mit denen ich durch die Höhen und Tiefen des Studiums gegangen bin. Danken möchte ich für die gegenseitige Unterstützung und für die schöne gemeinsame Zeit des Studentenlebens.
Danke auch an meine liebe Freundin Solveig, die mir vor allem beim Diplomarbeitschreiben eine große seelische Stütze war.
Danke allen meinen Freunden!

Contents

CHAPTER I: DRUG-TARGETING AND DRUG-RELOADING

Abstract

Although stents significantly reduce restenosis when compared with balloon angioplasty, restenosis rates in patients who receive stents are still 20% to 40% at 6 months[12]. Important is thereby the biocompatibility of the implant. Two categories of stent coatings may be distinguished: biocompatible coating to be less thrombogenic and inflammatory and drug-eluting coating. Drug-eluting stents (DES) were developed to release drugs site-specific and hence to prevent restenosis. With the aid of DES, the restenosis rate decreased to 16% [13]. However, after depletion the drug reservoir cannot be refilled.

Drugs may be administered encapsulated in nanoparticles (NPs), but display a typical distribution in corpora depending on the NP's surface. Equipped with a cognition domain such as antibodies (so called "target-oriented" structure), the drug-carriers are able to "dock" to the targeting-moiety and release their drugs site-specifically. The advantage of drug-targeting is the increase of bioavailability and the decrease of side effects. Another aspect is the controlled drug-release over a specific time. The degradation rate of the NPs and thus of the drug-release is determined by its hydrophobic/hydrophilic ratio. If the degradation products are also biocompatible, it is called biodegradable.

PLGA (Polyl(lactic-*co*-glycolic acid))-nanoparticles are commonly used as drug-carriers and have shown full biocompatibility both *in vitro* and *in vivo* studies[23]. Although PLGA-biomaterials are generally biocompatible and non-toxic, *several studies have reported inflammatory reactions*[11]. This was utilised to immunise rabbits with PLGA-NP and produce antibodies against it (PLGA-Ab). The surface of PLGA-NPs acts now as recognition domain for the PLGA-Ab. PLGA-antibodies immobilised on implants specifically bind the nanoparticles and enrich them site-specific.

PLGA-NPs are also biodegradable. By degradation to lactic and glycolic acid, the drugs are released; but also the conformation changes by and by. Thereby the binding to the PLGA-Ab weakens and the NPs dissociate again. New drug-carriers can now administered and reloading can take place.

This principle of the combination of drug-targeting and drug-reloading has the advantage that new drugs can - adjusted to the specific needs of patients - site-specifically target the moiety with simultaneous increase of the bioavailability and decrease of side effects.

Abstract (German)

Obwohl Stents signifikant das Risiko von Restenosis im Vergleich zur Ballon-Angioplastie herabsetzen, liegt die Restenosisrate bei Patienten mit Stents nach 6 Monaten noch immer bei 20-40%. Entscheidend dabei ist die Biokompatibilität des Implantats. Zwei Kategorien von Stentbeschichtungen können unterschieden werden: biokompatible Beschichtung und *drug-eluting* Beschichtung. *Drug-eluting* Stents (DES) wurden entwickelt, um Arzneimittel (drugs) ortsspezifisch freizusetzen und so einer Restenosis vorzubeugen. Mittels DES konnte die Restenosisrate auf 16% gesenkt werden. Sind die Arzneimittel jedoch aufgebraucht, können sie nicht wieder aufgefüllt werden.

Drugs können eingeschlossen in Nanopartikel (NP) verabreicht werden, zeigen aber eine für sie typische Verteilung im Körper abhängig von der NP-Oberfläche. Ausgestattet mit einer Erkennungsdomäne wie z.B. Antikörper (man spricht von einer "ziel-orientierten" Struktur) können die *drug-carrier* an das Zielgewebe "andocken" und ortsspezifisch ihre Arzneimittel freisetzen. Der Vorteil von *drug-targeting* ist die Steigerung der Bioverfügbarkeit und Reduzierung der Nebeneffekte. Ein anderer Aspekt ist die kontrollierte Freisetzung der Arzneimittel über einen bestimmten Zeitraum. Der Abbau der NP und somit die Arzneimittelfreisetzung wird beeinflußt durch dessen hydrophoben zu hydrophilen Anteils. Sind auch dessen Abbauprodukte biokompatibel spricht man von biodegradabel.

PLGA-Nanopartikel werden häufig als Arzneimittelträger genützt und sowohl *in vitro* als auch *in vivo* Studien haben deren vollständige Biokompatibilität gezeigt. Aber obwohl PLGA-Materialien im Allgemeinen biokompatibel und nicht toxisch sind, haben einige Studien Entzündungsreaktionen aufgezeigt. Dies wurde ausgenutzt, um Kaninchen mit PLGA-NP zu immunisieren und so Antikörper (PLGA-Ak) gegen diese zu produzieren. Die Oberfläche von PLGA-NP agiert nun als Erkennungsdomäne. Immobilisiert man PLGA-Ak auf Implantaten, binden diese spezifisch die NP und es kommt zu einer ortsspezifischen Anreicherung.

PLGA-NP sind auch biodegradabel. Bei der Degradation in Milch- und Glykolsäure werden die Wirkstoffe freigesetzt, aber auch die Konformation der NP ändert sich mit der Zeit. Dabei wird die Bindung an den Ak zunehmend schwächer und die NP dissoziieren. Neue *drug-carrier* können verabreicht werden, ein *Reloading* kann durchgeführt werden.

Diese Kombination von *drug-targeting* und *drug-reloading* hat den Vorteil, dass neue Arzneimittel – angepaßt an die spezifischen Bedürfnisse der Patienten – ortspezifisch an ihr Wirkungsfeld gelenkt werden können unter gleichzeitiger Steigerung der Bioverfügbarkeit und Reduzierung der Nebeneffekte.

1 Introduction

1.1 Stents, Biomaterials and Biocompatible Coatings

1.1.1 Stents – an Introduction

Arteriosclerosis is a hardening of connective tissue caused by plaque deposition (calcification and adiposis) in blood vessel walls. Stenosis at coronary heart disease (CHD) leads to angina pectoris and myocardial infarction, *which causes approximately half of all deaths of adults over age 60 in industrialized nations*[1]. If medical attendance lowering blood pressure, cholesterol and triglyceride and applying anticoagulant is not efficient, the stenotic (narrowed) artery has to be widened by percutaneous transluminal (coronary) angioplasty (PTA, PTCA) by means of balloon dilation. *In this procedure, a catheter having an inflatable balloon at its distal end is introduced into the coronary artery, the uninflated balloon is positioned at the stenotic site, and the balloon is then inflated*[2]. If stenosis fairly advanced, balloon dilation is not enough because the blood vessel walls would recoil and become smaller shortly after being enlarged. Hence, the walls have to be supported by stents. *A stent is a small, expandable wire mesh in a tube form that is used to maintain an artery open after balloon angioplasty*[3] possessing following properties:

- biocompatibility (non-toxic, non-carcinogenic and non-inflammatory)
- flexibility
- visibility under x-ray apparatus (optimal positioning is only possible at sufficiently radio-visibility)

Stent implantation, however, tends to cause injury to the blood vessel resulting in neointimal proliferation [development of a thick smooth muscle tissue inside the lumen, ed.]*, known as in-stent restenosis*[3]. Following risks and complications can appear inter alia:

- stent thrombosis
- cardiovascular trauma
- internal bleeding
- hypersensitivity to contrast medium and narcotics
- infections
- embolism (stroke, pulmonary embolism)

To reduce the risk of thrombosis the patient has to take coagulation inhibitors for at least three months.

1.1.2 Biomaterials, Biocompatibility and Biocompatible Coatings

A biomaterial is any pharmacologically inert material, viable or non viable, natural product or man made, that is a part of or is capable of interacting in a beneficial way with a living organism[4] (Black) or defined by Williams as *any nonliving materials used in medical devices intended to interact with biological systems*[5]. Cardiovascular biomaterials can be basically divided into three broad categories (Society for Biomaterials Special Interest Groups, SIG):

- adsorbable polymers (e.g. in case of surgical sutures, short-term applications)
- non-adsorbable polymers (are permanently implanted inside the patient's body for long-term applications, e.g. the biocompatible and under *in vivo* conditions stable polymer polyester is used.)
- metals (particular noble metals (like gold, platinum) and titanium)

Next to short-term and long-term applications, tissue engineering is an important application field for biomaterials.

Biomaterials are in direct contact with biological tissue by chemical, physical and biological interactions and thus the biological compatibility is an important factor for applications. The biocompatibility is defined as *The ability of a material to perform with an appropriate host response in a specific application*[6] or revisited by Williams himself for long-term applications: *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*[7]. For acceptance or rejection of the foreign material, the chemical, physical and structural properties of the biomaterial and the tissue response to it are critical factors.

The interaction between the material and tissue involves:

- the primary reaction on the material surface occurring within seconds to minutes. In most of the cases, the first contact takes place with blood resulting in adsorption of blood proteins by hydrophobic interactions between the nonpolar side chain of an amino acid and the hydrophobic material surface. Thus, the *Protein adsorption plays a central role in the short-term and long-term blood biocompatibility of foreign surface*[8].
- local tissue reaction caused by immune response. Inert materials show minimal or unverifiable reaction with the biological environment and the wound healing processes naturally beginning with inflammation and following repairing. At non-inert materials the local tissue reaction is significantly stronger and occurs, according to severity, in:

    - connective tissue encapsulation
    - chronic inflammation
    - creation of granuloma (*a compact (organized) collection of mature mononuclear phagocytes*[9] as inflammatory reaction of the immune system)
    - necrosis
    - implant rejection
- corrosion or degradation of the implant material. Minimal release of corrosion products as result of electrophoretic corrosion of the metal has already severe consequences, but also polymers may underlie degradation e.g. by hydrolysis, oxidation or enzymatic catabolism.
- systemic effect (long-term effect) as consecutive reaction of the effects occurring on the material surface.

An overview about the types of implant-tissue responses are given in (tab. 1).

If the material is toxic, the surrounding tissue dies.
If the material is nontoxic and biologically inactive (nearly inert), a fibrous tissue of variable thickness forms.
If the material is nontoxic and biologically active (bioactive), an interfacial bond forms.
If the material is nontoxic and dissolves, the surrounding tissue replaces it.

tab. 1 types of implant-tissue response[10]

Before surgical placement, all medical implants have to be sterilised *to reduce the risk of infections and associated complications. The most commonly used sterilization techniques utilize heat, steam, radiation or a combination of these methods*[11].

Two categories of stent coatings can be distinguished:

- biocompatible coating to be less thrombogenic and inflammatory, including inert materials like carbon, gold and silicon carbide or the neutrally charged phospholipid phosphorylcholine, which was found on animal plasma membranes.
- drug eluting coating (1.1.3)

Substances that have been examined as stent coatings are listed in (tab. 2).

Tests of biocompatibility take place *in vivo* 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. In the latter case, material properties in comparison to positive and negative controls of related materials are examined with regard to cell morphology, growth behaviour and metabolic activity.

1.1.3 Drug-Eluting Stents (DES)

Drug-eluting stents (DES) were developed to release drugs, which block the cell proliferation and hence prevent restenosis. The drugs, encapsulated in a polymer matrix, are delivered directly to the target site minimising the systemic effect. *An ideal drug delivery system should deliver a drug to a specific site in a specific time and release pattern*[12]. The drug release is determined by[3] physical mechanisms:

- diffusion-controlled drug-release (drug diffusion through a polymer layer)
- dissolution/degradation-controlled drug-release (the drug release is controlled by dissolution or degradation of the polymer matrix)
- ion exchange-based drug-release (ionised drugs bind to the matrix through electrostatic interactions and are replaced by ions of the same charge)
- other controlled release technologies

and chemical mechanisms:

- The covalent bonds of drug molecules on a delivery vehicle (so called prodrugs) are broken by chemical or enzymatic degradation with a high efficiency in controlling the drug-release kinetics.

Numerous anti-proliferative and anti-inflammatory drugs were assessed; some of them are listed in (tab. 2). As of 2004, *with DES, the pivotal clinical parameter TVF (target vessel failure) is in the upper single-digit range for "standard" lesions and 16% for long lesions*[13].

Coating	Mechanism of Action
Biocompatible coatings	
Carbon	Chemically inert and potentially noninflammatory
Gold	Chemically inert and potentially noninflammatory
Silicon carbide	Inert semiconductor potentially less thrombogenic and inflammatory
Phosphorylcholine	Organic polymer potentially less thrombogenic and inflammatory
Drug coatings	
Anticoagulants	
Heparin	Antithrombotic and possible direct inhibition of smooth muscle cell proliferation
Hirudin	Antithrombotic and potentially antiproliferative
Iloprost	Prostacyclin analogue that is antithrombotic and potentially antiproliferative
Corticosteroids	
Dexamethasone	Corticosteroid with potent anti-inflammatory properties
Methylprednisolone	Corticosteroid with potent anti-inflammatory properties
Antimitotic agents	
Paclitaxel	Interferes with microtubule function and thereby inhibits a variety of intracellular processes including mitosis and cell migration
Sirolimus	Inhibits production of proteins essential for cell division; has potent immunosuppressive and antimitotic properties
Angiopeptin	Somatostatin analogue that inhibits many cytokines and growth factors, and potentially has antiproliferative effects
Tyrosine kinase inhibitors	Interfere with intracellular cell signalling that regulate cell proliferation and differentiation

tab. 2 Substances that have been examined as stent coatings[12]

1.2 Nanoparticles, Drug-Targeting and Controlled Release

Drugs display a typical distribution in corpora caused by their physico-chemical properties. Hence, only a part of the applied drugs reaches the required target location whereas residual substances are responsible for side and toxicological effects. Thus, following requirements arise for pharmaceutical products:

- "drug-targeting" (drug carrier under well-directed conditions target to the site of (pharmacological) action)
- "controlled release" (of drugs after reaching the target location)
- large bioavailability
- storage stability (protection against environmental influence e.g. air, light and humidity)
- protection against instability in physiological media e.g. enzymatic degradation or acidic instability

Advantage of drug-targeting is the dose reduction with simultaneous improvement of therapeutic effectiveness and decrease of unwanted side effects.

In 1913, Paul Ehrlich (1854-1915) coined the term "Zauberkugel" (magic bullet):

Wenn wir z.B. eine bestimmte Art eines Empfängers oder Chemorezeptors auffinden würden, der nur im Parasiten vorkommt, den Organen des Körpers aber vollkommen fehlt, so würden wir einen Stoff haben, der an und für sich vollkommen unschädlich ist und dabei die Parasiten radikal vernichtet. Er würde in diesem Sinne genau den Immunprodukten des Organismus entsprechen, die ihrerseits nach Art von Zauberkugeln ihren Feind, den Parasiten, isoliert treffen[14].

With the aid of nanotechnology, the ability to control or manipulate structures at the atomic level was offered to develop effective drug-delivery systems[15]. *Nanoparticles are small polymeric colloidal particles varying in size from 10-100 nm with a therapeutic agent adsorbed, attached, dissolved, dispersed or encapsulated in its polymer matrix*[16]. Other definitions refer a size range of 1-1000 nm in diameter, *comparable to that of an antibody or virus*[20]. Thereby nanoparticles (NPs) provide a means for drug transport, envisaged as 'Trojan horses'. However, NPs show different properties in biological systems depending on their surface (see fig. 1). Thus, *their biodistribution is largely determined by their physical and biochemical properties, such as particle size, nature of the polymer and drug, and surface biochemical properties*[17]. The polymeric matrixes of "conventional structure" tend *to be hydrophobic and are readily coated by serum proteins, which act as opsonins. (...) However, coating these particles with flexible hydrophilic polymers, particularly polymers containing*

polyethylene glycol (PEG), can reduce opsonisation of the particles and allow them to remain in the circulation for much longer periods[18]. PEG is in the literature one of the most described polymer for "nonfouling" surfaces, which resists the adsorption of proteins and/or cells. By means of the hydrophobic/hydrophilic ratio, the biodegradation of NPs can be controlled and thus their drug-release over a specific period can be regulated. The release rates depend on:

- desorption of the surface-bound/adsorbed drug
- diffusion through the NP-matrix
- diffusion (in case of nanocapsules) through the polymer wall
- NP matrix erosion
- a combined erosion/diffusion process

Modifying the polymeric NP-matrix with cell-specific ligands such as antibodies, the structure becomes "target-oriented". This therapy form is commonly used in chemotherapy, too.

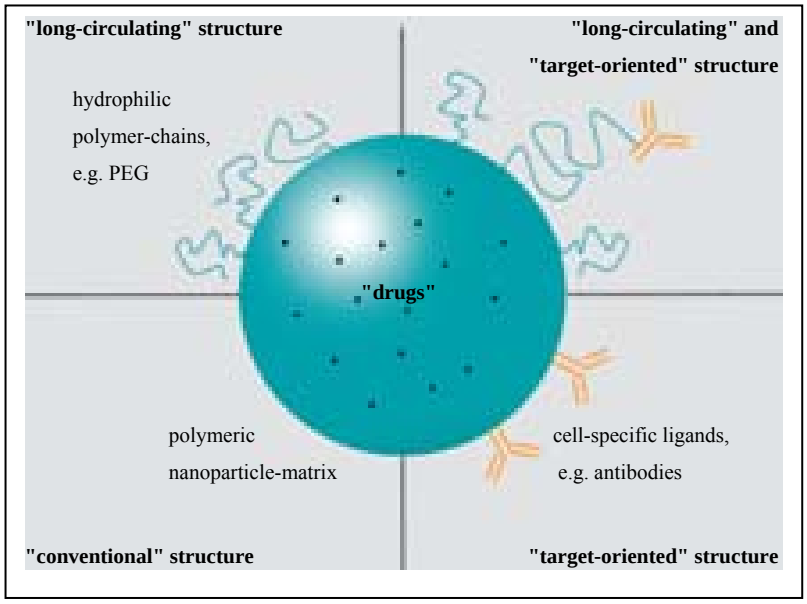


fig. 1 Variants of nanoparticle drug carriers[19]

Physiological, physicochemical and molecular aspects have to be taken into consideration to determine the toxicity of nano-carrier systems. *Nanoparticle exposures through the skin, the respiratory tract, the gastrointestinal tract and the lymphatics have been described*[20]. Next to the polymer matrix, the degradation products of biodegradable NPs and their metabolism are critical factors for the biocompatibility. Often described biodegradable NPs as effective drug-delivery devices consist of the polymers poly(D,L-lactide), poly(lactic acid) PLA, poly(D,L-glycolide) PLG, poly(lactide-co-glycolide), PLGA (1.2.1), and poly(cyanoacrylate) PCA[21].

1.2.1 Biodegradable PLGA-Nanoparticles

PLGA (poly(lactic-*co*-glycolic acid)) is a random copolymer of glycolic and lactic acid, soluble in dichlormethane, chloroform, acetone, tetrahydrofuran, ethyl acetate, dioxane, DMSO and toluene but insoluble in water, ethanol and petroleum ether. The lower the percentage of glycolic acid is, the more hydrophilic the polymer becomes. Hence, the ratio of glycolic acid to lactic acid determines the rate of degradation, which is generally considered as a hydrolytic mechanism. *Both in vivo and in vitro studies have identified a heterogeneous degradation in large-sized devices (...) characterized by a rate of degradation in the core which is greater than that at the surface of the device. (...) microspheres less than 300 microns in diameter undergo a homogeneous degradation with the rate of degradation of the core being equivalent to that at the surface*[23]. Next to the degradation mechanism and rate, the size influences the foreign body responses. It is long been known that there is a relationship between particle size and spleen uptake, whereas with the increase of size the removal increases[22]. *Microspheres greater than 5 to 10 microns in diameter may not be phagocytosed within macrophages and foreign body giant cell and thus a foreign body reaction is present at the surfaces of these microspheres. Microspheres smaller than 5 microns may undergo phagocytosis by macrophages, foreign body giant cells and other types of cells. Incorporation of these (...) lead to a more rapid degradation of the biodegradable polyester*[23].

PLGA degrades *in vivo by hydrolysis into lactic acid and glycolic acid, which are then incorporated into the tricarboxylic acid cycle and excreted*[11]. A large number of in vivo and in vitro studies have shown the full biocompatibility and biodegradability of PLGA-NPs and their degradation products [11, 23, 24].

PLGA-NPs are produced in laboratory scale by the water-in-oil-in-water (w/o/w) double emulsion method (3.10). In clinical applications, PLGA-NPs can be administered orally, parenterally or vascularly for site-specific delivery. *However, nonspecific adsorption of plasma proteins on PLGA micro- and nanospheres is a main limitation of drug targeting*[25]. Thereby, the study of Müller et al (2002) has shown 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, Fibrinogen, Fibrinonectin and IgG as representative for the human blood proteins.

1.3 Immune System and Immunisation

Antibodies against PLGA-nanoparticles were produced by immunisation of rabbits. Thus, a short introduction in the immune system is given in (1.3.1). As well for the understanding of biocompatibility of biomaterials (1.1.2), it is important to know how the immune system reacts.

1.3.1 Immune System

In the course of evolution, the immune system of vertebrates evolved "to detect and protect against danger". Thereby each immune response involves the recognition of and reaction against pathogens or other foreign matters, able to distinguish between foreign and body's own. The immune system can be divided into (fig. 2):

- innate immunity: unspecific acting cells form the first early phase of defence mechanism against pathogens.
- adaptive immunity: developed after some days and acts high specific, differing between self and non-self and forming a memory

The adaptive immunity reacts highly selective to a specific pathogen. 'Remembering' the pathogenic agent, the immune response is on the up and up at each infection. Mainly responsible for the immune response are:

- lymphocytes (recognise specific individual pathogens)
    - B-lymphocytes or B-cells
        - formed and matured in the bone marrow
        - able to produce antibodies (Ab)
    - T-lymphocytes or T-cells
        - formed in the bone marrow and matured in the thymus gland
        - control the development and Ab-production of B-cells
        - assist phagocytes to destroy the engulfed pathogens
        - recognise cells infected by viruses and destroy them
        - natural killer cells (NKC)
- phagocytes
    - monocytes, macrophages, granulocytes

The immune system is decentrally organised in primary lymphatic organs (bone marrow and thymus gland) – involved in production and maturation of lymphocytes – and in secondary lymph organs such as spleen and tonsils. The weight of the lymphatic system sums about 2%

of the body weight. About 10^{12} lymphocytes circulate in the body, whereby 10^{9} lymphocytes are newly-formed per diem.

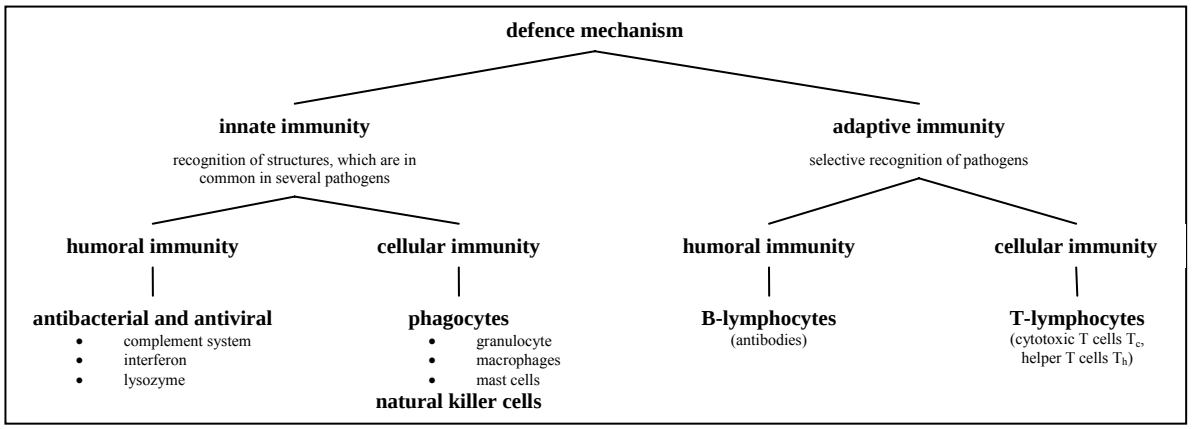


fig. 2 Diagram of the defence mechanism, both the innate and adaptive immunity[26]

As component of the unspecific immune defence, the epidermis with a low acidic pH constitutes the first barrier.

The stages of the specific immune response are illustrated in (fig. 3).

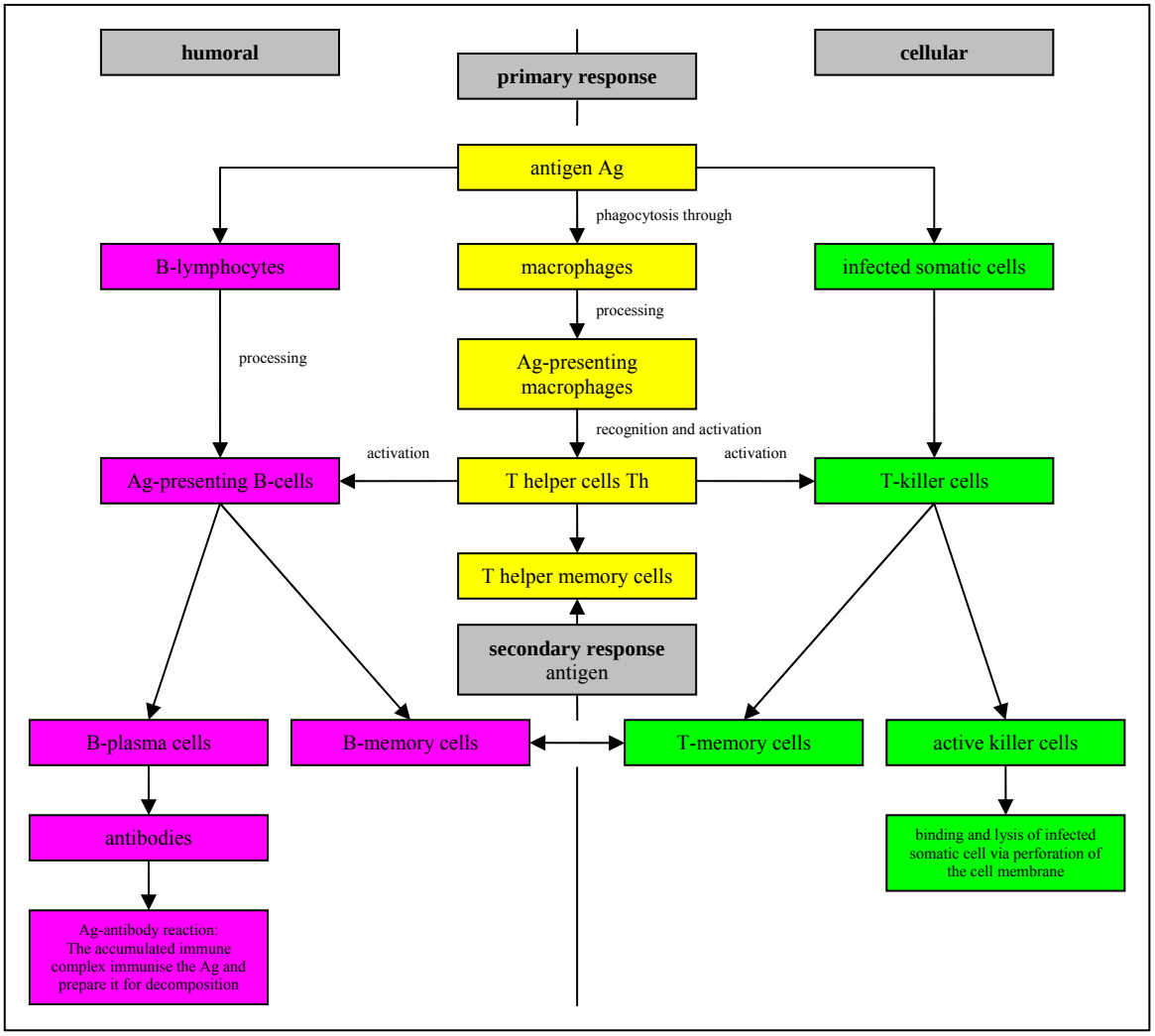


fig. 3 Stages of the specific immune response

Invaded pathogens are transported via lymph or blood to the specific cells in the lymph nodes and spleen. Antigen-presenting cells (APC) such as macrophages roam the body looking towards antigens, commonly a foreign protein molecule of bacteria or other pathogens. APC engulfs the antigen and decomposes it in antigenic peptides. The smaller fragments of the protein are presented in combination with major histocompatibility complex (MHC-II) on the surface of APC. $CD4^+$ T-cells bind specific to this peptide-MHC complex via its T-cell receptors (TCR). TCR is non-covalent associated with CD3 (CD = cluster of differentiation) and two ζ-polypeptides, which convey the signalling cascade. The T-cell receptor is not only specific to the antigenic peptide but also to the presenting MHC, this property is called self-restriction. By an additional co-stimulating signal of the T-cell receptor CD28 by APC-receptors of the B7-family (in brief B7:CD28), the T-lymphocytes are activated and the clonal expansion is initiated via the growth factor IL-2. The activated T-cells release lymphokines. This group of messenger activates B-lymphocytes among other elements of the immune system.

B-cells possess also specific receptors against antigens. However, these receptors recognise – in contrast to TCR – non-attached antigens or more precisely a specific configuration of the protein. Ag-presenting B-cells, activated through lymphokines, differentiate in antibody-producing plasma cells. Antibodies (Ab) are free released soluble B-cell-receptors with primarily three properties:

- membrane-bound Ab activate the complement system or other unspecific defence mechanisms
- opsonisation of antigens by free released Ab
- neutralisation of antigens

A couple of B- and T-lymphocytes become memory cells and circulate through the body. Thus, the immune system is able to response faster when reinfected.

1.3.2 Antibodies

Antibodies (also known as immunoglobulins, Ig) are free released soluble B-cell receptors as result of humoral immune response (1.3.1). As a group of glycoproteins, they are found in serum and body fluid of all mammalians. In human beings there are five isotypes of immunoglobulins (IgG, IgA, IgM, IgD and IgE) differing in size, electric charge, composition of amino acids, percentage of carbohydrate and in their function. An overview gives (tab. 3).

Ig isotypes	IgG	IgM	IgA	IgD	IgE
heavy chain	γ	μ	α	δ	ε
structure	monomer	pentamer	monomer (> 80%), dimer	monomer	monomer
average serum concentration (mg/mL)	14.5	1.5	3.5	0.03	0.00005
percentage of Ig	70-75%	10	15-20	<1	in traces
function	most important Ig of the 2nd immune response	predominant "early" Ig in 1st immune response	mainly in mucosae, e.g. saliva	unknown	parasites, allergy
molecular weight	146000	970000	160000	184000	188000
half-life (d)	7-21	10	6	3	2
distribution (% intravascular)	45	80	42	75	50
percentage of carbohydrates	2-3	12	7-11	9-14	12
sub-groups	IgG1, IgG2, IgG3, IgG4		IgA1, IgA2, sIgA		

tab. 3 Physical-chemical properties of human immunoglobulins[27]

The basic structure consists of four polypeptide chains – two small light chains (L) and two long heavy chains (H) – connected by disulfide bridges to a Y shape (fig. 4). The type of heavy chain (γ, μ, α, δ, ε) determines the isotypes and their subgroups. With mercaptoethanol and 6 M urea, the antibody splits into the four parts. Papain cleaves proteolytic in the "hinge" region whereby three parts are generated: two identical antigen-binding fragments F_{ab} and one crystallisable fragment F_c. Pepsin cleaves elsewhere in one $(F_{ab}')_2$ fragment and in several sub-fragments of F_c.

Each chain consists of a constant domain (c) and a variable domain (v). Additional to one variable domain per chain (v_L and v_H), the light chain composes of one constant domain (c_L) and the heavy chain of three (IgG, IgA) or four (IgM, IgE) constant domains (c_{H1}, c_{H2}, c_{H3}, c_{H4}).

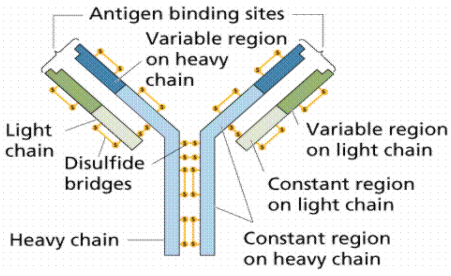


fig. 4 Structure of immunoglobulin[28]

The theoretical Ab-repertoire of human beings is guessed to about 10^{11} different specifities[26]. The high diversity is generated by somatic recombination of the variable gene segments (V), the diversity gene segments (D) and the joining gene segments (J), also known as V(D)J-recombination. The gene sequences are encoded on chromosome 14 for the heavy chain and on the chromosomes 2 and 22 for the light chains. The light chain is composed of V and J sequences and the heavy chain of V, D and J. In the course of the B-cell development in the bone marrow, the different gene sequences are stochastic recombined and form the coding domain of the variable regions on the light and heavy chain. In addition, the diversity increases by somatic mutations. The possibility of the immune system to form antibodies against any antigen is utilised in immunisation (1.3.3).

The variable regions v_L and v_H consists of 3 hypervariable regions with 5-15 amino acids, which form the antigen-binding site, the paratope. The reaction between the paratope and the antigen determinant, the epitope, is reversible and given by the affinity constant. The avidity is a measure of the binding strength as sum of the primary binding (electrostatic forces) and secondary binding (hydrogen bonds, Van der Waals forces and hydrophobic forces). An antibody binds specifically to an antigen in a complex mixture but shows also cross-reactivities to antigens with identical or similar determinants.

The high affinity and specificity is utilised in immunoanalytical methods (2.2) and in immunoaffinity chromatography (2.2.4).

1.3.3 Immunisation

Producing polyclonal antibodies, the natural immune reaction of the body against immunogenic foreign molecules with a sufficient size (antigens) is taken advance. The immune system is described above (1.3.1). Thereby the activation of antigen-presenting cells and in the end the differentiation in antibody-producing B-plasma cells and in B-memory cells is crucial. Plasma cells survive only a couple of days or several weeks. Hence, they have to be replaced continuously by reactivating of memory cells.

Rabbits, mice, rats, guinea pigs and chickens are commonly used as laboratory animals. Next to the ethical husbandry, the susceptibility to stress, the feasibility of applications and the desired amount of serum are essential for the choice of animal. Thereby the optimal yield of antibodies is achieved with young adults (rabbits 3 months, mice and rats 6 weeks) because very young animals are not yet fully immune competent.

For the formation of antibodies, the concentration of the antigen has to be in a certain range (about 10 µg – 1 mg). Out of range, the antibody production fails (suppression of immune response, tolerance)[29]. Antigens with a high immunogenicity generate a high antibody titre

and antibodies with a high affinity. However, adjuvants are for the most part necessary when immunising with native or soluble antigens or with only a low amount of antigens. Adjuvants are auxiliary substances for enhancement and/or extension of the immune response. Thereby the sterility and purity of the inoculum is important for the success of immunisation and for the stress reduction of animals. Injection methods are efficient when the antigen get rapid and well distributed in the lymph nodes and spleen. Subcutaneous is the preferred method for all animal species.

For the stimulation of the memory cells, a lower amount of antigen is necessary in comparison to the first immunisation. Booster injections are done without or, if need be, with low-stressing adjuvants. Basic procedural steps in immunisation with laboratory animals (rabbits) are shown in (fig. 5).

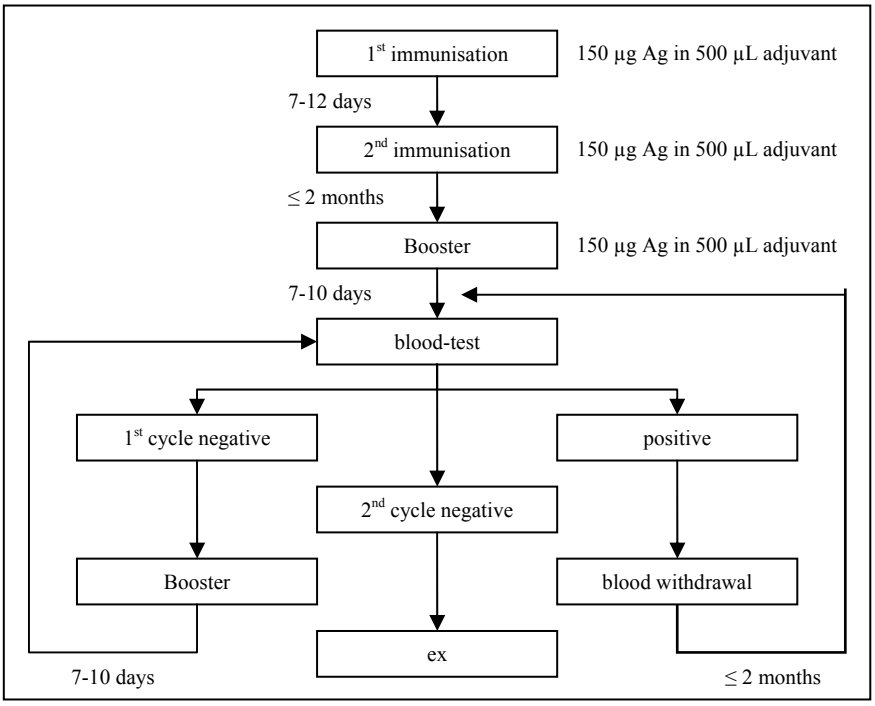


fig. 5 Basic procedural steps in immunisation with laboratory animals (rabbits)

1.3.3.1 Antibodies against PLGA-NPs

In summary, although PLA-PGA biomaterials are generally biocompatible and non-toxic, several studies have reported inflammatory reactions with the polylactide or polyglycolide implants, usually occuring 7-20 weeks after placement in the body[11]. The aim of this project was to produce antibodies against (biocompatible) PLGA-nanoparticles (1.2.1).

Rabbits were immunised with PLGA-nanoparticles by the company SCIOTEC in cooperation with Professor Marcela Hermann and the sera received of them. From the obtained sera, the titre was determined and the antibodies against PLGA (PLGA-Ab) were isolated.

1.3.3.2 Determination of Titre

The titre is a measure of concentration e.g. from antibodies or viruses. For determination, the serum is several times diluted and the serial dilutions are tested for the determinative agent with immunoanalytical methods such as ELISA (2.2). The titre is the lowest concentration, which yields yet a positive reading.

1.4 Drug-Targeting and Reloading with PLGA-Ab and PLGA-NP

Nanoparticles are solid colloidal drug-carriers. Depending on its surface, NPs possess different properties in biological systems. Equipped with a cognition domain such as antibodies (so called "target-oriented" structure), the drug carriers can "dock" to the targeting-moiety and release site-specific their drugs (1.2).

PLGA-NPs are biocompatible and one of the most described drug-carriers (1.2.1). By immunisation of rabbits, antibodies against PLGA-NP (PLGA-Antibodies) were formed (1.3.3). Thus, the surface of PLGA-NP itself acts now as recognition domain for the PLGA-Ab and no further surface modification of the NP is necessary for targeting.

However, PLGA-NPs are also biodegradable. Targeted specific to the implant surface via recognition by PLGA-Ab, they degrade in lactic and glycolic acid releasing the encapsulated drugs. Thereby the conformation changes by and by. The binding to the antibody weakens and the nanoparticles dissociate again. Now, the PLGA-Ab is free to bind another drug-carrier – drug-reloading can take place. The principle of drug-targeting and drug-reloading with PLGA-NPs is schematic shown in (fig. 6).

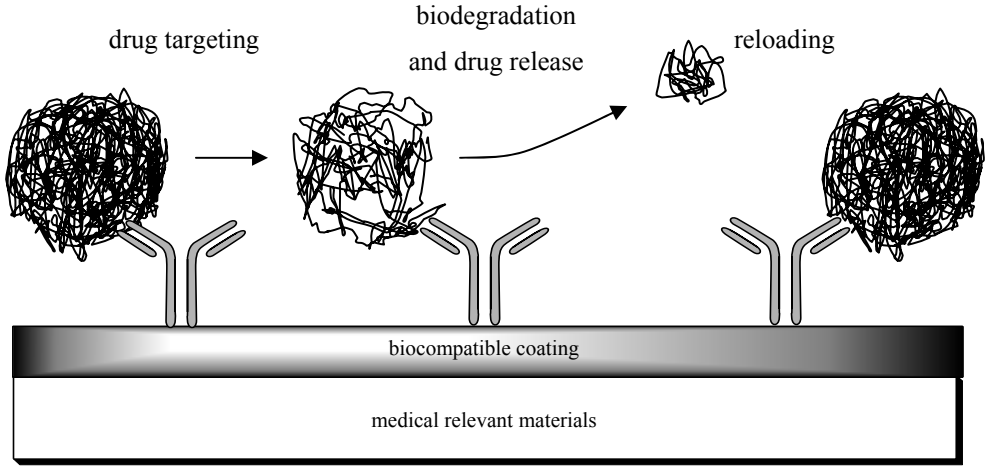


fig. 6 Principle of the setup for binding PLGA-NPs to implant surfaces via recognition by antibodies

Next to drug-targeting and decreasing of side effect, new drugs can administered. Thus, reloading has - compared with drug-eluting stents (1.1.3) – the main advantage that the medication can be adjusted to the specific needs of patients.

2 Methods

2.1 Spectroscopic Methods

2.1.1 Theoretical Foundations

The interaction of electromagnetic waves of a certain wavelength and intensity with matter is the basis of all spectroscopic methods. As a quantum phenomenon, it is described by wave-particle dualism. The wave model interprets the electromagnetic waves as a sinus curve with the parameter wavelength, frequency, velocity and amplitude; but by this the phenomenon of absorption and emission is not proper interpretable. The particle model describes the electromagnetic wave as flow of discrete particles, the photons, with the energy E and the unit Joule (J) or for the sake of clarity electron Volt (1 eV = $1.6 \cdot 10^{-19}$ J):

$$E = h \cdot \nu$$

h = Planck's constant ($6.62 \cdot 10^{-34}$ J s)

ν = oscillation frequency

The spectrum ranges from long-wave radio- and microwaves (exciting spins) to infrared (inducing molecule vibrations) to the short-wave visible and ultraviolet light and X-rays (resulting in excitation of electrons). The number of wave trains per cm gives the wave number:

$$wave\ number\ (cm^{-1}) = \frac{frequency\ (s^{-1})}{light\ velocitiy\ (3 \cdot 10^{10}\ cm\ s^{-1})}$$

An overview of the spectrum and applications in spectroscopic methods gives (tab. 4).

spectrum	wavelength	wave number [cm^{-1}]	photon energy [eV]	excitation of	applications
radio waves	10cm-100m	10^{-1}~10^{-4}	10^{-5}-10^{-8}	nuclear spin	NMR
microwaves	1000-10µm	10-0.1	10^{-3}-10^{-5}	electron spin	ESR
far-infrared (FIR)	30-1000µm	330-10	0.04-0.001	molecular rotation	
mid-infrared (MIR)	3-30µm	3300-330	0.4-0.04	molecular vibration	IR/Raman
near-infrared (NIR)	760-3000nm	13000-3300	1.6-0.4	overtone	
visible (VIS)	400-760nm	25000-13000	3.1-1.6	electrons	absorption
ultraviolet-A (UV-A)	320-400nm	30000-25000	3.9-3.1	electrons	absorption
ultraviolet-B (UV-B)	280-320nm	35000-30000	4.4-3.9	electrons	absorption
ultraviolet-C (UV-C)	100-280nm	100000-35000	12.4-4.4	electrons	absorption
X-ray	0.01-100nm	10^{9}-10^{5}	10^{5}-10	core atomic electrons	structure identification

tab. 4 Spectrum of electromagnetic radiation and their application in spectroscopic methods[30]

By irradiation of the sample with ultraviolet or visible light, the absorption of a photon effects an energy transfer onto the absorbent medium, resulting in an electronic transition. Thereby, an electron is raised from ground state S_0 (single state) to higher excited discrete states (S_1, S_2 ...) with corresponding vibration levels v while maintaining the spin configuration. Under specific conditions, a spin conversion can occur from singlet to triplet state T.

Return of the excited molecules to the ground state can happen radiationless in consequence of internal conversion (IC) or intersystem crossing (ISC) or by emission e.g. fluorescence or phosphorescence. The transitions between the energy levels are illustrated in the simplified Jablonski diagram (fig. 7).

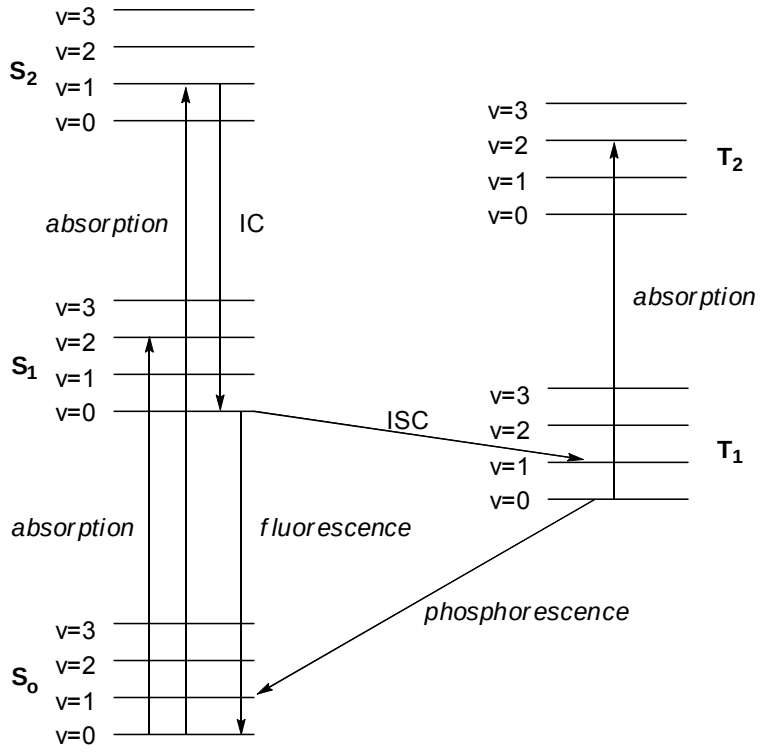


fig. 7 Jablonski diagram (IC = internal conversion, ISC = intersystem crossing, S = singlet state, T = triplet state, v = vibration level)

2.1.2 UV/VIS-Spectroscopy

UV/VIS-Spectroscopy is an absorption method based on the attenuation of a light beam caused by electron excitation of atoms and molecules. Atoms absorb photons exclusive by excitation of electrons in frontier orbitals. In molecules and complexes, the visible light excites non-bonding electrons, π-bonding electron pairs or d-electrons of the central atom.

Lambert-Beer's law describes the absorbance A of ultraviolet and visible light (UV/VIS):

$$A = \log\left(\frac{I_o}{I}\right) = \varepsilon \cdot c \cdot d$$
$$\Rightarrow I = I_0 \cdot e^{-\varepsilon \cdot c \cdot d}$$

I_0 = intensity of the incident light

I = intensity of the light after passing the material

ε = molar absorption coefficient ($L \cdot mol^{-1} \cdot cm^{-1}$)

c = concentration of the absorbent material ($mol \cdot L^{-1}$)

d = distance the light has to travel through (cm)

The intensity of the light I passed the material decreases exponentially with the concentration c of the absorbent material. For characterisation of the absorbance's intensity transmission T

$$T = \frac{I}{I_0}$$

is often used.

The absorbance measurement in the sample and reference cuvette is simultaneous in a two-beam photometer. As a light source in the UV-range, a deuterium lamp is usually used and in the visible-range a tungsten lamp. The wavelength is determined by a monochromator and the beam is divided into two parts by a beam splitter, for reference and sample. Plastic cuvettes for visible range absorb ultraviolet light, so use silica cuvettes for absorption below 350nm. The signal is detected by photomultiplier.

2.1.3 Fluorescence-Spectroscopy

The excitation of S_0, v = 0 results in excited states S_1 and S_2 with vibration levels v = 0 or higher. By internal conversion (IC) thermal equilibration occurs to S_1, v = 0 before relaxing to the ground state is possible (fig. 7). Hence the fluorescence spectrum is shifted to lower energies and thus to higher wavelengths in contrast to the absorbance spectrum, called Stoke-shift. The fluorescence lifetime refers to the average time the molecules stay in the excited state before emitting a photon and ranges in the order of magnitude 10^{-9} – 10^{-7} s. The fluorescence intensity F is depending on the concentration c of the molecule to be analysed:

$$F = I_0 \cdot \phi \cdot (2.303 \cdot \varepsilon \cdot c \cdot d)$$

I_0 = intensity of the intrinsic light

ϕ = quantum efficiency

ε = molar absorption coefficient ($L \cdot mol^{-1} \cdot cm^{-1}$)

d = thickness of the cuvette (cm)

The most intensive fluorescence is observed at molecules with functional aromatic groups low-energetic $\pi \rightarrow \pi^*$ transition states. Fluorescence extinction occurs by collision of excited molecules with other particles like low-molecular compounds, ions or solvent itself and the transfer of energy; thus, the probability of emission decreases - the phenomenon of quenching. Too high concentrations of fluorescent molecules also result in extinction by self-relaxation.
As light source, mercury or xenon high-pressure lamps are used. The wavelengths for excitation (λ_{exc}) and emission (λ_{em}) are selected by monochromators before and after the sample. The photomultiplier is aligned perpendicular to the intrinsic light beam.

2.2 Immunoanalytical Methods

2.2.1 Theoretical Foundations

Immunoanalytical methods are based on the specific interactions between immunoglobulins (Ig) and antigens (Ag); hence, in most of the cases no sample preparation is necessary. Utilising the high affinity, immunoaffinity chromatography (2.2.4) is one of the most efficient separation methods in biochemistry. Immunoassays (IA) are rapid tests, divided in:

- Immunoprecipitation
- competitive IA
- non-competitive IA

Immunoprecipitation requires at least two or three antigen determinants. Loss of solubility caused by stoichiometric binding of antibodies (Ab) to the paratopes results in agglutination used e.g. in Ouchterlony double immuno diffusion.
The competitive assay is mainly applied for antigens with only one paratope. The immobilised antibody is incubated with a solution of antigen and labelled antigen, then both compete for binding. The detection occurs by measuring the signal of the increase of labelled antigen bound or by its decrease in solution.
Working with non-competitive IA either Ab or Ag can be immobilised. When adding the analyte, a direct correlation between the concentration and the signal is observed. Working with labelled analytes allows for direct detection of the signal. However, the indirect method requires a second labelled antibody to target the bound analyte. At the "sandwich assay", the Ag is encased between the immobilised and labelled secondary antibody.
The homogeneous assay creates the signal by formation of Ab-Ag complex, so no further separation is necessary. In contrast, with a heterogeneous assay, the labelled analyte bound and free in solution would give the same signal and therefore separation is essential before detection is possible. The separation can be carried out e.g. by immobilisation onto a solid

phase and removing the supernatant by washing. 96-well-Microtiter plates are commercially used binding proteins unspecific onto a polystyrol layer. The automation makes screening of a large sample number possible. Another possibility is the immobilisation onto polystyrol-coated beads with iron core; the bound analytes are removed simply by magnet.
By means of labelling and type of detection, it is possible to distinguish:

- radio immunoassay (RIA)
- enzyme-linked immunosorbent assay (EIA, ELISA) (2.2.2)
- fluoro immunoassay (FIA)
- cluster-linked immunosorbent assay (CLISA) (2.2.3)

Important for development of immunoassays is the type of antibody, monoclonal or polyclonal, and its affinity and cross reactivity (specificity).

2.2.2 ELISA

The principles of enzyme-linked immunosorbent assay (ELISA) are described above (2.2). The labelling of analyte with enzyme has many advantages over RIA and FIA e.g. by avoiding radioactivity or photobleaching. The most commonly used enzymes are alkaline phosphatase (ALP, 2.2.2.1) and horseradish peroxidase (HRP, 2.2.2.2), which convert a colourless substrate into a dyed product. Immunohistochemical staining occurs by an insoluble product.
Important for the choice of enzyme to be labelled is the conjugation on molecules, its specificity and turn over rate and the detection limit of the product, but also its robustness and independence from reaction parameters like pH and temperature.

2.2.2.1 Alkaline Phosphatase (ALP)

Alkaline phosphatase (Orthophosphoric-monoester phosphohydrolase (alkaline optimum); EC 3.1.3.1)[31] is an ubiquitous isoenzyme with the highest activity at pH 8-10, but instable and inhibited in acidic solution. The most utilised isoenzyme is isolated from calf intestine with a molecular weight of 140 kDa and a turn over rate of 3500 s^{-1}. ALP *is a metalloenzyme with two* Zn^{2+} *and one* Mg^{2+} *at each active site*[32], hence metal chelators like EDTA act as inhibitors. The enzyme catalyses the phosphoric ester hydrolysis of an orthophosphoric monoester while creating alcohol and orthophosphate.
As a very sensitive detection of ALP, a solution of BCIP (5-bromo-4-chloro-3-indolylphosphate) and NBT (nitro blue tetrazolium chloride) can be used (fig. 8).

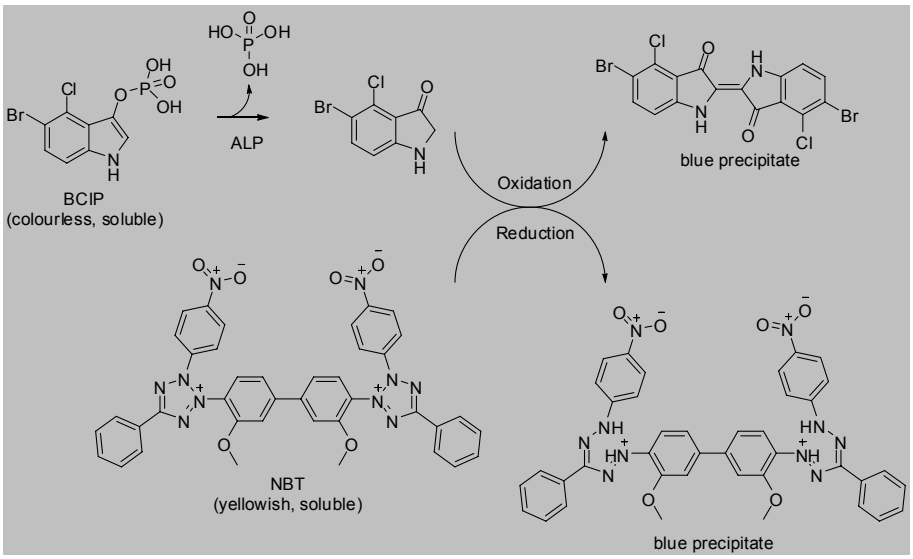


fig. 8 Staining with BCIP and NBT

Both with a very little solubility generate insoluble products applied for the ALP-detection in immunoblotting and immunohistochemical assays. BCIP, the substrate for ALP, is dephosphorylated under hydrolysis and reacts further by dimerisation to a dark-blue insoluble indigo dye, an oxidation product reducing NBT to the blue precipitate diformazan. Thereby NBT intensifies the colour and makes detection more sensitive.

2.2.2.2 Horseradish Peroxidase (HRP)

Horseradish Peroxidase (Donor: hydrogen-peroxide oxidoreductase; EC 1.11.1.7)[33] is one of the most popular enzymes used in antibody labelling. With a molecular weight of 40 kDa it is comparatively small and stable. HRP is a glycoprotein with at least seven isoenzymes. It catalyses the redox reaction of hydrogen peroxide with electron-donating substrates under formation of water and oxidised donor, its optimum pH is 7. The oxidation of an electron donor is utilised to create a coloured product.

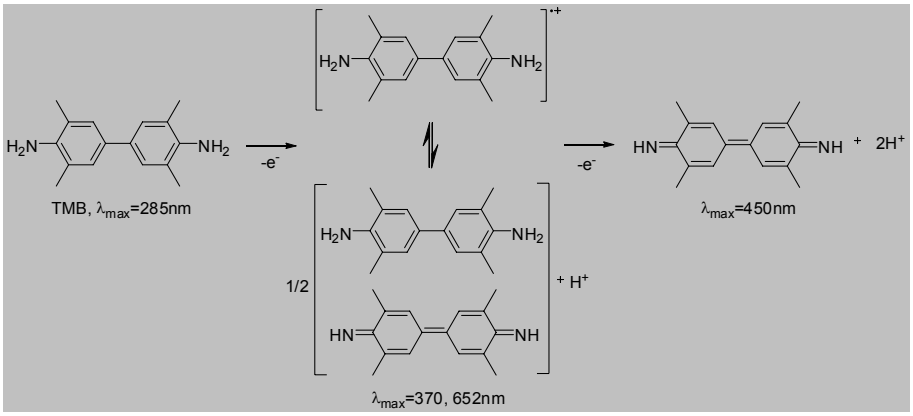


fig. 9 Oxidation of TMB to a blue and a yellow product

TMB (3,3',5,5'-tetramethylbenzidine) is a chromogenic solution and oxidised during the enzymatic degradation of hydrogen peroxide forming a blue colour with wavelength of 652nm, *a blue charge-transfer complex of the parent diamine and the diimine oxidation product. This species exists in rapid equilibrium with the radical cation*[34]. After stopping the reaction with acidic solution, a stable yellow diimine is formed and can be measured at 450nm (fig. 9).

2.2.3 Gold Cluster Labelling as an Alternative to ELISA

The gold cluster colloids (GCC) were prepared according to Frens [35] by reduction of chloroauric acid ($HAuCl_4$) with trisodium citrate leading to particles with negatively charged surface, so-called ζ-potential. The size of GCC decreases with the increase of the power and concentration of the reducing agent. The DLVO theory (named after Derjaguin, Landau, Verwey and Overbeek) describes the stability of colloidal suspensions based on attractive forces and negatively charged repulsion. Electrolytes like sodium chloride in buffer destroy the ζ-potential leading to coagulation. The colloids can be stabilised by adding proteins or blocking agents like fish gelatine or Tween20 (4.2.1).

The adsorption of proteins on GCC occurs spontaneously. Preparation of stable protein-gold complexes depends on several interactions[36]:

a) the electronic attraction between the negatively charged gold particles and the abundant positively charged sites on the protein molecule,

b) an adsorption phenomena involving hydrophobic pockets on the protein binding to the metal surface, and

c) the potential for covalent binding of gold to free sulfhydryl groups, if present.

The cluster linked immunosorbent assay (CLISA, fig. 10) was developed by Haifa Al-Dubai in the research group of Professor Pittner and constitutes an inexpensive alternative to ELISA.

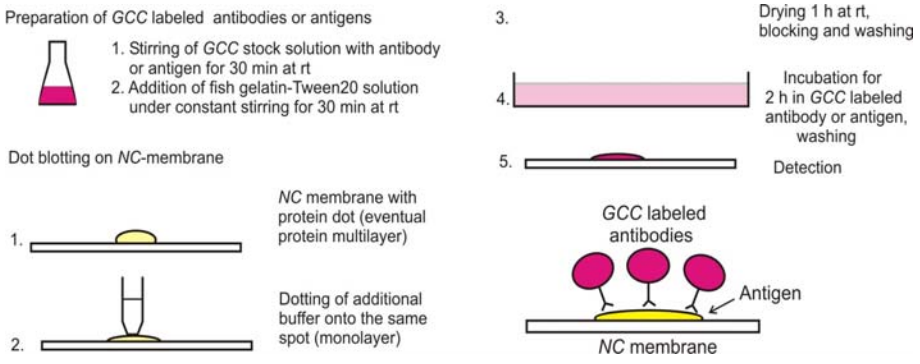


fig. 10 CLISA (cluster linked immunosorbent assay): A dot-blot test using gold colloid cluster technology[37] (see also attachment)

Dotting of additional buffer onto the nitrocellulose membrane with a protein dot, results in a protein-monolayer. After drying for 1h at rt, the membrane has to be blocked and washed. Thereafter, incubation takes place in GCC labelled antibody solution for 2h. Washing again the signal is detectable by visual colourimetry. Comparing with the signal of protein dots of known concentration, a semi-quantitative valuation is possible. *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*[37].

2.2.3.1 Signal Amplification with Protein G

An additional signal amplification is achieved by means of protein G, a protein of the Streptococcal bacteria cell wall. It has three homologous domains, which bind with a high affinitiy to the F_c fragment of immunoglobulins, primary IgG. Protein G is commonly used in affinity chromatography to purify antibodies, too.

Adding the protein G to the GCC solution it binds to the negatively charged surface of the gold clusters with simultaneous preventing coagulationg by blocking the surface. Now, the immunoglobulins can be bound to the protein G, with theoretically three antibodies per protein. In practise, the GCC are more stable and the signal is several times amplified.

2.2.4 Affinity Chromatography

The affinity chromatography is a highly selective separation method based on reversible interaction of the ligand with its target molecule such as between antigen (Ag), protein A or protein G with antibody (Ab) and substrate or cofactor with enzyme. Biospecific interactions for affinity chromatography typically show affinity constants in the range 10^{-5} – 10^{-7} M. At higher values the binding is too weak to efficiently retain the target molecule and lower than 10^{-8} M the elution is difficult. In immunoaffinity chromatography, an Ag as ligand is used to separate antibodies.

The ligand is covalently coupled to a chemically and physically inert matrix, the stationary phase, via a spacer arm to overcome sterical hindrance and thus to improve the binding between ligand and target molecule. Packed into a column, equilibration occurs with binding buffer, which is optimised for effective interactions. Loading the sample, the target molecule is retained by the affinity medium while unbound molecules are washed through the column with several of column volume of washing buffer (often the binding buffer is used to wash).

The elution buffer reverses the interaction and hence elutes the target molecules. The weakening of the binding can occur either by changing pH, ionic strength or polarity and thus

changing the Ab conformation or by adding a substance that competes for binding. The elution should be done fast to achieve an enrichment of the analyte. Afterwards, the eluate has immediately reconditioned to avoid denaturation.

The detection of the analyte can be unspecific e.g. by UV-absorption of aromatic side chains of proteins at 280nm, or by Bradford Assay (3.5.1) or can be specific by measurement of the biological activity e.g. by enzymatic assays and immunoassays.

After regeneration of the matrix, the column can be reused several times.

Important purification parameters are

$$specific\ activity = \frac{biological\ unit\ of\ the\ protein}{mg\ protein}$$

$$total\ activity = (specific\ activity) \cdot (total\ amount\ of\ proteins)$$

$$recovery = \frac{total\ activity\ in\ fraction}{total\ activity\ of\ original\ sample}$$

Ideally the specific activity increases and the recovery = 1.

2.3 PAGE - Polyacrylamide Gel Electrophoresis

2.3.1 Theoretical Foundations

The electrophoretic process is the separation of charged particles in an electric field. The balance between the accelerative forces F_e affecting to the charge q

$$F_e = q \cdot E$$

E = electric field strength

and the friction force F_{fr}

$$F_{fr} = f_c \cdot v$$

f_c = frictional coefficient

v = particle's velocity of migration

effects a constant velocity of the particle:

$$F_e = F_{fr};\ q \cdot E = f_c \cdot v$$
$$\Rightarrow v = \frac{q \cdot E}{f_c} = u \cdot E$$

u = electrophoretic mobility, substance specific.

Stoke's law describes the friction force for small spherical objects:

$$f_c = 6\pi \cdot \eta \cdot r$$

η = fluid's dynamic viscosity

r = radius of the spherical object

Therefore, for the velocity v follows from above:

$$v = \frac{q \cdot E}{6\pi \cdot \eta \cdot r}$$

This means that the velocity of the particle is proportional to its total charge and the applied field strength and reciprocally proportional to its size and the fluid's viscosity. In an electric field, particles differing in charge-to-radius ratio migrate with different velocities in the gel and can thus be separated. Using supporting materials with high viscosity and defined pore size (e.g. agarose and polyacrylamide gels), the separation by size of the molecules is improved.

For non-spherical molecules like peptides and proteins an empirical relationship between the mobility u and the molecular weight MW is given by:

$$u \approx \frac{q}{MW^{2/3}}$$

The spectrum of electricity suppliers ranges from 200V at 400mA to 500V at 150mA depending on the applied method. The current is maintained by electrolysis on both electrodes, changing the buffer by and by:

$$2e^- + 2H_2O \xrightarrow{\text{cathode}} 2OH^- + H_2$$

$$H_2O \xrightarrow{\text{anode}} 2H^+ + 1/_2O_2 + 2e^-$$

During electrophoresis, Joule heating W is developed per unit of volume:

$$W = E^2 \cdot \lambda \cdot c$$

λ = equivalent conductivity

c = molar concentration of the electrolyte

With increase of the temperature, the resistance decreases. In addition, the fluid evaporates faster and the concentration of the electrolytes increases, which results in a slowdown of the sample movement and band broadening. Another aspect of the heat is the denaturation of the proteins and an altered electrophoretic mobility.

Besides the pore size and temperature, the buffer system and the salt concentration are important factors for an optimal separation influencing not only the current via ion strength but also the charge and conformation of the proteins.

2.3.2 Polyacrylamide Gel

Polyacrylamide is formed by radical polymerisation of acrylamide. The chain reaction, started by ammoniumpersulfate (APS), is catalysed by tetramethylethylenediamine (TEMED). N,N´-methylenebisacrylamide (Bis) cross-links the linear polyacrylamide chains (fig. 11). The polymerisation occurs under exclusion of air because oxygen leads to chain reaction interruption.

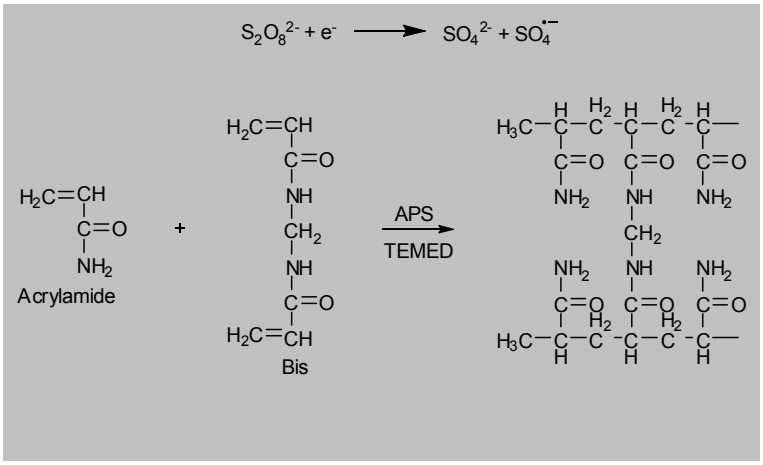


fig. 11 Polymerisation and crosslinking of acrylamide

The pore size is determined by the total amount of acrylamide *T:*

$$T = \frac{(a+b)}{m} \cdot 100\%$$

and the amount of crosslinker *C:*

$$C = \frac{b}{(a+b)} \cdot 100\%$$

a = acrylamide [g]

b = crosslinker [g]

m = volume of buffer [cm^3]

A discontinuous electrophoresis is applied to narrow bands of the separated proteins using two gel systems differing in pore size and pH. The samples are loaded onto the short and large-pore stacking gel with pH 6.8, about two units lower than the running buffer. By the process of isotachophoresis, a stacking effect occurs resulting in a pre-separation and enrichment. Arriving at the small-pore separating gel with higher ionic strength (pH 8.4) a compression and an additional band narrowing is the consequence. In the separating gel, the proteins are separated then by electrophoretic principles.

To monitor the electrophoretic process bromophenol blue is used as a front marker.

2.3.3 Native-PAGE

Native gel electrophoresis is a technique that separates the proteins by their mass-to-charge ratio. Avoiding detergents like SDS, the separated proteins are not denatured and keep their physical properties. Hence, specific properties of the proteins can be utilised for selective and sensitive detection. For example, the enzyme activity or the antibody-antigen interaction can be detected directly in the gel or on nitrocellulose membrane after Western Blot by enzymatic or immunological methods.

2.3.4 SDS-PAGE

SDS (sodium dodecylsulfate) is an anionic detergent, denaturing secondary and non-disulfide-linked tertiary protein structure. In excess over the proteins, it covers their charges accumulating micelles with negative charge with about 1.4 g SDS per g protein. During sample preparation, the hydrogen bridges are broken by heating to 95°C for 5min and the molecule is stretched. Adding reducing agents like dithiothreitol (DTT), the disulfide bonds are reduced, too. The uncurled and with SDS loaded peptide chains can be separated now by their molecular weight.

2.3.5 Detection and Quantification of the Separated Proteins

After separation, the proteins have to be fixed in the gel with methanol/acetic acid/water solution, which denature and precipitate the proteins or the proteins can be blotted onto nitrocellulose membrane by Western Blot (2.4).

The proteins can be visualised by staining with e.g. Coomassie Brilliant Blue (CBB) or PonceauS (fig. 12). In acidic solution, CBB binds unspecific to basic amino acids, primary arginin, with an absorbance shift from 465nm to 595nm. CBB is also utilised for Bradford-Assays (3.5.1). PonceauS binds to positively charged amino acids, staining proteins reversibly. Hence they can be used for further applications.

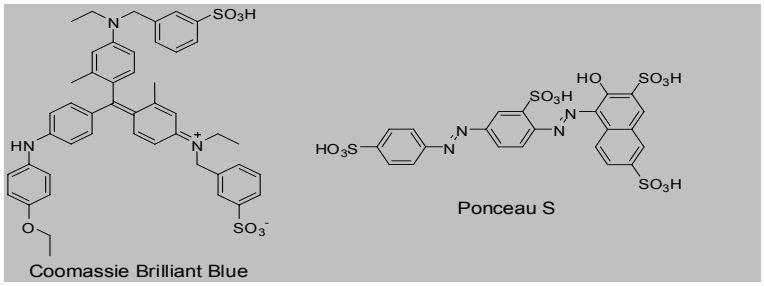


fig. 12: Stains: CBB and PonceausS

Furthermore, enzymatic or immunological methods (2.2) take advantage of specific properties of the proteins for sensitive and specific detections.

2.4 Western Blot

After electrophoresis, the separated proteins can be transferred from the polyacrylamide gel to nitrocellulose or polyvinylidene difluoride (PVDF) membrane by diffusion, capillary action or electroblotting. For the electrophoretic transfer a standard tank or semi-dry blotting system is used.

For semi-dry blotting, the blot sandwich soaked with transfer buffer is placed - free from air bubbles - between two graphite plates in following set-up: anode/ 3 sheets of Whatman® filter paper/ gel/ nitrocellulose membrane/ 3 sheets of Whatman® filter paper/ cathode. An electric field vertical to the gel elutes the proteins out of the polyacrylamide gel and transfers them onto the membrane. The experiment runs at constant current of 1mA per cm^2 blot area. For a quantitative elution, pH and ionic strength of the SDS-free transfer buffer should agree with the running buffer of electrophoresis. At beginning, low voltages (<5V) are generated but increase by and by with the increase of ohmic resistance up to 20-50V. By electrolysis of water, a pH gradient is generated (ranging from pH 12 on the cathode to pH 2 on the anode) as well as gas evolution[38]. To avoid non-uniform voltage caused by pushing apart the blot sandwich because of gassing, weight down the blotting equipment.

The proteins adhere on the nitrocellulose membrane by hydrophobic interactions and can be detected directly on the membrane by immunological and enzymatic methods (2.2.4).

3 Experimental Section

3.1 Gold Cluster Labelling Test

a) Gold Cluster labelling of antigen (or antibody):

Prepare 1 mL 1% $HAuCl_4$ in ddH_2O and dissolve it in a very clean Erlenmeyer flask covered with aluminium foil in 100 mL ddH_2O under stirring until boiling. Add rapidly 4 mL of filtered 1% trisodium citrate solution and heat for another 10 min while the colour changes to red. Cool down the solution at rt.

For labelling the gold cluster colloid (GCC) take 10 mL of the solution and add about 1 to 10 µL of the respective antigen or antibody. Check the correct concentration by adding an aliquot of NaCl to a portion of the GCC solution – if coagulation occurs, add more of the respective proteins to stabilise the GCC. To block the residual surface of the cluster add 1 mL of the blocking solution (1% fish gelatine in water, avoid electrolytes like sodium chloride).

For signal amplification, protein G can be added first to the GCC solution before labelling with antibodies.

The labelled and blocked GCC can further be concentrated by centrifugation at 14000 rpm for 10 min and resolving the precipitate in an aliquot of the supernatant.

b) Dotting of antibody (or antigen) onto nitrocellulose membrane:

In the meantime, dot 1 µL of the respective antibody (or antigen) onto the nitrocellulose membrane. Dotting of additional 5 µL buffer onto the same spot enlarges the spot obtaining a protein monolayer. After drying for 1 h at rt, block for 30 min with the blocking solution and wash 3x for 5 min with the washing solution and 3x shortly with ddH_2O. In this case, the washing steps are important to remove electrolytes such as sodium chloride to prevent coagulation of GCC.

Now incubate with the gold cluster labelled antigen (or antibody) as prepared according to a) for 2 h. To remove unspecific bindings of gold cluster with the nitrocellulose membrane wash 3x 5 min with the washing solution.

Blocking solution:

gelatine from cold water fish skin	2 g
Tween20	100 µL
50 mM Tris/HCl, 0.15 M NaCl, pH 7.3	100 mL

Washing solution:

Tween20	1 mL
50 mM Tris/HCl, 0.15 M NaCl, pH 7.3	200 mL

3.2 Biocompatible Coating

3.2.1 Etching of Aluminium, Steel and Titanium

To obtain hydroxyl groups for further aminosilanisation (3.2.2), etch the different materials like aluminium, steel (Fe/Cr18/Ni8) or titanium with 3% HNO_3 solution for 10 min. After repeated washing with ddH_2O, soak them for two days in water.

3.2.1.1 Detection of Iron – Verification of Effective Etching

Prepare a Whatman® filter paper moistened with 5% NaSCN and then dry at rt. Take 1 mL of the supernatant of the 3% HNO_3 solution over steel (3.2.1) and add 50 µL of 1% H_2O_2. Dot a few µL of this solution onto the Whatman® filter paper. If the reaction is positive, the colour will turn to red.

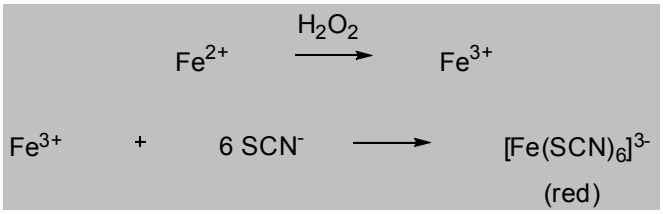


fig. 13 Detection of iron

3.2.2 Coupling of Organosilane to Inorganic Surface

Hydroxyl groups of an inorganic surface can be reacted with APTS (3-Aminopropyltriethoxysilane), a popular organosilane to create functional amino groups on inorganic surfaces. The reaction can be performed either by aqueous or by organic solvent deposition.

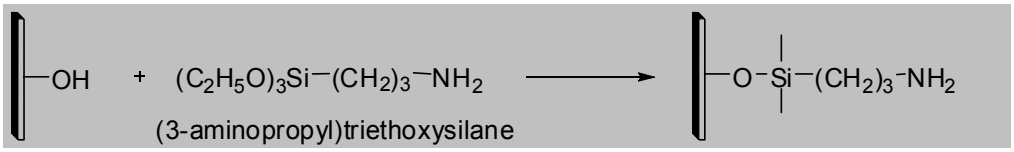


fig. 14 Hydroxyl groups of inorganic surface reacted with APTS

3.2.2.1 Organic Solvent Deposition

At first, wash the slides, glass powder or the etched materials respectively (3.2.1) with isopropanol and ddH_2O. In the meantime, prepare a 5% (3-aminopropyl)triethoxysilane solution in 95% ethanol. Shake the slides for 1 h in the solution and wash 3x with 95% ethanol afterwards. Keep o/n at 110°C.

3.2.2.2 Aqueous Deposition

Wash the slides, glass powder or the etched materials respectively (3.2.1) with isopropanol and ddH_2O. In the meantime, prepare a 10% (3-aminopropyl)triethoxysilane solution with ddH_2O and adjust with HCl to pH 3.5. Shake the slides in the solution for 2 h at 75°C and thereafter wash 3x with ddH_2O. Keep o/n at 110°C.

3.2.3 Introduction of Carboxylate Groups

Prepare a 0.4 M succinic anhydride solution in 50 mM PBS, pH 6 and add this to the aminopropylsilane coated slides (3.2.2). Shake the slides in the solution o/n and wash then 3x 5min with 50 mM PBS, pH 6 and 3x with ddH_2O.

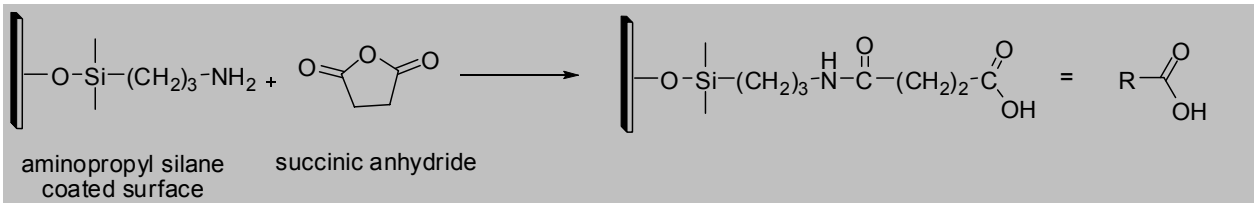


fig. 15 Reaction of amino groups with succinic anhydride

3.2.4 EDC – A Zero-Length Crosslinker

EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) is an often used carbodiimide for coupling of carboxylates with amines. Avoid buffers containing free amines like Tris or glycine.

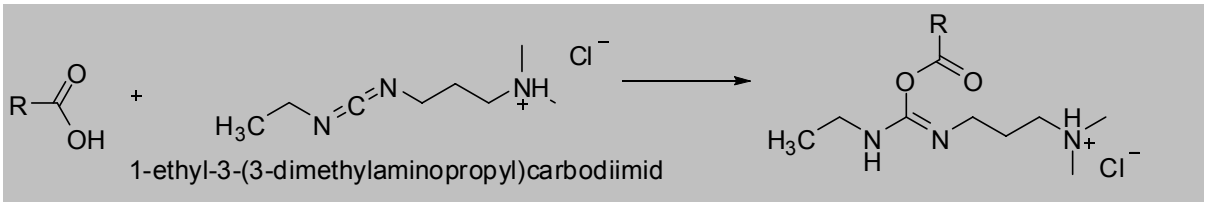


fig. 16 Activation of carboxyl groups with EDC

Dissolve 400 mg EDC in 50 mL ddH_2O or buffer and adjust with NaOH to pH 10. Shake the slides prepared with succinic anhydride (3.2.3) in this solution for 1.5 h. Wash the slides with ddH_2O repeatedly.

3.2.4.1 EDC plus Sulfo-NHS

Sulfo-NHS (sulfo-N-hydroxysulfosuccinimide) increases the yield of EDC-mediated reactions several times over.

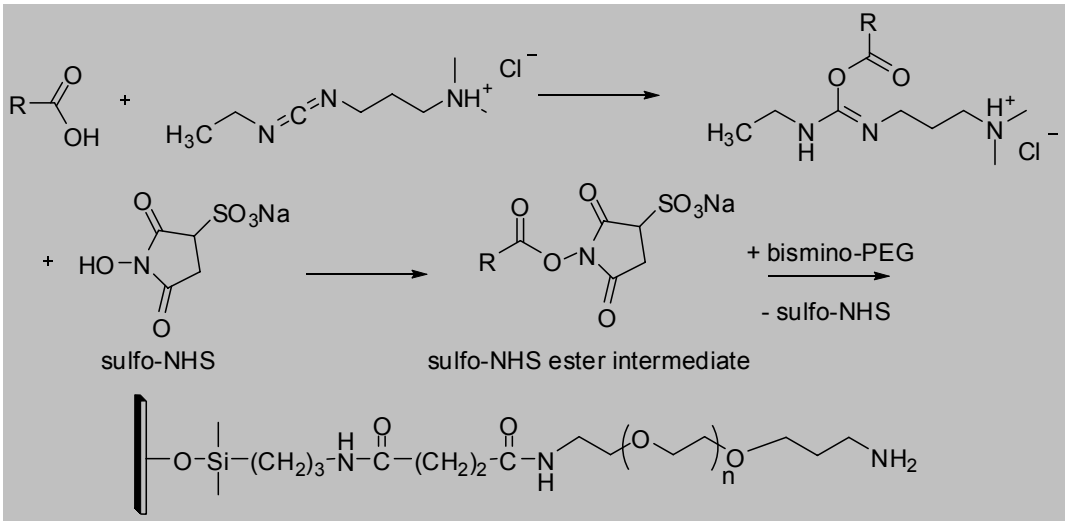


fig. 17 Activation of carboxyl groups with EDC and sulfo-NHS

Dissolve 400 mg EDC and sulfo-NHS (final concentration 5 mM) in 50 mL 0.05 M PBS, pH 6. Shake the slides prepared with succinic anhydride (3.2.3) in this solution for 1.5 h. Wash the slides with ddH_2O repeatedly.

3.2.5 Pegylation

Dissolve 1 g O,O′-bis(3-aminopropyl)polyethylene glycol (bisamino-PEG) in 50 mL carbonate-bicarbonate buffer, pH 10 or in 50 mL citric acid-sodium citrate buffer, pH 5. Shake the slides prepared with EDC or EDC plus sulfo-NHS respectively in this solution o/n. Wash the slides with ddH_2O repeatedly.

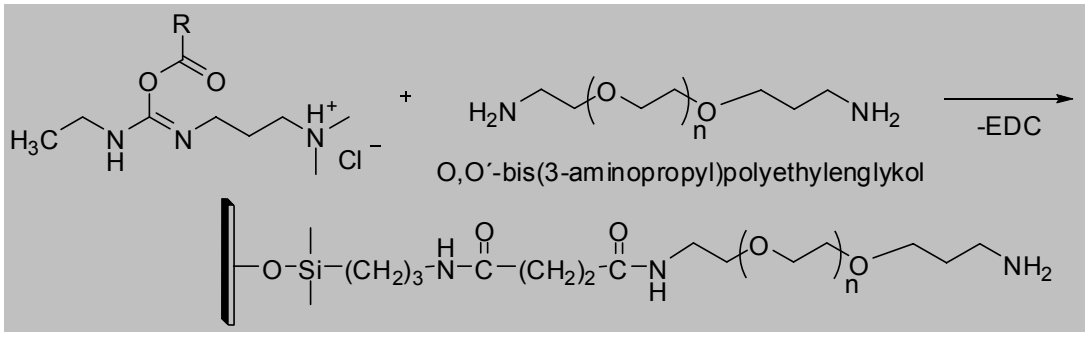


fig. 18 Pegylation

3.3 Verification of Biocompatible Coating by Surface Labelling

3.3.1 Staining with TNBS

TNBS (2,4,6-trinitrobenzenesulfonic acid solution) reacts with molecules forming highly chromogenic derivatives orange-coloured with an absorbance of 335nm. TNBS was used to stain the amino groups of the biocompatible coating on slides (3.2). As shown in (fig. 19), *TNBS reacts under relatively mild alkaline conditions to produce an unstable Meisenheimer complex. Subsequent acidification to pH 1 rapidly converts the orange unstable product to a yellow stable trinitrophenol (TNP) derivative*[39].

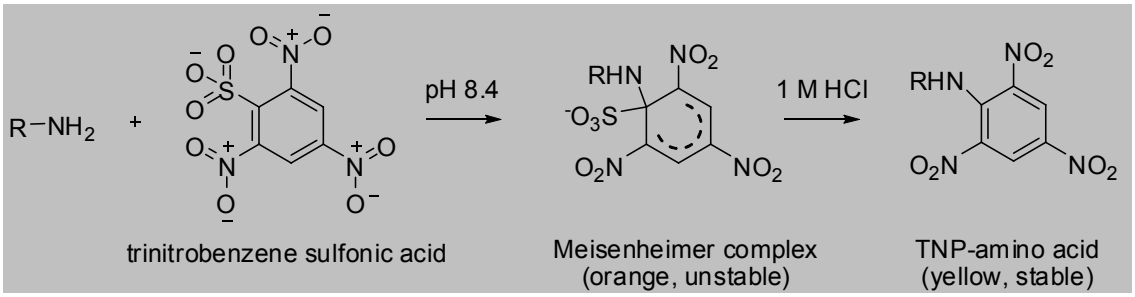


fig. 19 Detection of amino groups with TNBS

Prepare freshly a 0.01% (w/v) TNBS-solution by dilution of 20 µL 5% (w/v) stock solution (Sigma) with 0.1 M sodium bicarbonate buffer, pH 8.4. Shake the (amino group containing) slides for 2h at 37°C in this solution Add 1 mL 10% SDS and 0.5 mL 1 M HCl to each sample.

3.3.2 Staining with OPA

OPA (O-phthaldialdehyde) was used to stain the biocompatible coating on slides (3.2). It reacts with amino groups in presence of thiol-containing molecules like 2-mercaptoethanol to a fluorescence product with an excited wavelength λ_{exc} = 360nm and an emission wavelength of λ_{em} = 455nm. Detection limits for proteins in liquid are in the range of µg/mL.

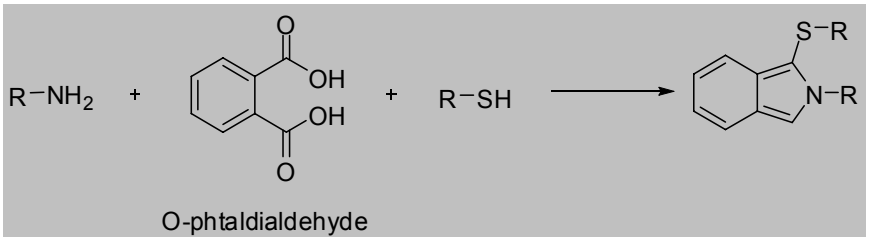


fig. 20 OPA - fluorescence reaction for amino groups

Add to 1 mL 50 mM borate buffer, pH 9.2 250 µL OPA (20 mg/mL) and 250 µL 2-mercaptoethanol or cleaved antibodies respectively (3.6.3). React the slides in this solution for 1h and wash them with ethanol thereafter. The marking of amines from the biocompatible coating (3.2) by OPA was observed under the fluorescence microscope Olympus BX41 using an excitation wavelength of 360nm and an emission wavelength of 436nm. The photos were taken by ColorView (Soft Imaging System) with resolutions 10x (N.A 0,25), 20x (N.A 0,40), 40x (N.A 0,65) and 60x (N.A 0,80) and edited with the Cell^D life science documentation software.

3.4 PLGA-Antibody Purification

3.4.1 Immobilisation of PLGA onto Glass Powder

Dissolve 450 mg PLGA (poly(lactic-*co*-glycolic acid, 50:50, MW 07462) in 20 mL dioxane and 100 mg EDC and 100 mg sulfo-NHS in 10 mL ddH_2O. Pool both solutions and stir for 1 h. Add 5 g aminosilanised glass powder (3.2.2) and react o/n. Wash the glass powder in a suction filter, pore size 4, with 3x dioxane, 3x dioxane/ddH_2O and 3x ddH_2O.

3.4.2 Purification of PLGA-Ab with PLGA-Affinity Chromatography

Fill a 3 mL column with PLGA conjugated glass powder (3.4.1) and wash with at least 4 column volumes of binding buffer (5 mM PBS, 0.15 M NaCl, pH 7.2). Dilute the rabbit serum immunised with PLGA 1:5 with binding buffer and apply this on the column with 0.15 mL/min. Wash the column with binding buffer until no more proteins can be detected by Bradford protein assay (3.5.1). Elute with the elution buffer (0.1 M acetic acid/ 0.1 M NaOAc, pH 3) and a flow rate of 1.7 mL/min. Collect the fractions in micro wells, put in first the neutralisation buffer (0.5 M $NaHCO_3$). For neutralisation about 8 µL neutralisation buffer are in need of 2 drops elution buffer. The proteins are detected with Bradford protein assay (3.5.1), this fractions pooled and adjusted to pH 7. The concentration was determined with Quant-iT™ Protein Assay Kits (for use with the Qubit™ fluorometer, Invitrogen, 3.5.2).
If necessary, the sample can be concentrated by adding dry Sephadex25 and suck off.

3.5 Protein Assays

3.5.1 Bradford Protein Assay

Coomassie Brilliant Blue (see also 2.3.5) stains proteins in acidic solution by unspecific binding to cationic and non-polar, hydrophobic side chains of proteins resulting in an absorbance shift from 465nm to 595nm. The method is named after M. M. Bradford.

Add your sample to 1 mL Bio-Rad Protein Assay (Bio-Rad Laboratories, 1:5 diluted). After 10 min incubation, measure the colour change at 595nm against a blank value. The calibration curve is prepared with BSA-standards.

For a qualitative determination of the proteins, to find the fractions after elution from the affinity chromatography (3.4.2), 20 µL Bio-Rad Protein Assay 1:5 diluted were added to 10 µL of each fractions and the colour change to blue observed. These fractions were pooled.

3.5.2 Quant-iT™ Protein Assay Kit (Invitrogen)

The Qubit™ Fluorometer is calibrated with three BSA standards (0 ng/µL, 200 ng/µL and 400 ng/µL in TE buffer with 2mM sodium azide). 10 µL of each standard is 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 rt.

Dilute 1-20 µL of your sample in Quant-iT™ protein buffer to obtain finally 200 µL solution. Mix well and measure the protein concentration after 15 min incubation with the Qubit™ Fluorometer. The concentration of the sample is 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 = the number of microlitres of sample you added to the assay tube.

3.6 Cleaving Disulfide with Immobilised Dihydrolipoamide

Immobilised dihydrolipoamide for the reduction of disulfides was used first by Gorecki and Patchornick in 1973 [40]. Lipoic acid (6,8-dithiooctanoic acid) can be immobilised to aminosilanised glass powder via the carboxylic acid group with the help of EDC. Afterwards the cyclic disulfide is reduced with $NaBH_4$ (or $Na_2S_2O_4$ respectively) to the thiol dihydrolipoamide, which is able to reduce disulfides and antibodies. This method has the advantage of batch processing and the reagent dihydrolipoamide can easily be removed and thus no contaminating by-products are produced. Furthermore, after regeneration with $NaBH_4$ ($Na_2S_2O_4$) the reaction with immobilised dihydrolipoamide can be repeated several times[41].

3.6.1 Immobilisation of Lipoic Acid onto Glass Powder with EDC

Lipoic acid is not soluble in aqueous solution in contrast to EDC. Therefore, I decided to use a dioxane/water mixture to bring both of the reagents in one phase.

Dissolve 1 mmol lipoic acid in 10 mL dioxane and 2 mmol EDC in 10 mL ddH_2O. After both reagents are dissolved, mix the solutions and stir for 1h at rt. Now add 5g amino-silanised glass powder (3.2.2) and stir o/n. By that procedure, some of the lipoic acid will precipitate when added to the aqueous solution of EDC but the result is much better than by using only an aqueous solution or buffer. Finally wash several times with dioxane and water.

3.6.2 Reduction of Lipoamide to Dihydrolipoamide

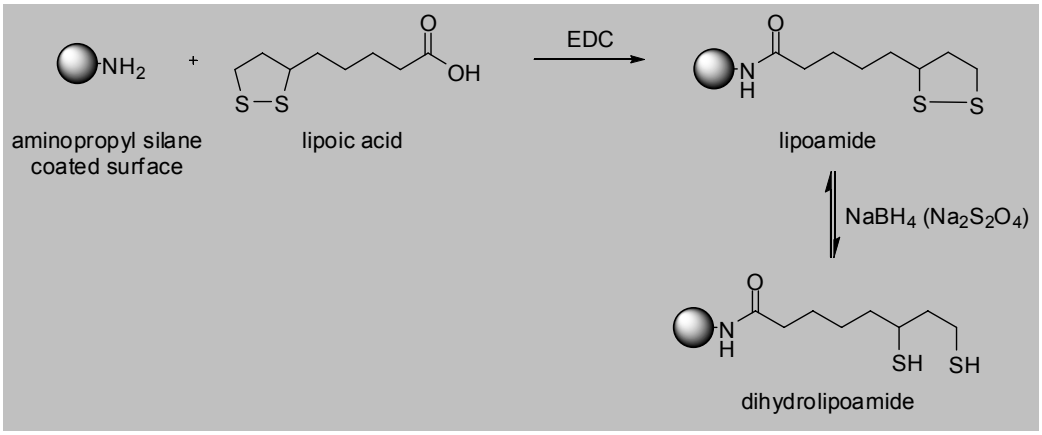


fig. 21 Immobilisation of lipoic acid and reduction to dihydrolipoamide

Wash the glass powder coated with lipoic acid (3.6.1) 3x with dH_2O and ethanol. Add 10 mL ddH_2O, 2.5 mL EtOH and 200 mg $NaBH_4$. Shake for 1 h at rt, add another 200 mg $NaBH_4$ portion to the solution and shake for further 5h. Reduce the rest of $NaBH_4$ with 6N HCl until the development of gas has stopped. Wash the glass powder with ddH_2O. $Na_2S_2O_4$ can also be utilised as a reducing agent instead of $NaBH_4$.

The proof of the immobilisation of lipoic acid on glass powder is given by Ellman's assay (3.7): Adding 50 µL Ellman's reagent to 50 mg on glass immobilised lipoic acid and 1 mL 0.1M PBS, pH 8 results in yellow staining with an absorbance of A=0.255. The negative control without lipoic acid shows an absorbance of A=0.101.

3.6.3 Cleaving Antibodies with Immobilised Dihydrolipoamide

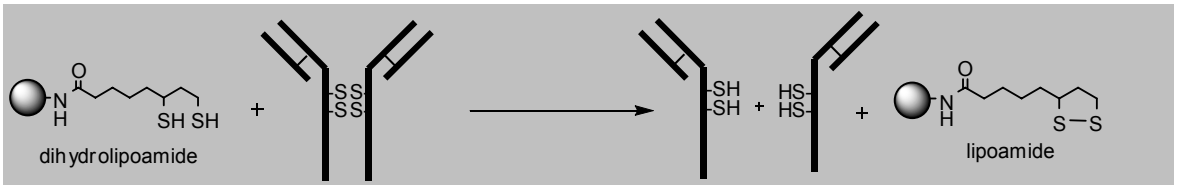


fig. 22 Antibody split by immobilised dihydrolipoamide

Add 1 µL of the antibody to 1 mL 0.1 M PBS, pH 8 and 0.5 g glass powder with immobilised dihydrolipoamide (3.6.2) and shake for 30 min. The supernatant with the split antibodies is used for further experiments.

3.7 Ellman's Assay

Ellman's reagent (5,5´-dithio-bis-(2-nitrobenzoic acid), DTNB, Pierce) reacts with sulfhydryl containing molecules under slightly alkaline conditions releasing TNB (5-thio-2-nitrobenzoic acid), a highly chromophoric compound with an absorbance at 412nm. The calibration curve is prepared by cysteine standards with a detection limit of 10 nM cysteine.

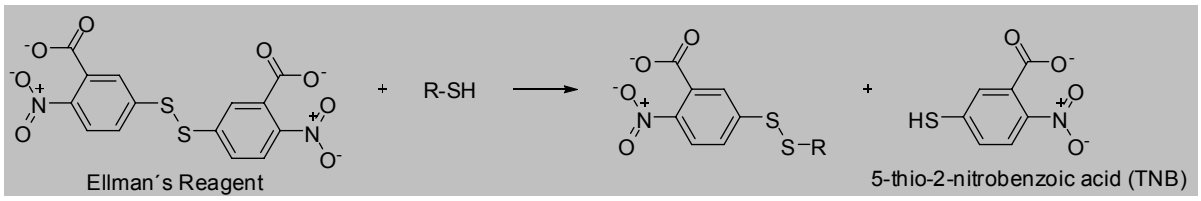


fig. 23 Ellman's assay: detection of SH-groups

Dissolve 5.4 mg Ellman's reagent in 1 mL 0.1 M PBS, pH 8 and add 50 µL to 1 mL of the standard solutions of N-acetyl-L-cysteine (Sigma) or respectively your sample. Measure the absorbance of the yellow product after incubation at rt for 15 min at 412nm. The concentration of the sulfhydryl groups of the sample is determined by comparison to the standard curve.

3.8 Immobilisation of LDH and Serum

Different coupling methods were studied by immobilisation of LDH testing its activity when bound to the slides to find the most efficient immobilisation method. Furthermore rabbit serum immunised with PLGA-NP as well as preserum as a negative control were immobilised to study the interaction with PLGA-nanoparticles and the possibility of reloading.

Organic silanised glass cover slides (3.2.2.2) were utilised for the following activations.

3.8.1 Cross-Linking of Two Amine-Groups

3.8.1.1 Glutaraldehyde Activation

Glutaraldehyde (Pentane-1,5-dial) is a homobifunctional crosslinker and one of the best and easiest ways to immobilise biocomponents. The disadvantage is the aging by polymerisation based on aldol condensation[42]. Crosslinking proteins can be made a point of doing but it is also the reason for its toxicity acting as a hapten when incorporated. Work in flue and under light protection.

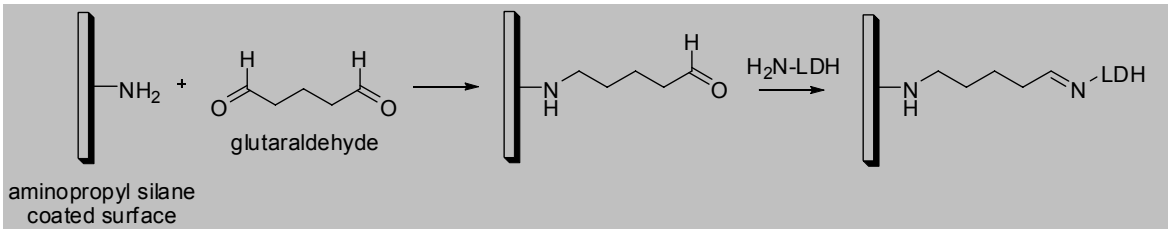


fig. 24 Immobilisation of LDH with glutaraldehyde

Add a 2.5% glutaraldehyde solution to the aminosilanised slides and shake for 1h. Wash 4x with ice-cold water.

3.8.1.2 p-Chloranil Activation

In addition to p-chloranil, also other p-halogenanils and related quinones may be in use to activate the amino-silanised surfaces.

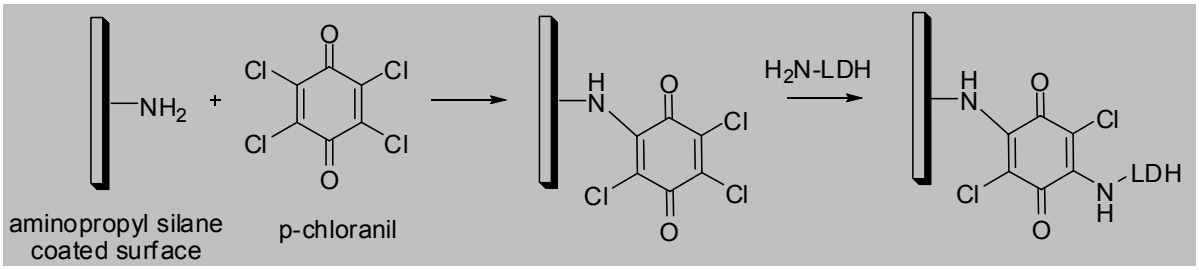


fig. 25: Immobilisation of LDH with chloranil

Wash the slides 2x with toluene and shake them in 1% solution of p-chloranil in toluene under light protection. Wash 2x with toluene, 1x with acetone and 1x with ddH_2O.

3.8.1.3 BS^3 Activation

BS^3 (bis(sulfosuccinimidyl)suberate) is a homobifunctional noncleavable crosslinker. It contains two sulfo-NHS ends that couple two amine-containing molecules irreversibly[43].

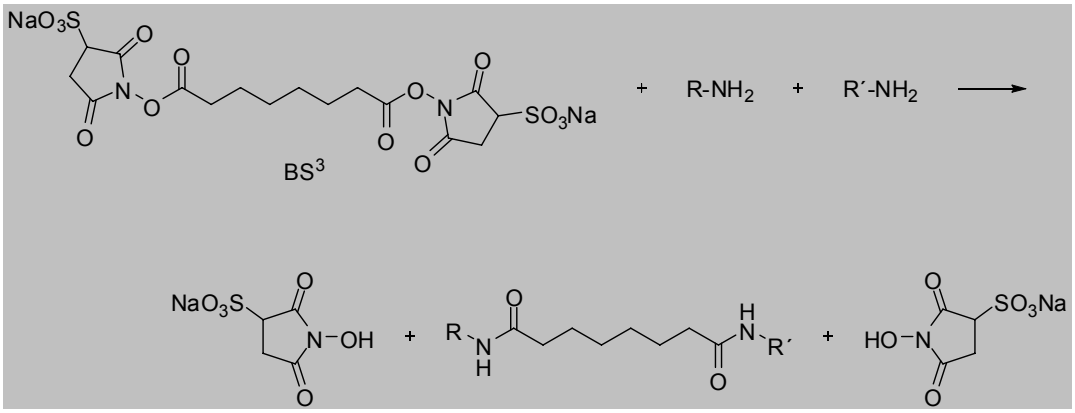


fig. 26: BS^3 reacts with two amine-containing molecules to form amide bond cross-links

Dissolve 2.2 mg BS^3 in 30 mL 0.1 M PBS, pH 7.2 and shake the cover slides for 2h in this solution. Wash 3x with buffer.

3.8.1.4 Immobilisation of LDH onto the Activated Slides

Dissolve 3 µL LDH (12.6 mg/ml, 643 units/mg, Sigma) in 300 µL PBS isotonic, pH 7.2. Pipet 20 µL of this solution onto a object slide and deposit the cover slides with the activated side (3.8.1.1, 3.8.1.2, 3.8.1.3) on it. Incubate in a wet chamber o/n at 4°C and wash 3x with buffer.

3.8.2 Cross-Linking Amine- With Sulfhydryl-Groups

Sulfo-SMCC (sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate) and sulfo-SMPB (sulfosuccinimidyl-4-(p-maleimidophenyl)butyrate) are heterobifunctional crosslinkers coupling amine-containing molecules with sulfhydryl-containing ones. After activation of the aminosilanised slides, the split to half antibodies cleaved before with immobilised dihydrolipoamide (3.6.3) can be immobilised.

3.8.2.1 Activation with Sulfo-SMCC

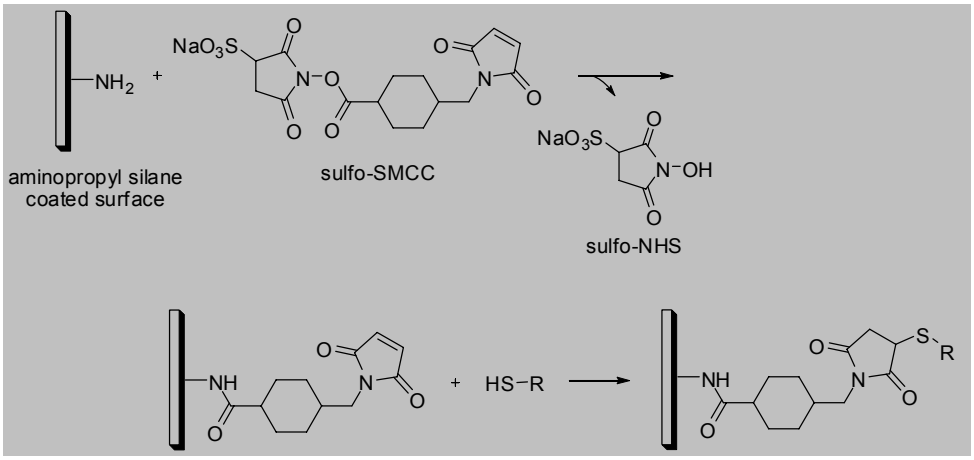


fig. 27: Immobilisation with sulfo-SMCC

Wash the slides with 0.1 M PBS, 15 mM NaCl, pH 7.2. Add 4.8 mg sulfo-SMCC dissolved in 1 mL ddH_2O to 15 mL of the buffer and shake the slides for 30 min in this solution. Wash the slides 3x with buffer.

3.8.2.2 Sulfo-SMPB Activation

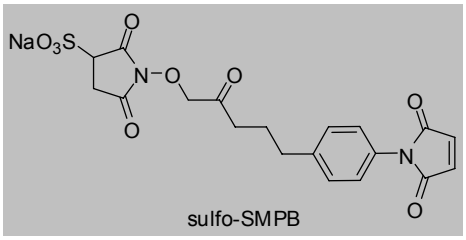


fig. 28: Sulfo-SMPB

Dissolve 10 mg sulfo-SMPB in 15 mL 0.1 M PBS, pH 7.2 and shake the aminosilanised slides for 1 h in this solution. Wash 3x with buffer.

3.8.2.3 Immobilisation of Half Antibodies onto the Activated Slides

Pipet 20 µL of the split antibodies reduced with immobilised dihydrolipoamide (3.6.3) onto a object slide. Deposit the cover slides with the activated side (3.8.2.1, 3.8.2.2) on it. Incubate in a wet chamber o/n at 4°C and thereafter wash 3x with buffer.

3.9 Derivatisation of PLGA

PLGA itself does not bind to gold particles. The goal of the derivatisation of PLGA was to introduce a free sulfhydryl group that binds covalently to the metal surface and that way to achieve the Gold cluster Labelling Test (3.1).

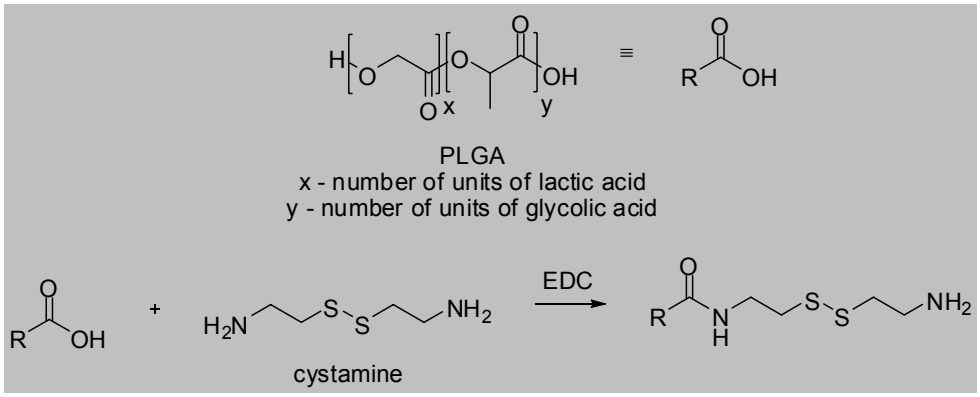


fig. 29: Derivatisation of PLGA with cystamine

Dissolve 20 mg PLGA in 1 mL dioxane and 4 mg cystamine and 20 mg EDC in 1 mL ddH_2O. Mix both solutions together and react for 2 h. Extract 4x with 2 mL ethyl acetate. After evaporating under pressure, a yellow oil will be left.

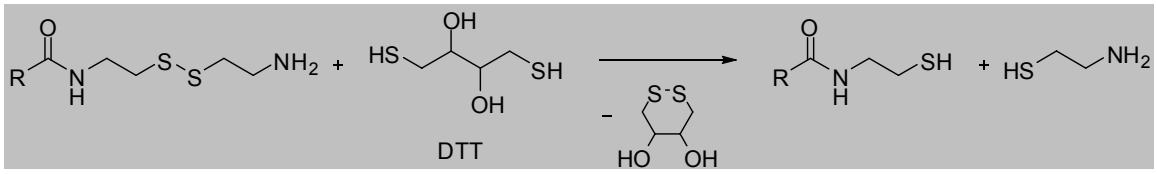


fig. 30: Cleaving of disulfides with DTT

Add 5 mg dithiothreitol (DTT) to the oil. After 30 min extract with ddH_2O and evaporate again.

3.10 Production of PLGA-Nanoparticles[44]

The NPs were produced using the water-in-oil-in-water (w/o/w) double emulsion method.

Dissolve 400 mg polymer RG 503H (= 40 mg f-PLGA + 360 mg PLGA) in 2 g ethyl acetate under stirring at 4°C. Add 400 µL ddH_2O or respectively your drugs if you want your nanoparticles medicated. Treat the solution for 60 sec with ultrasonic (sonifier, Bandelin electronic UW 70/HD 70; tip, MS 72/D, Berlin, Germany) under cooling by means of a water bath. Add rapidly 6 mL 10% Pluronic F-68 solution and treat again with ultrasonic for 50 sec under cooling to prepare the double emulsion. Decant the emulsion rapidly in 1% Pluronic F-68 solution, however, avoid dropping but rather abstain from the rest of the emulsion. Stir with 600 U/min for 1 h at rt. Then evaporate with 170 U/min and under reduced pressure (100 mbar) for 30 min and finally for further 30 min under absolute vacuum. Filter the suspension

through a micro pore filter (1µm glassfiber Prefilter, Millipore Filter). The mean size of the NPs and their distribution are determined by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Instruments Ltd, U.K.) at 20°C. The concentration is given by the weight after the lyophilisation of 1 mL NP solution.

Freezing the emulsion at -80°C keeps it stable for a very long time. For further use the NP solution has to be washed free from pluronic with 7x an ultra filtration membrane (Vivaflow 50; 100000 MWCO PES, Sartorius Vivascience Gmbh) operated by a peristaltic pump (MV-CA 8, Ismatec, Glattbrugg) at a system pressure of 2.5bar. Wash 20 mL 7x with the same amount of ddH_2O to remove the most part of Pluronic. However, the washed NPs are less stable and have to be used as soon as possible.

3.10.1 Nanoparticles Containing Alkaline Phosphatase

After dissolving 400 mg polymer RG 503H (= 40 mg f-PLGA + 360 mg PLGA) in 2 g ethyl acetate under stirring at 4°C, add the alkaline phosphatase (0,02 mL, 22 mg protein/mL, 5590 units/mg protein, Sigma-Aldrich) instead of 400 µL ddH_2O to probe the NPs. The remaining protocol is the same like the water-in-oil-in-water (w/o/w) double emulsion method described above (3.10).

A zeta-potential was measured of -36.1 mV (-26.0 mV after washing with Vivaflow50) and a mean diameter of 191.6 nm (167.0 nm). After washing, a concentration was determined of 9.7 mg/mL.

3.11 PAGE - Polyacrylamide Gel Electrophoresis

For the SDS- and native PAGE, the Mini-ProteanII BioRad electrophoretic system was in use. For the different solutions of the both types of PAGEs, see below.

Pour the separating gel and overlay with isopropanol. Remove the isopropanol after polymerisation has finished. Pour the stacking gel onto the separating gel and insert the 10-slot comb. After polymerisation of the gel, remove the comb and wash the slots with running buffer. Fill the cathodic and anodic buffer tank with running buffer.

The samples have to be prepared with sample buffer. Working with SDS-PAGE the sample has additionally to be heated 5 min at 95°C. 5-20 µL of the prepared samples are loaded into the slots and 2-5 µL of the protein ladder.

The separation of the proteins occurs at 200 V and 100 mA for 45 min.

3.11.1 SDS-PAGE

Stacking gel (4%):

30% acrylamide/0.8% Bis	1.3 mL
ddH_2O	6.1 mL
0.5 M Tris/HCl, pH 6.8	2.5 mL
10% (w/v) SDS	100 µL
10% (w/v) APS	50 µL
TEMED	10 µL

Separating gel (10%):

30% acrylamide/0.8% Bis	6.7 mL
ddH_2O	8 mL
1.5 M Tris/HCl, pH 8.8	5 mL
10% (w/v) SDS	200 µL
10% (w/v) APS	100 µL
TEMED	10 µL

Running buffer:

Tris	3.03 g
glycine	14.6 g
SDS	1 g
ddH_2O	ad 1 L

Sample buffer:

0.5 M Tris/HCl, pH 6.8	2.5 mL
20% (v/v) glycerine	2 mL
SDS	0.4 g
DTT	0.31 g
bromophenol blue	1 mg
ddH_2O	ad 20 mL

Protein ladder:

PageRuler^{TM} Prestained Protein Ladder, Fermentas
(10, 17, 26, 34, 43, 55, 72, 95, 130, 170 kDa)

3.11.2 Native PAGE

Stacking gel:

30% acrylamide/0.8% Bis	1 mL
ddH_2O	4.8 mL
0.5 M Tris/HCl, pH 6.8	2 mL
10% (w/v) APS	80 µL
TEMED	8 µL

Separating gel:

30% acrylamide/0.8% Bis	6 mL
ddH_2O	11.8 mL
1.5 M Tris/HCl, pH 8.8	6 mL
10% (w/v) APS	200 µL
TEMED	20 µL

Running buffer

Tris	3.0 g
glycine	14.4 g
ddH_2O	ad 1 L

Sample buffer:

125 mM Tris/HCl, pH 6.8	2.5 mL
20% (v/v) glycerine	2 mL
bromophenol blue	1 mg
ddH_2O	ad 10 mL

3.11.3 Staining of Gels with CBB-R250

The separated proteins can be detected directly in the gel after PAGE by visualisation with CoomassieBrilliantBlue-R250 (CBB-R250). Incubate the gel in staining solution under shaking for 30 min and afterwards in destaining solution o/n.

Staining solution:

CBB-R250	1.25 g
acetic acid	5 mL
methanol	15 mL
ddH_2O	30 mL

Destaining solution:

acetic acid	25 mL
methanol	60 mL
ddH_2O	ad 200 mL

3.11.4 Drying of Gels

To preserve the stained gel, incubate it in ddH_2O with 2-5% glycerin for 10 min. Place the gel onto a moistened filter paper and cover with cellophane sheet carefully avoiding air bubbles. Dry the gel at 70°C for 2 h on a gel dryer (Slab Gel dryer CGD 4050, Savant).

3.12 Western Blot

Semidry-blotting (NovaBlot, Pharmacia, Multiphor II SemiDry) transfers the by PAGE (2.3) separated proteins from the gel to the nitrocellulose membrane. Between two graphite plates, the blotsandwich soaked with transfer buffer is placed free from air bubbles in following direction: anode/ 3 sheets of Whatman® filter paper/ gel/ nitrocellulose membrane/ 3 sheets of Whatman® filter paper/ cathode.

The transfer of the proteins occurs at 10 V and 150 mA for 1.5 h.

transfer buffer:

Tris	5.8 g
glycine	2.9 g
ddH_2O	ad 1 L
adjust with 6 M HCl ad pH 6.5	

3.12.1 Western Blot and Elisa

After Western Blot, block the nitrocellulose membrane with blocking solution for 30min and wash 3x 5 min with washing solution (preparation of solutions see 3.1). Incubate the membrane with anti-Rabbit-IgG-ALP (1:10000 thinned in buffer) o/n and wash 3x 5 min with ALP-washing solution. Add the ALP-staining solution carefully; do not shake. Within minutes, a blue precipitate is formed where the anti-Rabbit-IgG-ALP has bound. Decant the ALP-staining solution and wash with ddH_2O. Preparations of ALP-solutions see below.

3.12.2 Western Blot and NP containing ALP

After Western Blot, block the nitrocellulose membrane with blocking solution for 30 min and wash 3x 5 min with washing solution. Incubate the membrane with solution of nanoparticles containing ALP (3.10.1) o/n and wash 3x 5 min with ALP-washing solution. Add carefully the ALP-staining solution; do not shake. Within minutes, a blue precipitate is formed where the nanoparticles have bound. Decant the ALP-staining solution and wash with ddH_2O.

ALP-buffer solution, pH 9.5:

Tris	12.11 g
NaCl	5.84 g
$MgCl_2 \cdot 6H_2O$	20.33 g
ddH_2O	ad 1 L

ALP-blocking solution:

gelatine from fish skin	2 g
Tween20	100 µL
ALP-buffer	ad 100 mL

ALP-washing solution:

Tween20	100 µL
ALP-buffer	ad 100 mL

ALP-staining solution:

NBT (50 mg/mL 70% DMF)	1 mL
BCIP (50 mg/mL 70% DMF)	500 µL
ALP-buffer	ad 50 mL

3.13 Determination of Titre

PLGA-nanoparticles with a mean diameter of 200 µm and a concentration of 5.11 mg/mL were diluted with 50 mM carbonate buffer, pH 9.6 to a concentration of 6.25 µg/mL and 3.125 µg/mL. Dot 100 µL of each solution in a well plate and incubate o/n at 40°C. After blocking for 1 h with blocking solution, wash 3x 5 min with washing solution. The sera or respectively the antibodies purified by PLGA-affinity chromatography (3.4.2) are diluted 1:100, 1:500 and 1:2500. As negative controls, only buffer was used as well as the preserum. Add the thinned samples to the dotted nanoparticles and incubated o/n at 4°C. Wash 3x 5 min with washing solution and add anti-rabbit IgG-HRP 1:10000 thinned. Incubate for 2 h at rt and wash again 3x 5 min. Adding the substrate TMB (3,3',5,5'-Tetramethylbenzidine, SCIOTEC) the colour will change to blue, measured at 370nm or 655nm. Stop the reaction after 15 min with 50 µL sulfuric acid. The colour changes to yellow and is measured at 450nm.

4 Results and Discussions

4.1 Biocompatible Coating

4.1.1 Staining with OPA

In the literature, Poly(ethylene glycol) (PEG) is one of the best described polymer for non-fouling surfaces. By staining the biocompatible coating of slides (3.2) with the fluorescent molecule OPA (3.3.2), the amino groups of APTS and PEG were visualised under the fluorescence microscope Olympus BX41, with a resolution of 10x (N.A 0,25) and an exposure time of 500 ms. Thus, the success and the preferably area-wide coating should be examined. The different coupling methods were studied under several pH conditions.

Following (coated) slides were studied by means of OPA:

I. uncoated slides as negative control (no amino groups)

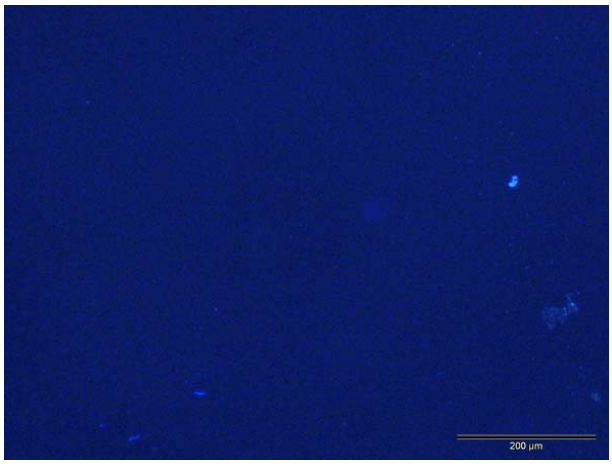


fig. 31 Uncoated slides

II. slides coated with APTS, organic solvent deposition (3.2.2.2)

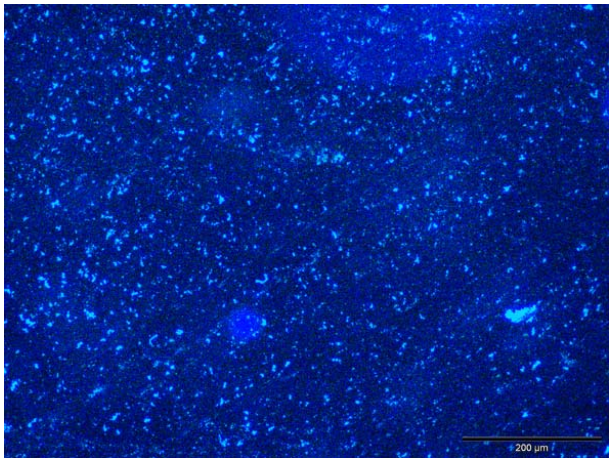


fig. 32 Aminosilanisation, organic deposition

III. slides coated with APTS, aqueous deposition (3.2.2.2)

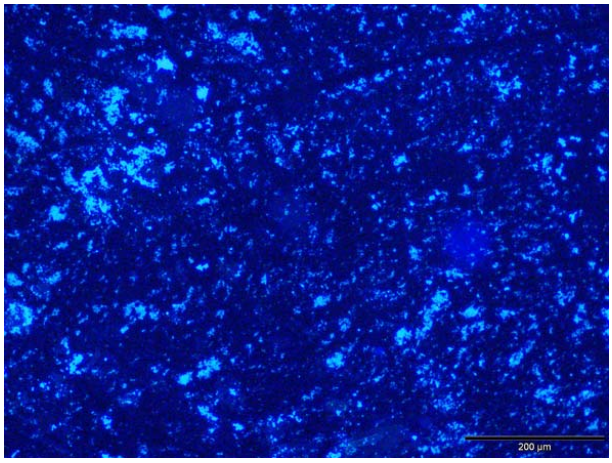


fig. 33 Aminosilanisation, aqueous deposition

IV. IgG-HRP was cleaved with immobilised dihydrolipoamide (3.6.3). The splitted antibodies were immobilised onto the slides coated with APTS, organic deposition (3.2.2.2), via the sulfhydryl groups in the hinge region reacting with OPA.

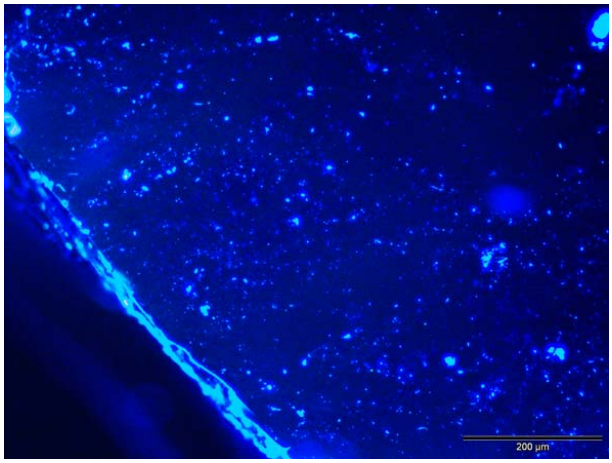


fig. 34 IgG-HRP splitted and immobilised onto aminosilanised slides

Adding the substrate TMB (2.2.2.2) a blue staining was observed, too (4.1.2).

V. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH 6 (3.2.3)

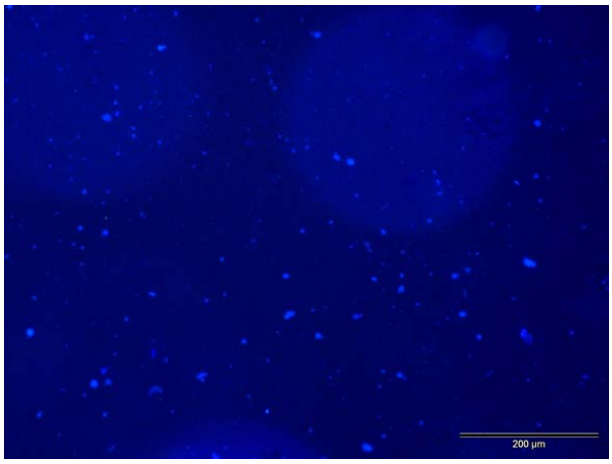


fig. 35 Succinic anhydride, pH 6

VI. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH 8.4 (3.2.3)

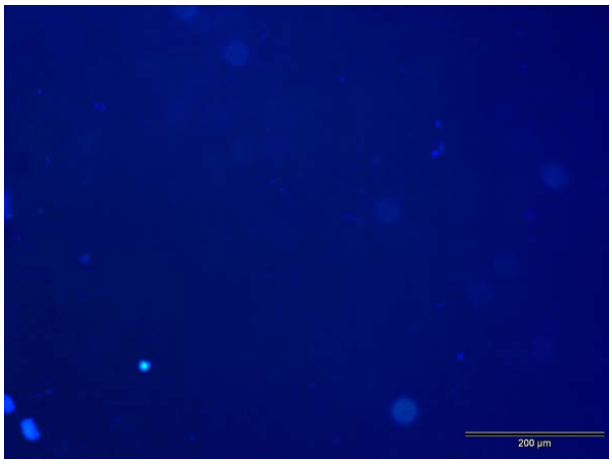


fig. 36 Succinic anhydride, pH 8.4

VII. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH6 (3.2.3)
- EDC, pH 6 (3.2.4)
- bisamino-PEG in 50 mL carbonate-bicarbonate buffer, pH 5 (3.2.5)

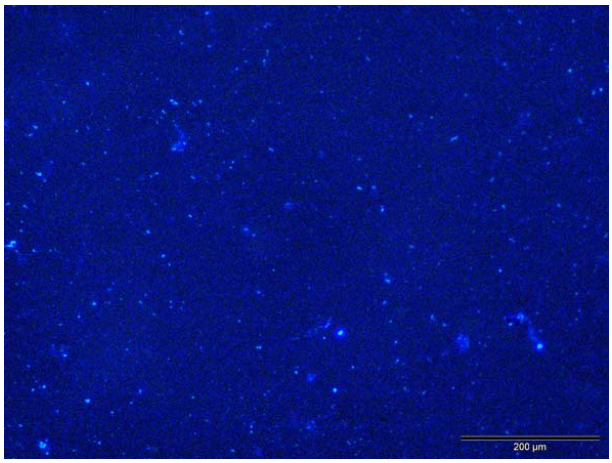


fig. 37 Pegylation

VIII. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH6 (3.2.3)
- EDC, pH 6 (3.2.4)
- bisamino-PEG in 50 mL carbonate-bicarbonate buffer, pH 5 (3.2.5)
- splitted IgG-HRP (3.6.3) immobilised via its sulfhydryl groups in the hinge region reacting with OPA

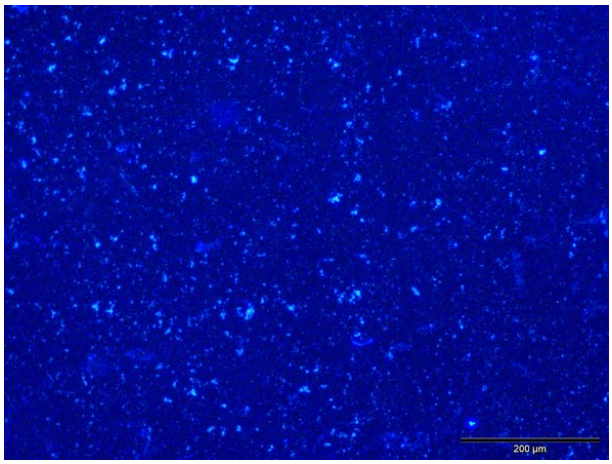


fig. 38 Splitted IgG-HRP immobilised onto pegylated slides

IX. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH6 (3.2.3)
- EDC and sulfo-NHS, pH 6 (3.2.4)
- bisamino-PEG in 50 mL carbonate-bicarbonate buffer, pH 5 (3.2.5)

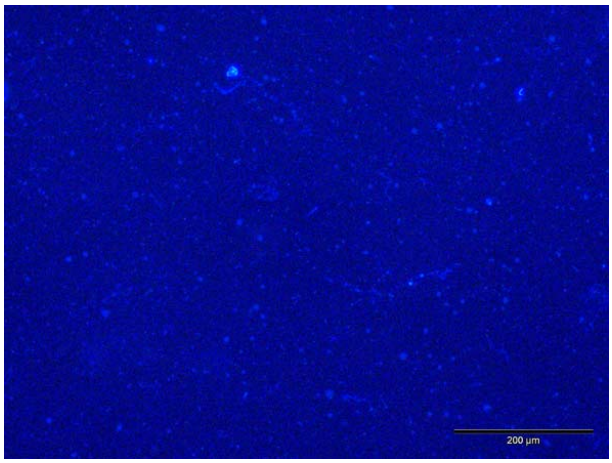


fig. 39 Pegylation

X. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH6 (3.2.3)
- EDC and sulfo-NHS, pH 6 (3.2.4)
- bisamino-PEG in 50 mL carbonate-bicarbonate buffer, pH 5 (3.2.5)
- splitted IgG-HRP (3.6.3) immobilised via its sulfhydryl groups in the hinge region reacting with OPA

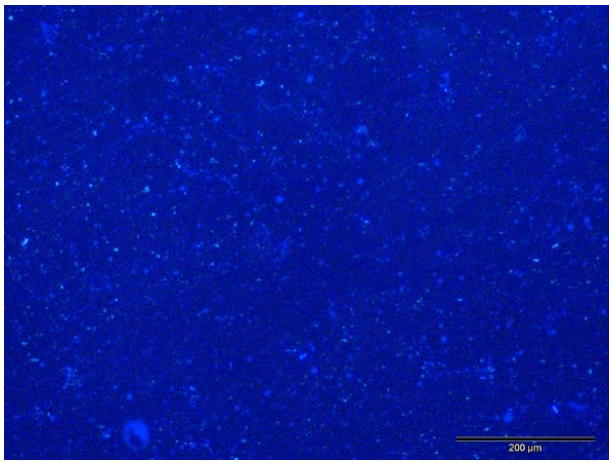


fig. 40 Splitted IgG-HRP immobilised onto pegylated slides

XI. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH6 (3.2.3)
- EDC, pH 8 (3.2.4)
- bisamino-PEG in 50 mL carbonate-bicarbonate buffer, pH 8 (3.2.5)

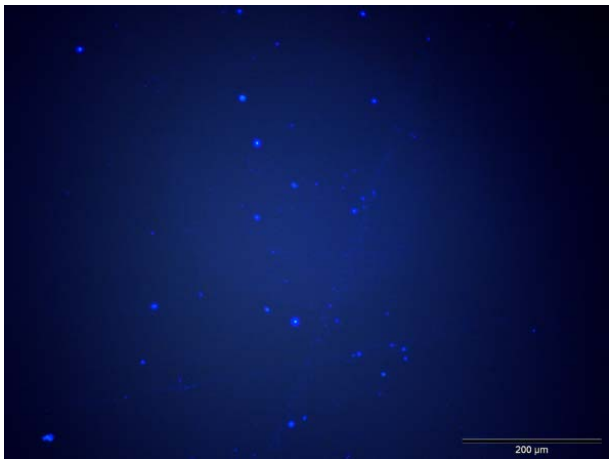


fig. 41 Pegylation

XII. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH 6 (3.2.3)
- EDC, pH 10 (3.2.4)
- bisamino-PEG in 50 mL carbonate-bicarbonate buffer, pH 8 (3.2.5)

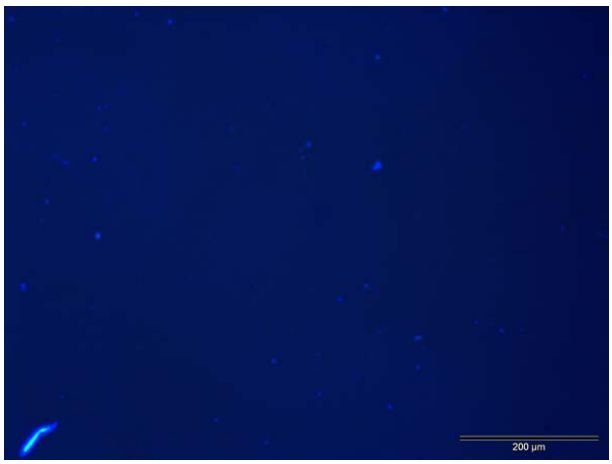


fig. 42 Pegylation

XIII. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH 8.4 (3.2.3)
- EDC, pH 8 (3.2.4)
- bisamino-PEG in 50 mL carbonate-bicarbonate buffer, pH 8 (3.2.5)

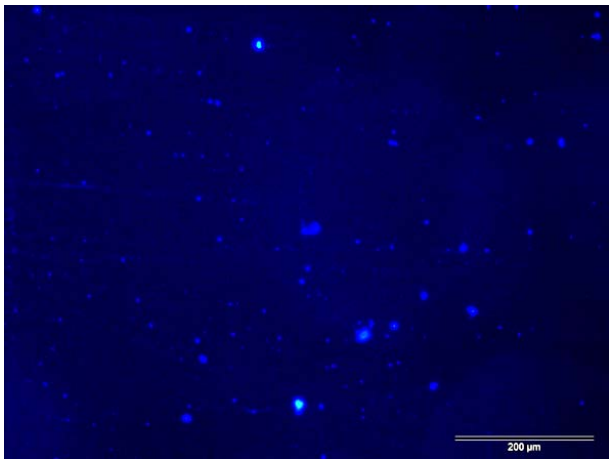


fig. 43 Pegylation

XIV. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH 8.4 (3.2.3)
- EDC, pH 10 (3.2.4)
- bisamino-PEG in 50 mL carbonate-bicarbonate buffer, pH 8 (3.2.5)

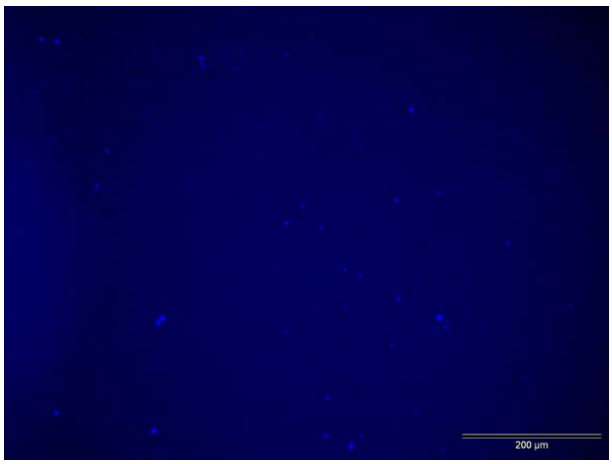


fig. 44 Pegylation

XV. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH 8.4 (3.2.3)
- EDC, pH 10 (3.2.4)
- bisamino-PEG in 50 mL carbonate-bicarbonate buffer, pH 10 (3.2.5)

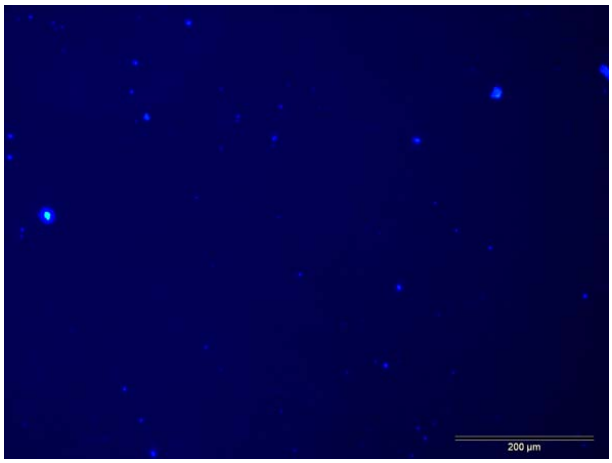


fig. 45 Pegylation

XVI. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4M succinic anhydride solution in 50 mM PBS, pH 8.4 (3.2.3)
- EDC, pH 8 (3.2.4)
- bisamino-PEG in 50 mL carbonate-bicarbonate buffer, pH 10 (3.2.5)

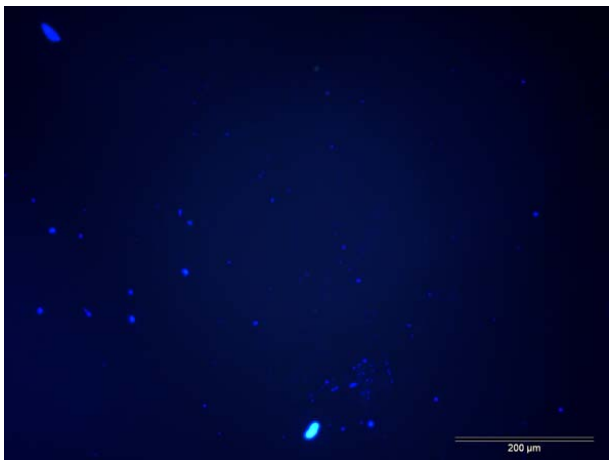


fig. 46 Pegylation

XVII. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH 8.4 (3.2.3)
- EDC, pH 10 (3.2.4)
- bisamino-PEG in 50 mL carbonate-bicarbonate buffer, pH 10 (3.2.5)
- splitted IgG-HRP (3.6.3) immobilised via its sulfhydryl groups in the hinge region reacting with OPA

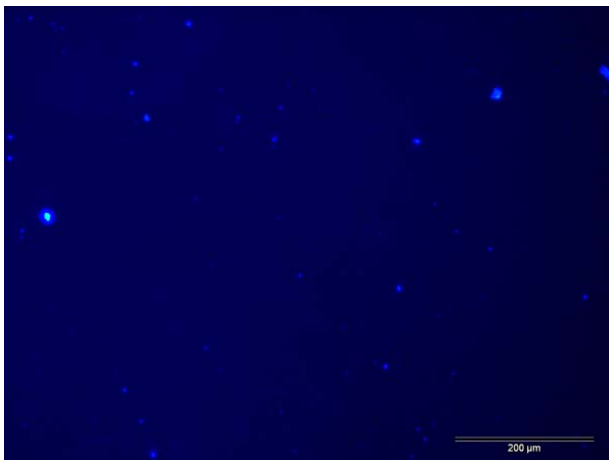


fig. 47 Splitted IgG-HRP immobilised onto pegylated slides

XVIII. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH 8.4 (3.2.3)
- EDC and sulfo-NHS, pH 10 (3.2.4.1)
- bisamino-PEG in 50 mL carbonate-bicarbonate buffer, pH 10 (3.2.5)

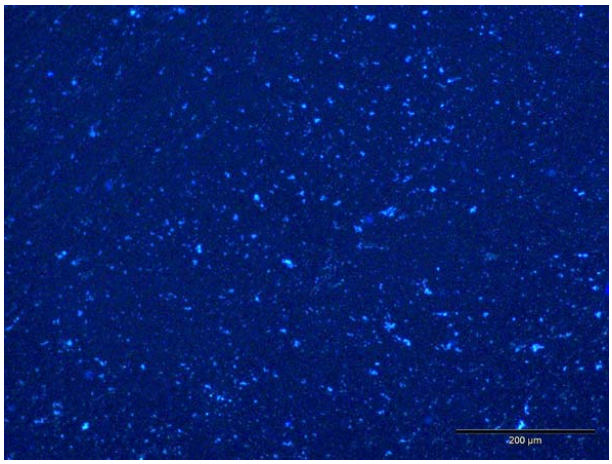


fig. 48 Pegylation

XIX. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH 8.4 (3.2.3)
- EDC and sulfo-NHS, pH10 (3.2.4)
- bisamino-PEG in citric acid-sodiumcitrate buffer, pH 5 (3.2.5)
- splitted IgG-HRP (3.6.3) immobilised via its sulfhydryl groups in the hinge region reacting with OPA

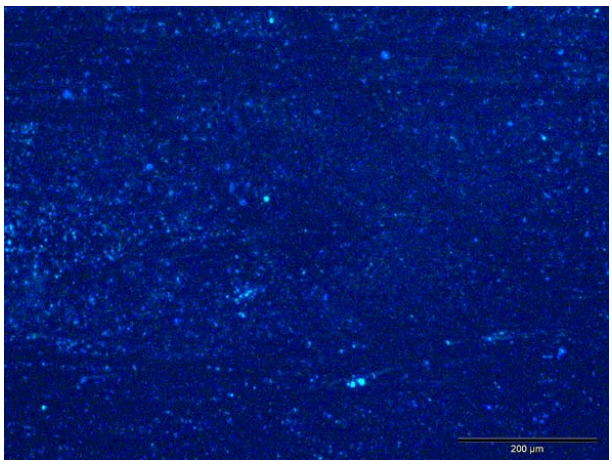


fig. 49 Splitted IgG-HRP immobilised onto pegylated slides

XX. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH 8.4 (3.2.3)
- EDC and sulfo-NHS, pH 10 (3.2.4)
- bisamino-PEG in carbonate-bicarbonate buffer, pH 10 (3.2.5)
- splitted IgG-HRP (3.6.3) immobilised via its sulfhydryl groups in the hinge region reacting with OPA

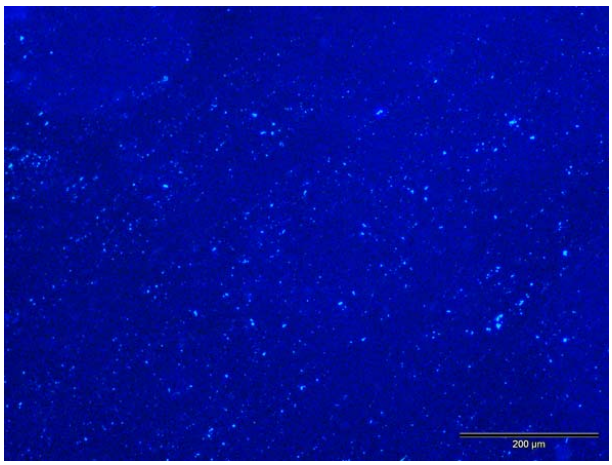


fig. 50 Splitted IgG-HRP immobilised onto pegylated slides

XXI. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH 8.4 (3.2.3)
- EDC, pH 6 (3.2.4)
- bisamino-PEG in carbonate-bicarbonate buffer, pH 10 (3.2.5)
- splitted IgG-HRP (3.6.3) immobilised via its sulfhydryl groups in the hinge region reacting with OPA

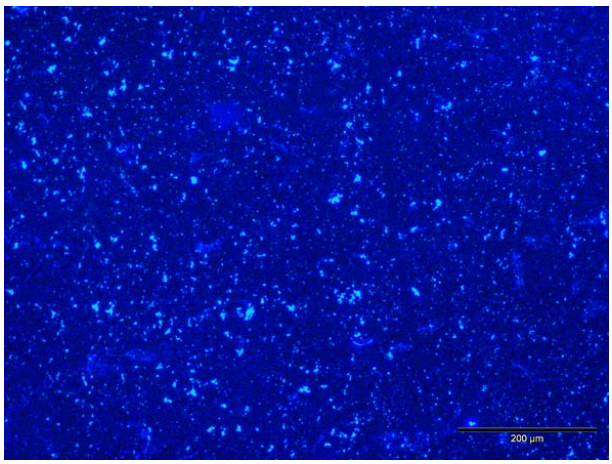


fig. 51 Splitted IgG-HRP immobilised onto pegylated slides

XXII. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH 8.4 (3.2.3)
- EDC, pH10 (3.2.4)
- bisamino-PEG in carbonate-bicarbonate buffer, pH 10 (3.2.5)
- splitted IgG-HRP (3.6.3) immobilised via its sulfhydryl groups in the hinge region reacting with OPA

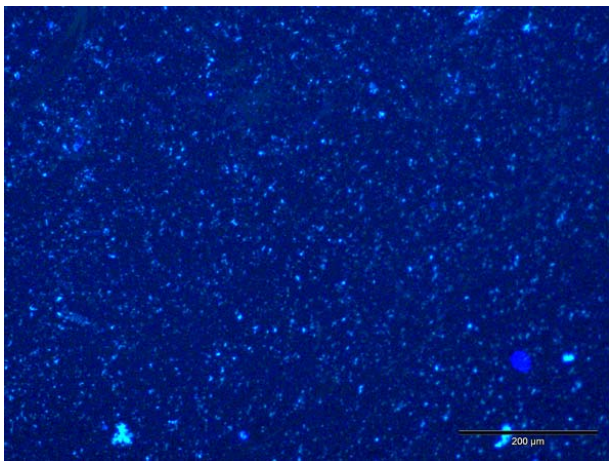


fig. 52 Splitted IgG-HRP immobilised onto pegylated slides

XXIII. slides coated with

- APTS, organic deposition (3.2.2.2)
- 0.4 M succinic anhydride solution in 50 mM PBS, pH 8.4 (3.2.3)
- EDC, pH 10 (3.2.4)
- amino-methoxy-PEG ($CH_3(OCH_2CH_2)_nNH_2$) in 50 mL carbonate-bicarbonate buffer, pH 10 (3.2.5) instead of bisamino-PEG

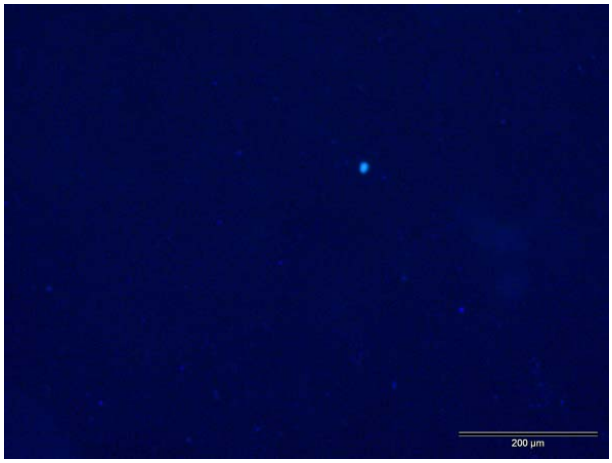


fig. 53 Pegylation with methoxy-PEG

XXIV. Coupling with chloranil (3.8.1.2) interferes under the fluorescent microscope. This coupling method was further studied by immobilisation of LDH (4.1.3)

The aminosilanisation with organic (I) and aqueous (II) deposition are approximate. However, the aminosilanisation by organic deposition is a more simple and faster method, thus it was preferred for the further coatings.

Coupling of splitted IgG-HRP via its sulfhydryl groups in the hinge region onto aminosilanised slides by means of OPA (III) shows a positive signal proving that the immobilisation of antibody onto aminosilanised slides is done successfully as well as the previous splitting of antibodies with immobilised dihydrolipoamide (3.6.3).

After coating with succinic anhydride under the condition of pH 8.4 (VI) a signal is no longer observable. By coupling of succinic anhydride with pH 6 (V), the signal decreases also in contrast to aminosilanised slides but not that much as (VI). Higher pH results in more positively charged amino groups, thus the reaction occurs more effectively and these conditions are preferable.

The comparison of pegylation by means of EDC (VII, XI, XII, XIII, XIV, XV, XVI, XXI) under different pH conditions is difficult, as the unbound amino group of bisamino-PEG is not only straightened outward, but PEG also forms coils or both amino ends can be bound via

EDC. However, coupling of bisamino-PEG with EDC and sulfo-NHS (IX, XVIII) shows an unique coating signal, which reconfirms that sulfo-NHS significantly increases the reaction yield. The coupling of bisamino-PEG in alkaline solution (pH 10) seems to be more successfully than in acid solution (pH 5), so this method was preferred for further immobilisations.

The immobilisation of splitted IgG-HRP via its sulfhydryl groups in the hinge region onto pegylated slides by means of OPA is successfully for both bisamino-PEG coupled only with EDC and bisamino-PEG coupled with EDC and sulfo-NHS. This is also important for the further immobilisation of PLGA-Ab onto the biocompatible coating of medical relevant materials with regard to drug-targeting and drug-reloading.

The last picture (XXIII) shows amino-methoxy-PEG coupled with EDC. This was done as a negative control where no amino groups are available on the surface. An effective coating with PEG is pointed out with it.

4.1.2 Staining with TNBS

Following slides were stained with TNBS (3.3.1):

- slides after organic solvent deposition (3.2.2.2)
- slides after aqueous deposition (3.2.2.1)
- slides after pegylation in carbonate-bicarbonate buffer, pH 10 (3.2.5)
- slides after pegylation in citric acid-sodium citrate buffer, pH 5 (3.2.5)

Add 1 mL 10% SDS and 0.5 mL 1 M HCl to each sample.

A faint yellow staining was observed on the slides after organic deposition.

4.1.3 Activity Measurement of Immobilised Lactate Dehydrogenase (LDH)

L-Lactate dehydrogenase ((S)-Lactate: NAD^+ oxidoreductase, E.C. 1.1.1.27) catalyses the redox reaction from pyruvate and $NADH/H^+$ to (S)-lactate and NAD^+ with a turn over rate of 14000 min^{-1} (pyruvate + NADH)[45] (fig. 54) and its reverse reaction. The pH optimum ranges between pH 6 and pH 8.

LDH *is an ubiquitous enzyme among all vertebrates. It catalyzes the final reaction of glycolysis, the formation of L-(+)-lactic acid. The enzyme activity found in serum or plasma usually originates from liver, heart, skeletal muscle erythrocytes and tumours. (...) To obtain serum samples (...) large haemolysis must be prevented in any case*[46]. LDH is a tetrameric molecule with five isoenzymes found in humans. The concentration of LDH in serum is used as clinical laboratory parameter e.g. for indication of myocardial infarction.

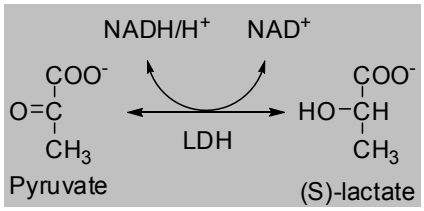


fig. 54 LDH catalyses the redox reaction from pyruvate and NADH/H$^+$ to (S)-lactate and NAD$^+$

The subunits of LDH have a molecular weight of 36000. This molecular weight was found in SDS-PAGE, too, for the rabbit serum as well as for the serum purified with affinity chromatography. This suggested the conclusion that LDH binds via its active site to PLGA-nanoparticles.

Coupling methods with chloranil and glutaraldehyde were used to immobilise lactate dehydrogenase (LDH, 5 mg/mL, Roche) onto aminosilanised slides, organic deposition (3.2.2.2), and the activity of the immobilised enzyme was measured in a long-term test. Immobilising LDH, different orientations of the active site are possible. Hence, some samples of the enzyme were first incubated with PLGA-NP o/n at 4°C to block the active site. Coupling then the LDH-nanoparticle complex onto the carrier material, the active site is adjusted outwards due to the sterical hindrance (fig. 55).

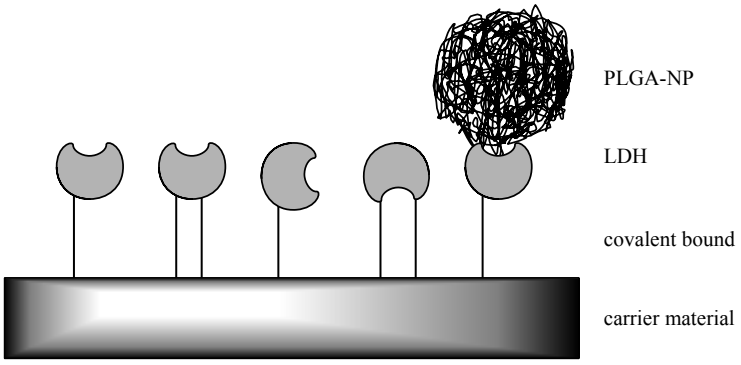


fig. 55 Immobilisation and orientation of LDH

In the group, Haifa Al-Dubai and Martina Strobl further investigated the interactions with PLGA-NP of bound LDH and purified (PLGA-)antibodies from serum.

4.1.3.1 Standard Curve

Solution for maximum activity:

isotonic PBS-buffer, pH 7.3	1.4 mL
pyruvate (8.28 µmol/mL)	50 µL
NADH (0.233 µmol/mL)	25 µL
LDH (different concentrations)	35 µL

The absorbance was measured after 1 min at 339nm against a blank value.

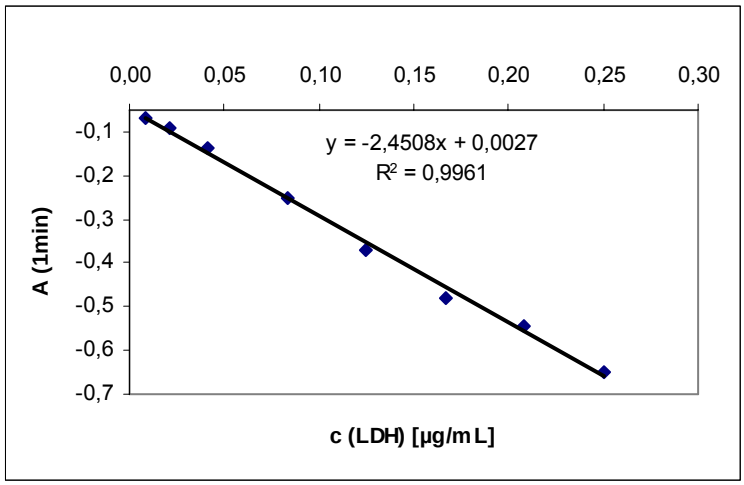


fig. 56 standard curve of LDH activity measurement

4.1.3.2 Activity Measurement of Immobilised LDH

The immobilisation methods are described in (3.8). Following methods were investigated:
LDH immobilised with

- chloranil (LDH Cl)
- chloranil and afterwards incubated with PLGA-NP (LDH Cl Nano)
- chloranil and afterwards incubated with HSA (LDH Cl HSA)
- glutaraldehyde (LDH GA)
- glutaraldehyde and afterwards incubated with PLGA-NP (LDH GA Nano)
- glutaraldehyde and afterwards incubated with HSA (LDH GA HSA)

LDH previously incubated o/n at 4°C with PLGA-NP and afterwards immobilised with

- chloranil (LDH + Nano Cl)
- glutaraldehyde (LDH + Nano GA)

The slides were shaken in 1.5 mL of following solution

isotonic PBS-buffer, pH 7.3	1.435 mL
pyruvate (8.28 µmol/mL)	50 µL
NADH (0.233 µmol/mL)	25 µL

and the activity was measured in a long-term test.
A measurable activity is only observable with LDH previously incubated with PLGA-NPs (fig. 57). This reconfirms that LDH binds via its active site to PLGA-NP, because the active site is oriented outward and an activity can be measured. Thereby, the coupling method with glutaraldehyde is about twice as effective as the coupling method with chloranil.

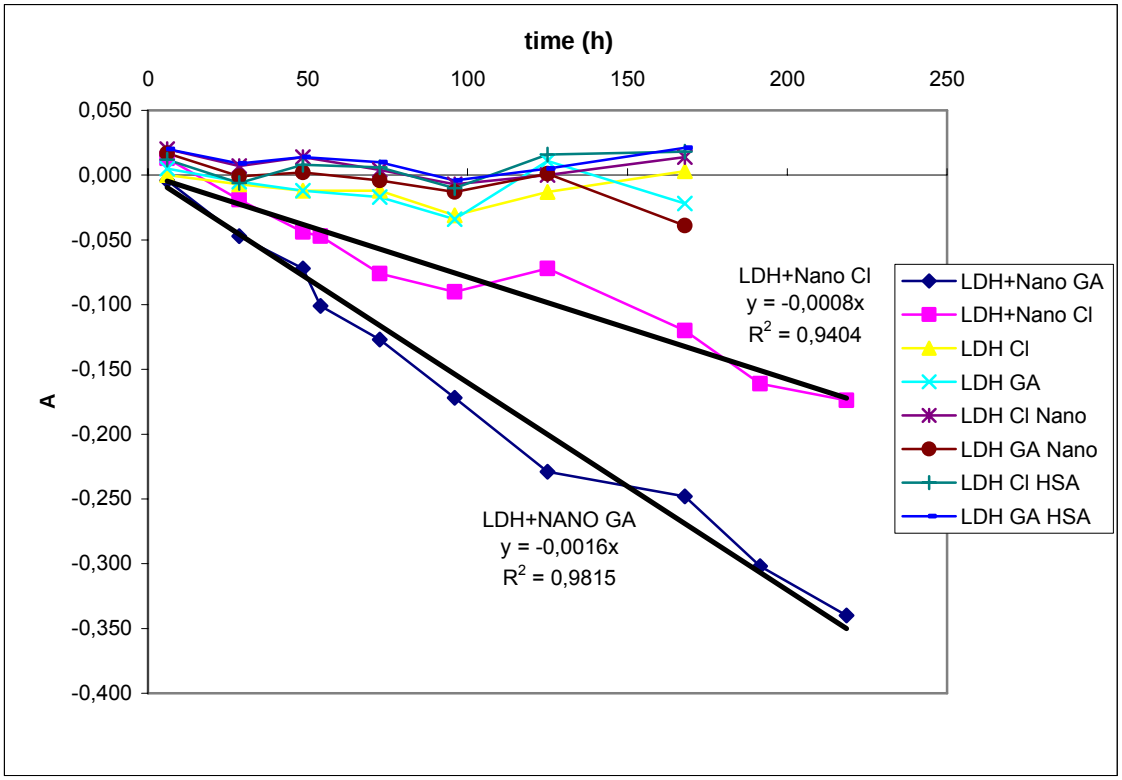


fig. 57 Activity measurement of immobilised LDH

An idea of this result that LDH binds to PLGA-NP was also to use immobilised LDH for drug-targeting and drug-reloading, but the interaction between LDH and the nanoparticles is too weak for effective drug-targeting.

However, this experiment demonstrated the importance to block previously the active site of the enzyme (as well as the cognitive domains of antibodies) before immobilisation to obtain the most effective orientations.

4.2 Gold Cluster Labelling Test

4.2.1 Stability Check of GCC Solution

The gold cluster colloid solution (3.1) shows an absorbance maximum at 520nm. Adding electrolytes like sodium chloride to the GCC solution leads to coagulation. However, the spontaneous absorption of proteins (in this case tested with 1 µL BSA-antibody) stabilise already the GCC measurable by the decrease of the absorbance maximum. Blocking with agents such as 1% fish gelatine (FG) stabilise additionally and prevents coagulation adding then electrolytes.

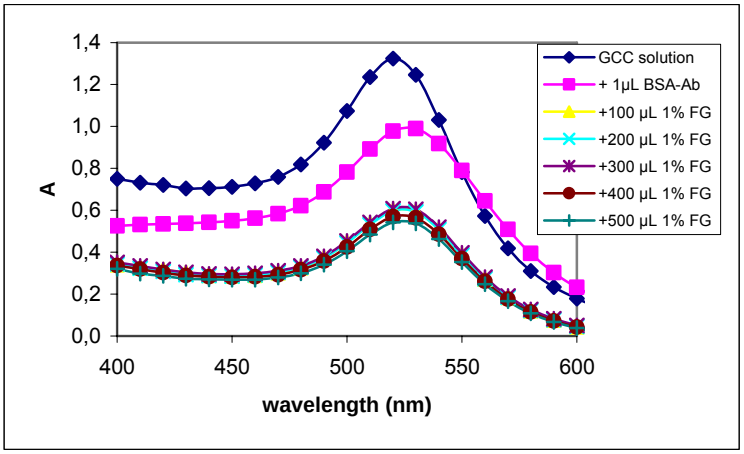


fig. 58 stability check of GCC solution

4.2.2 Pretests

As pretests for determination of titre, 2 µL PLGA solutions (5, 2.5, 1, 0.5, 0.1, 0.05 mg PLGA/mL) were dotted onto nitrocellulose membrane and the membrane was blocked and washed thereafter.

The GCC were labelled with:

- preserum
- serum of rabbit H52
- serum of rabbit H53
- serum of rabbit H52 purified with PLGA-affinity chromatography

Proteins in the sera labelled on gold cluster colloids bind to the dotted PLGA (fig. 59). With this experiment, the interactions between serum proteins and PLGA were visualised. However, several immunoglobulins as well as albumins and other serum proteins bind unspecifically to PLGA.

With the serum of rabbit H52 purified with PLGA-affinity chromatography, no signal was observable. This is may be a question of the concentrations.

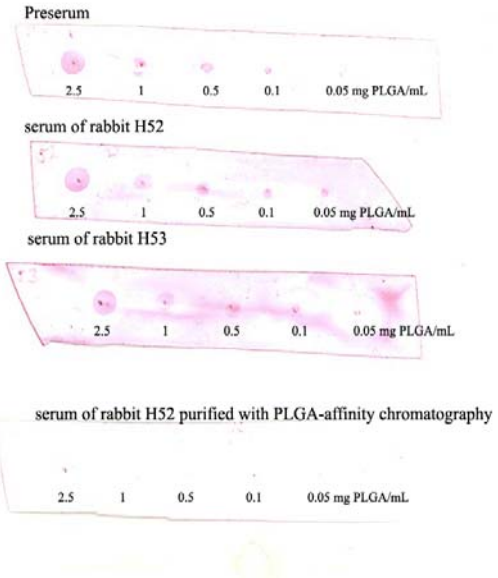


fig. 59 Gold Cluster Labelling Test with serum labelled on GCC and with dotted PLGA

For signal amplification, the Gold Cluster Labelling Test was conducted with protein G, which binds with high affinity to the F_c fragment of IgG (and with a weak binding to albumins). The GCC were first labelled with protein G (1 - 5 µL) and thereafter with the same amount of serum (fig. 60). As negative control a GCC solution only with protein G and blocked was used.

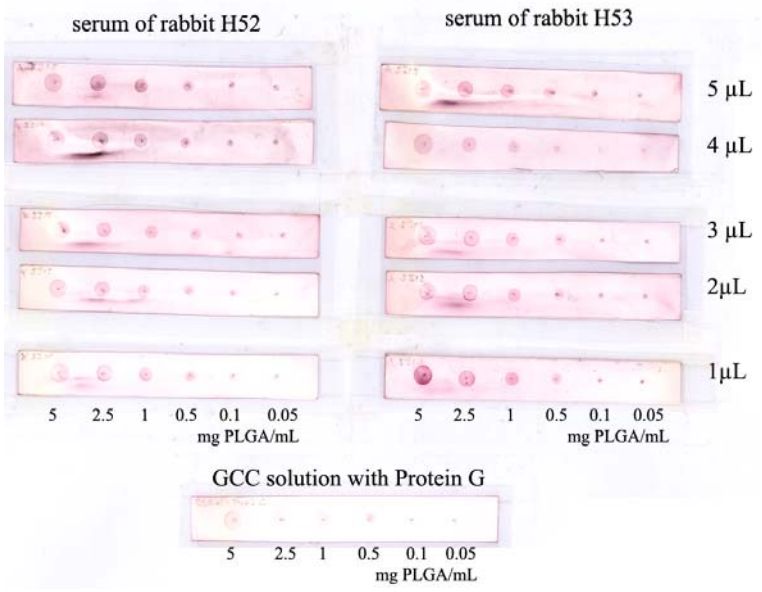


fig. 60 signal amplification with protein G

The signal decreases by the decrease of the PLGA concentration dotted (2 µL of 5 – 0.05 mg/mL) as well as by the decrease of serum labelled to the GCC (5 – 1 µL). This reconfirms the interaction between serum proteins and PLGA. However to determine the kind of proteins

and whether (PLGA-)antibodies or not, ELISA with the secondary antibody anti-Rabbit-IgG (4.5.3.1) was conducted.

4.2.3 Gold Cluster Labelling Test with Modified PLGA

For preparation of the solutions and the standard operation procedure of the Gold Cluster Labelling Test see (3.1).

a) Dotting:

Following probes were dotted onto nitrocellulose membrane, each with 1 µL:

- LDH (1:100 diluted),
- HSA (50 µg/mL),
- BSA (50 µg/mL),
- sera,
- sera after affinity chromatography (3.4.2)
- the flow of the affinity chromatography
- PLGA (3 mg/ml dioxane)
- NADH

Dry for 1h at rt. After blocking with blocking solution, wash 3x5min with washing solution and 3x with ddH_2O.

b) Gold cluster labelling of modified PLGA

Add 1 µL modified PLGA (3.9) (1:100 diluted) to 10 mL gold cluster solution and shake for 30 min. Block with blocking (1% fish gelatine in ddH_2O) solution for 15 min. As negative controls 2 µL PLGA (3mg/ml dioxane) were used instead of modified PLGA and gold cluster solution only blocked.

Incubate the dotted probes with the labelled gold cluster solutions for 2h. Wash 3x 5min with washing solution.

However, no relevant signal was observed with modified PLGA labelled GCC in comparison to the negative control of blocked GCC.

4.3 Bradford Protein Assay

standard curve

50 µL of the sample were added to 2.5 mL Bio-Rad Protein Assay (1:5 diluted) and the absorbance was measured after 10 min at 595nm against a blank value.

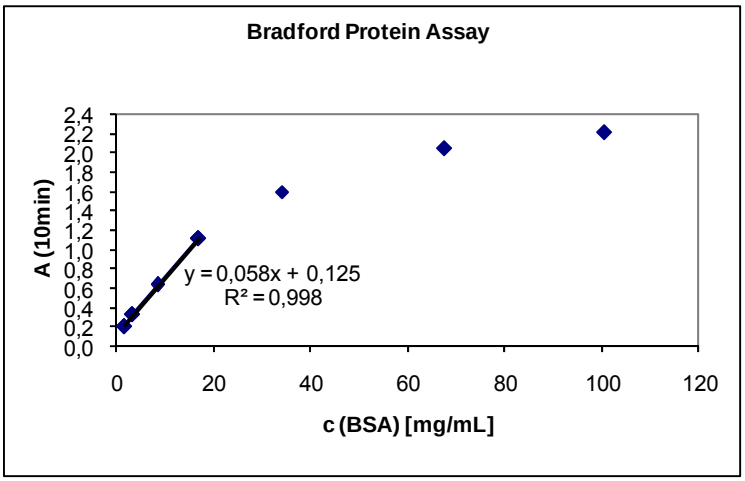


fig. 61 Bradford Protein Assay – standard curve

4.4 Ellman's Assay

standard curve

To 1 mL of the standard solution of N-Acetyl-L-Cystein 50 µL Ellman's Reagent (5.4 mg/mL 0.1 M PBS, pH8) were added and the absorbance was measured after 15 min at 412nm against a blank value.

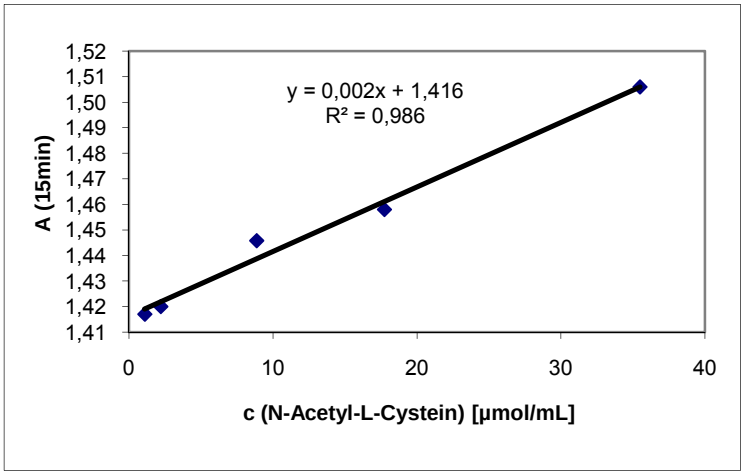


fig. 62 Ellman's Assay – standard curve

4.5 Purification and Verification of PLGA-Ab

4.5.1 Determination of Titre

For determination of the titre, different amounts of PLGA-NP (final amount of 6.25 µg and 3.125 µg) were dotted into micro wells and the wells thereafter blocked and washed. Sera 1:100, 1:500 and 1:2500 diluted were incubated and the bound IgG was detected with anti-rabbit-IgG-HRP and stained with TMB (fig. 63, fig. 64).

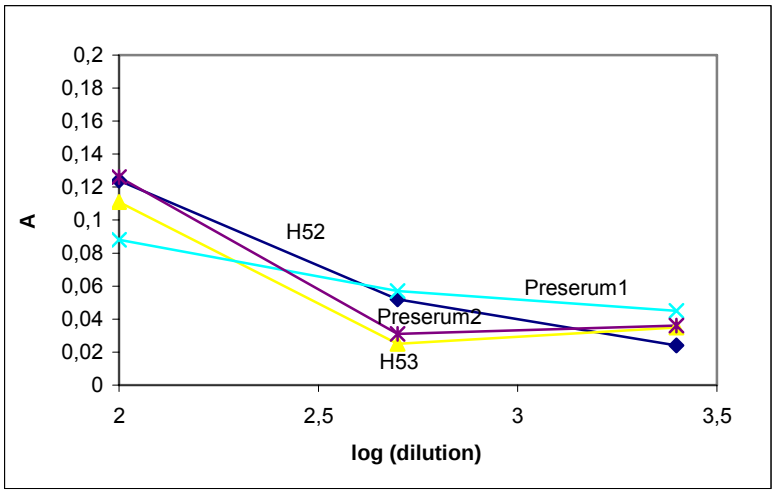


fig. 63 Determination of titre (with 6.25 µg PLGA dotted)

The absorbance decreases. Thus, IgG bind to the dotted PLGA, but no relevant titre is observable in comparison with preserum. It is known that several immunoglobulins bind unspecifically to PLGA-NP.

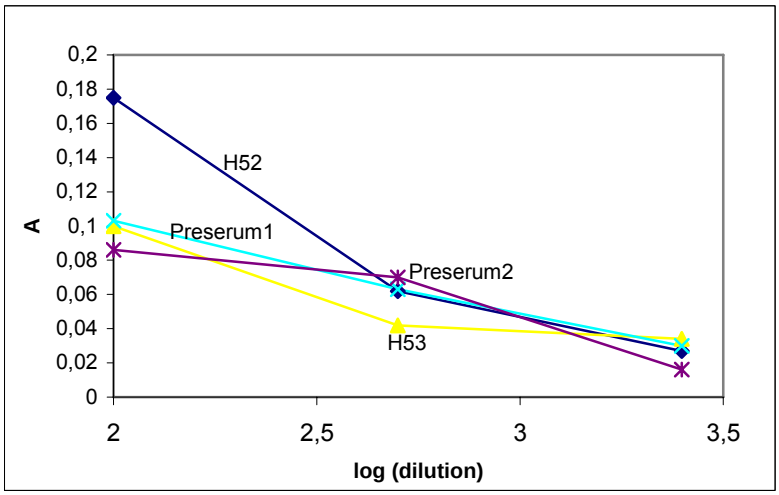


fig. 64 Determination of titre (with 3.125 µg PLGA dotted)

The same procedure only with 3.125 µg PLGA dotted, but also no relevant titre is observable. Rather at serum of rabbit H52 a titre is determined than the other ones, which would agree with other experiments like Western Blot and ELISA (4.5.3.1).

4.5.2 Polyacrylamide Gel Electrophoresis

4.5.2.1 SDS PAGE

Following samples were separated with the aid of SDS-PAGE (3.11.1):

(1) preserum (as negative control)

(2) serum of rabbit H52 immunised with PLGA-NP

(3) serum of rabbit H53 immunised with PLGA-NP

(4) serum of rabbit H52 immunised with PLGA-NP and purified with PLGA-affinity chromatography. Therefrom was loaded onto the gel:

   a. flow of the serum, which was not bound by the affinity chromatography

   b. eluate

(5) the samples marked with Haifa are from serum of rabbit H52 immunised with PLGA-NP and purified with different chromatographic methods conducted by Haifa Al-Dubai

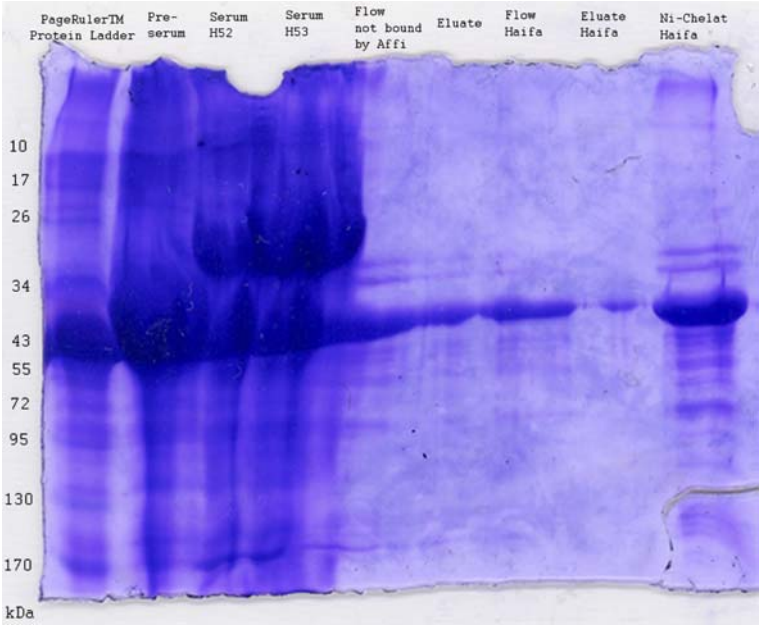


fig. 65 SDS-PAGE

The gel was stained with CBB. All samples show a signal with a molecular weight of about 36000 to 38000. This suggested the conclusion of LDH (for that purpose see 4.1.3).

4.5.2.2 Native PAGE

Following samples were separated with the aid of native PAGE (2.3.3):

(1) serum of rabbit H52 immunised with PLGA-NP
(2) serum of rabbit H53 immunised with PLGA-NP
(3) preserum
(4) serum of rabbit H52 immunised with PLGA-NP and purified with PLGA-affinity chromatography. Therefrom was loaded onto the gel
    a. eluate
    b. flow of the serum, which was not bound by the affinity chromatography
    c. eluate
(5) preserum purified with PLGA-affinity chromatography (as negative control). Therefrom was loaded onto the gel
    a. flow of the serum, which was not bound by the affinity chromatography
    b. eluate
(6) serum of rabbit H52 immunised with PLGA-NP and purified with proteinA-affinity chromatography, conducted by Haifa Al-Dubai

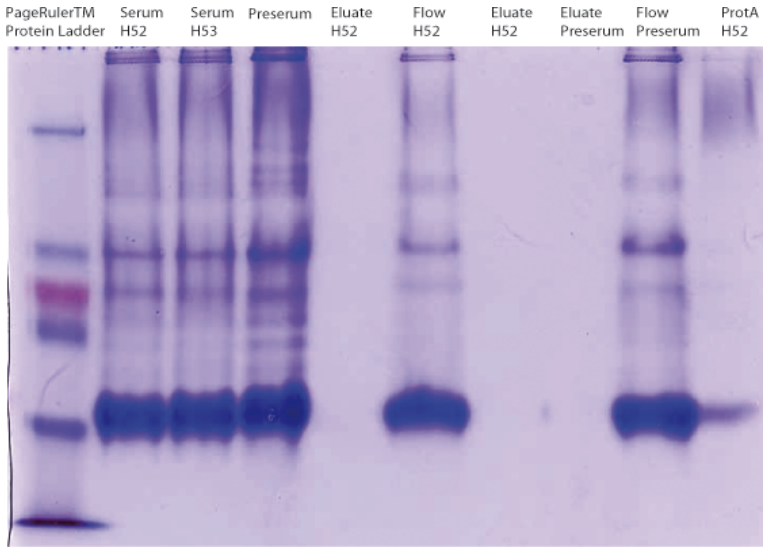


fig. 66 native PAGE

The gel was stained with CBB. No bands are visible at the eluates both H52 and preserum. The affinity chromatography was conducted with only 100 µL serum to purify PLGA-antibodies, thus the concentration is too low for staining with CBB. Hence, Western Blot (4.5.3) with the same samples was accomplished to detect then with the more sensitive ELISA.

4.5.3 Western Blot

After separating the proteins with native PAGE (4.5.2.2), the proteins were transferred onto a nitrocellulose membrane with Western Blot. The proteins were detected by ELISA and by PLGA-NP containing ALP.

4.5.3.1 Western Blot und Elisa anti-Rabbit IgG-ALP

The blotted proteins were detected with anti-rabbit-IgG-ALP (3.12.1).

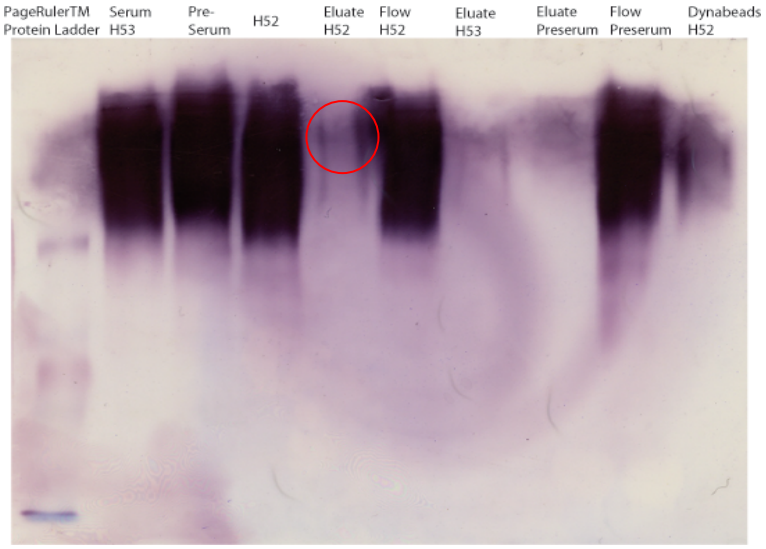


fig. 67 Western Blot and Elisa anti-rabbit IgG-ALP

Staining with BCIP and NBT, IgGs were detected in the eluate. However, immunoglobulins bind also unspecifically to PLGA-NP. For future perspectives, the purification of immunised rabbit serum has to be done with higher concentrations and the antibodies have to be verified afterwards with mass spectroscopy to determine if PLGA-Ab or not.

4.5.3.2 Western Blot and PLGA-NP containing ALP

The blotted proteins were incubated with a solution of PLGA-NP containing ALP (3.12.2).

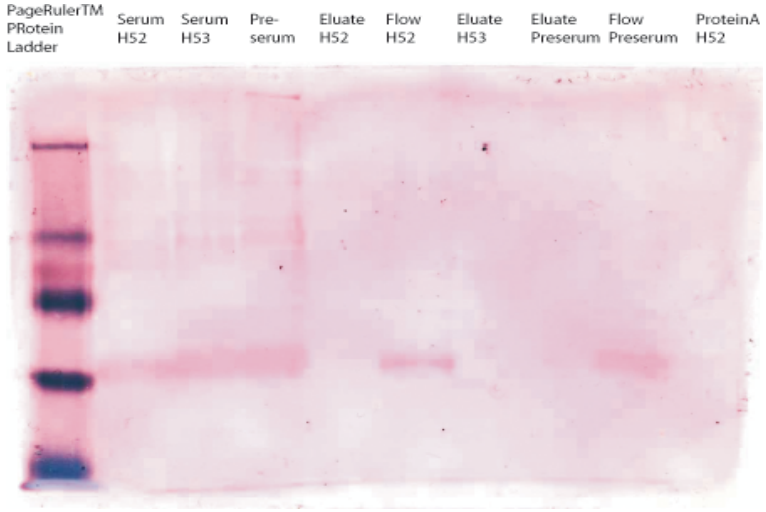


fig. 68 Western Blot and NP containing ALP

Staining with BCIP and NBT shows several bands visible at serum samples as well as one band in the flow, which is in the range of albumins. This reconfirms the interaction between albumins and PLGA-NP, which is a main limitation of drug targeting (see introduction).

The eluate shows no visible band. However, IgG purified with PLGA-affinity chromatography was detected with ELISA on Western Blot. Thus, the detection with PLGA-NP containing ALP is not sensitive enough and the method has to repeated with higher serum concentrations.

As negative control a Western Blot with the same types of samples was shaken in ALP-staining solution, however, no staining was observed.

5 Summary and Future Perspectives

The main focus of this master thesis was based on biocompatible coatings of medically relevant materials and its verification as well as on the purification of PLGA-antibodies from sera and its detection by titre determination, PAGE and Western Blot.

Biocompatible coating was studied by staining with the fluorescent molecule OPA. By this method, the immobilisation of splitted antibodies could also be shown. By immobilisation of LDH onto aminosilanised slides, the importance to block previously the active site was demonstrated to obtain the most efficient orientation of the enzyme (and of antibodies).

Sera of the immunised rabbits H52 and H53 were purified with PLGA-affinity chromatography. By the determination of titre, no relevant titre was observed in comparison to preserum. However, the detection of IgG with Western Blot and Elisa was successful, primary with the serum of rabbit H52. This might derive from the fact that also additives giving during the course of immunisation cause IgG production specific to their surface structure. Eventually, the same procedure should be done with higher concentrations, means with more serum to purify the (PLGA-)antibodies.

After working on my master thesis, another two rabbits were immunised. With these sera, the titre determination seemed to be more successfully.

It is known, that immunoglobulins bind unspecific to PLGA-NP. Therefore the next step is the identification of the separated immunoglobulins IgG by means of mass spectrometry. If antibodies specific to PLGA-NPs can be identified, a monoclonal antibody has to be cloned and the experiments of drug-targeting and drug-reloading have to be repeated with it.

The experiments of drug-targeting and drug-reloading in a long-term experiment were accomplished by Haifa Al-Dubai and show great promise.

In spite of all troubles, the project seems to succeed!

5.1 Summary and Future Perspectives (German)

Der Schwerpunkt dieser Diplomarbeit lag, neben der biokompatiblen Beschichtung medizinisch relevanter Materialien und deren Verifizierung, auf der Reinigung von PLGA-Antikörpern aus Seren und deren Nachweis mittels Titerbestimmung, PAGE und Western Blot.

Die Untersuchung der biokompatiblen Beschichtung erfolgte mit Hilfe des Fluoreszenzmoleküls OPA. Damit konnte auch die Immobilisierung gespaltener Antikörper gezeigt werden. Bei der Immobilisierung von LDH auf aminosilanisierten Deckgläsern wurde die Bedeutung aufgezeigt, die Aktivitätsstelle vorher zu blocken, um eine möglichst effektive Orientierung von Enzymen (und auch von Antikörpern) zu erhalten.

Die Seren von den immunisierten Kaninchen H52 und H53 wurden mittels PLGA-Affinitätschromatographie gereinigt. Bei der Titerbestimmung war jedoch im Vergleich zum Präserum kein relevanter Titer bestimmbar. Der Nachweis von IgG mit Western Blot und Elisa war jedoch positiv, insbesondere beim Serum vom Kaninchen H52. Dies kann von der Bildung von IgG´s gegen die verschiedenen Additive, die bei der Immunisierung verwendet wurden, kommen. Eventuell sollten die Versuche mit höheren Konzentrationen, sprich mit mehr gereinigtem Serum, nochmals durchgeführt werden.

Nach der praktischen Arbeit an meiner Diplomarbeit wurden zwei neue Kaninchen immunisiert. Bei diesen schien die Titerbestimmung erfolgreicher zu sein.

Es ist bekannt, daß Immunglobuline unspezifisch an PLGA-NP binden. Deshalb wäre der nächste Schritt die Identifizierung der gereinigten Immunglobuline IgG mittels Massenspektrometrie. Falls Antikörper spezifisch gegen PLGA-NP nachgewiesen werden können, müßten daraus monoklonale Antikörper hergestellt werden und die Versuche *drug-targeting* und *drug-reloading* damit wiederholt werden.

Die Experimente für *drug-targeting* und *drug-reloading* in einem Langzeitversuch wurden von Haifa Al-Dubai durchgeführt und sind vielversprechend.

Trotz aller Schwierigkeiten ist das Projekt erfolgreich!

CHAPTER II: DEVELOPMENT OF AN ALLERGY TEST

Abstract

An *in vitro* allergy test in microtitre plates was developed in the working group of Professor Pittner by means of Gold Cluster Labelling Test (2.2.3). The detection of the allergen occurs by gold cluster colloids labelled with the serum of the patient, which contains IgE specific to the proteins the patient is allergic to.

The advantages over commonly used ELSA tests are the inexpensive and simple application and the colour stability for long-term documentation as well as no hazardous reagents are necessary. The GCC Labelling Test can be also made as kits and the application of ELISA reader makes automation possible. Thereby the sensitivity of this test is comparable to ELISA techniques.

Abstract (German)

Ein *in vitro* Allergentest in Mikrotiterplattenformat mittels Goldcluster Labelling Test wurde in der Arbeitsgruppe von Professor Pittner entwickelt. Der Nachweis der Allergene erfolgte mit Goldcluster-gelabeltem Serum von Allergikern. Dieses enthält IgE spezifisch gegen das nachzuweisende Allergen.

Der Vorteil gegenüber herkömmlich genutzten ELISA Tests sind die kostengünstige und einfache Handhabung und die Farbstabilität für Langzeitdokumentation. Außerdem sind keine gefährlichen Reagentien nötig. Der GCC Labelling Test kann auch als Kit gestaltet werden und durch Anwendung von ELISA Readern ist eine Automatisierung möglich. Die Sensitivität dieses Tests ist dabei vergleichbar mit denen von ELISA.

1 Introduction

1.1 Allergy

Allergies are an inadequate and an enhanced immune reaction concerning normally harmless substances. Characteristic features are[47]:

- allergy develops only after several contacts with the allergen
- only a part of humans, who are in contact with the allergen, fall ill
- the immunoglobulin IgE is involved
- mast cells release active agents (mediators)

The most allergy-causing substances are proteins (antigens) elicited inter alia by mite, pollen, fungi, domestic animal hairs, stinger, chemical substances, medicaments and food. The question *when does a protein become an allergen?* is reviewed by Mari A. and for further information be referred on it. Important is the *Evaluation of the IgE reactivity of naturally purified molecule within a general population, in one or more geographical areas (...). Defining the symptomatic/asymptomatic ratio*[48].

The "classical" allergy is one of the four forms of hypersensitivity (*type I – IV*) and called *type I* (immediate hypersensitivity, IgE-mediated). More than 20% of persons in industrialised countries are affected.

During the first contact with an allergen, the immunopathogenic key processes are[49]:

- the activation of T_{H2}-cells with an increased production of the cytokines IL-4, IL-5, IL-9 and IL-13
- the differentiation (class switching) to IgE or respectively IgG1 expressing B-cells
- the production of these immunoglobulins through plasma cells
- binding of IgE via high affine F_c receptors ($F_c\varepsilon RI$) on the surface of mast cells (about 300000 IgE per cell) and of basophil granulocytes. Mast cells are located with a high density in mucosae.

In a second contact, the immunoglobulins IgE bound on the surface of mast cells are crosslinked by the allergen. Thus, the granula changes and the mast cell degranulates and releases mediators such as histamine. Each mast cell contains up to 5 pg histamines, a vasoactive amine that cause the anaphylactic reactions and the undesirable symptoms of an allergy (e.g. asthma or allergic rhinitis). In a later secondary reaction, activated mast cells set free cytokines, which affect inflammatory with a dead time of 5 to 6 hours. The stages of the first and second contact leading in the end to the release of histamine are shown in (fig. 69).

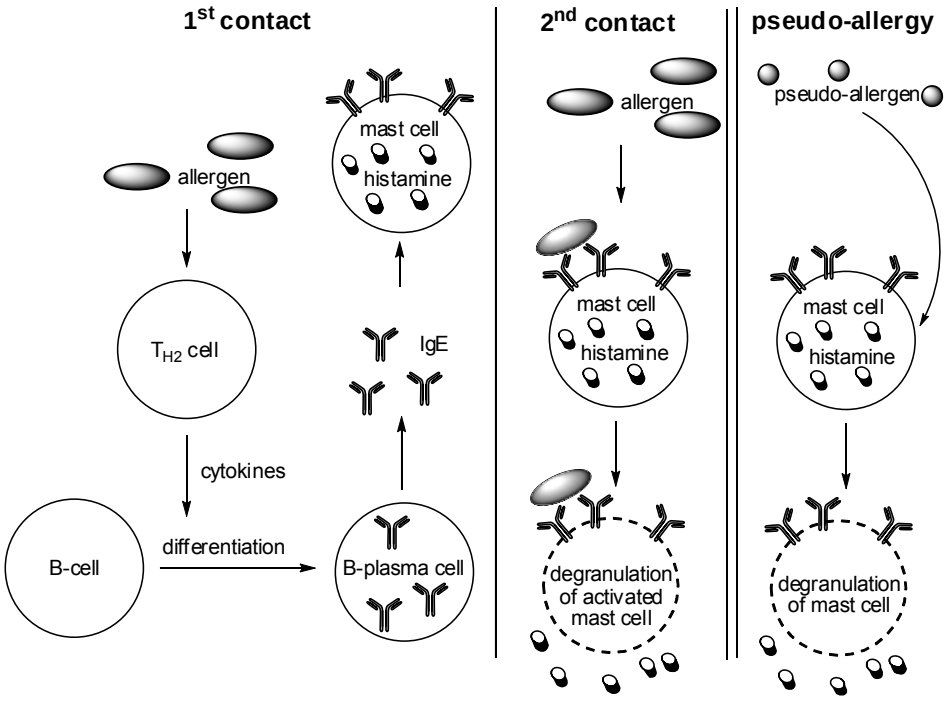


fig. 69 First contact: production of IgE by activation of T_{H2} and class switching of B-cells; second contact: releasing of histamine caused by crosslinking of IgE bound on the surface of the mast cells. In comparison to it: the pseudo-allergy to the right.

From allergy are to differentiate pseudo-allergy and intolerance. Certain substances (pseudo-allergens) act directly on the degranulation of mast cells and thus effect the release of histamine (fig. 69) with symptoms similar to the allergic ones. However, the intolerance is based on metabolic disorders, mostly caused by enzyme deficiency (e.g. lactose intolerance).

The cross-reactivity of IgE is not only of interests by patients, who mostly react to more than a single allergen, but also for the development of an allergy test. *Cross-reactivity has a clear structural basis: no relevant cross-reactivity without structural similarity. For globular proteins (i.e., for most allergens), structural similarity requires similarity in folding*[50].

The increased IgE level of allergic persons is measured *in vitro* allergy tests.

1.2 Allergy tests

Tests for the allergen, which causes the allergic response, can be made by:

- skin tests: small amounts of possible allergens are placed on or below the skin. After 20 min, the reaction (wheal development) is compared with a positive control (histamine) and a negative control (agent-free).
    - skin prick test: one of the most commonly used allergy test. Suspected allergens are dropped on the skin and with little scratches allowed to enter the skin.
- elimination tests: used to check food allergy.
    - In a diet, foods with possible allergens are avoided for several weeks. Afterwards, they are administered again under medical control looking at possible symptoms.
    - Another possibility is a double-blind study giving foods and harmless substances in a disguised form.
- blood tests: measuring of the IgE concentration in serum. Thereby, the free IgE as a whole or the allergen-specific IgE can be determined. Commonly used methods are:
    - fluoro immunoassay (FIA)
    - enzyme-linked immunosorbent assay (EIA, ELISA)
    - radioallergosorbent test (RAST)
    - radioimmunosorbent test (RIST)

The principle of the immunoassays are described in (2.2).

The skin test is a rapid, inexpensive test but cannot carry out with patients having hives or eczema. Another obstacle is taking medicine such as antihistamines, which falsify the result by preventing or reducing the allergic reaction. Reacts the person allergic to several allergens e.g. in food allergy, the main allergen can be found out with *in vitro* tests. Above all, a blood test has to be done instead of a skin test at patients with a high risk of anaphylaxis.

2 Methods and Experimental Section

2.1 Cohn-Fractionation of Blood Plasma

The plasma fractionation is used to separate and purify proteins from blood. Thereby the different solubility concerning pH, temperature, ionic strength, behaviour in solvents and interactions with solid phases is utilised.

Slowly unfreezing deep-frozen plasma to about 1°C, blood coagulation factors remain insoluble and are centrifuged to obtain the cryoprecipitate. The serum contains about 70 mg/mL proteins therefrom 12 mg/mL γ-globulins. The percentage of immunoglobulins with 17% is relatively low in contrast to albumins with 63.7%[51].

The proteins in the cryo-poor plasma are further separated by fractionation with increasing concentrations of ethanol and a temperature below 0°C, established by Edwin Joseph Cohn (1892-1953):

If alcohol precipitation is carried out at sufficiently low temperature (close to -5°C), denaturation is often prevented. (...) Alcohol precipitation methods (...) have the decided advantage that precipitates from ethanol-water mixtures at low temperatures can be readily dried under vacuum. This yields solid, salt-free, water-soluble, purified proteins[52].

The principle of the plasma fractionation is shown in (fig. 70).

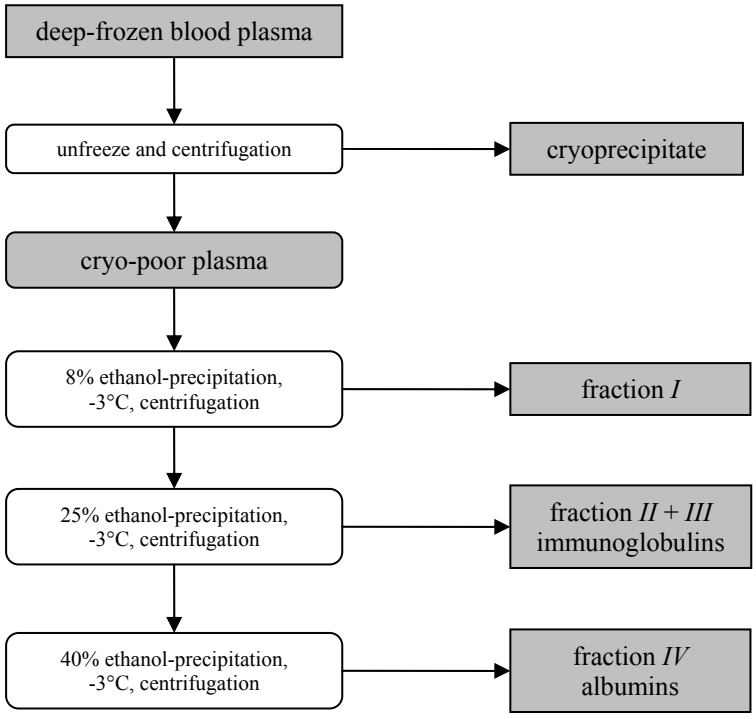


fig. 70 Plasma fractionation

After separation of fraction *I* by 8% ethanol-precipitation, immunoglobulins are precipitated with 20-25% ethanol and centrifuged. The last step with 40% ethanol yields albumins with the highest solubility and lowest isoelectric point of the plasma proteins. The separated fractions can be further purified e.g. by chromatographic methods to obtain the desired proteins.

2.2 Cohn-Fractionation

Unfreeze slowly 1 mL deep-frozen blood plasma of an allergic person to about 1°C. The sample is centrifuged in a cold room for 10 min at 14000 rpm. The cryoprecipitate is removed. Add ethanol to the cryo-poor supernatant (final concentration 8%). Cool down to about -3°C and centrifuge for 15 min at 14000 rpm and with a temperature of -3°C. The pellet is removed and ethanol is added to the supernatant to obtain a final concentration of 25% ethanol. Cool down again under 0°C and centrifuge for 15 min at 14000 rpm and -3°C.

A part of the ethanol can be removed from the precipitates by speed vacuum, however avoid a complete dehydration, which would denature the proteins.

The precipitates were dissolved in 500 µL ddH_2O and a Gold Cluster Labelling Test (2.2.3, 3.1) was conducted.

3 Results and Discussion

3.1 ELISA to Recheck the Cohn-Fractionation

The detection of IgG in the Cohn-fractions was accomplished by ELISA.

100 µL of the fractions were incubated in a wet chamber o/n at 4°C in micro wells. After blocking for 30 min with blocking solution (0.1% BSA/0.1% Tween20/PBS, pH 7.2), wash 3x 5 min with washing solution (0.5% Tween20/PBS). Incubate with 100 µL anti-human-IgG-HRP (SB2D40-05, #C248-X549J) for 2 h in a wet chamber at rt. Wash 3x 5min with washing solution and stain thereafter with a TMB-solution (SCIOTEC).

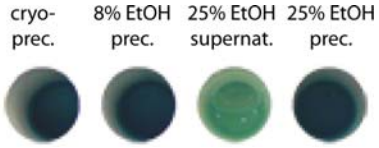


fig. 71 ELISA to recheck the Cohn-fractionation (prec. = precipitation, supernat. = supernatant)

The most IgG were found in the fraction with 25% ethanol precipitation, which reconfirms the blood plasma fractionation, but also in the fractions before immunoglobulins could be detected.

3.2 Allergy Test in vitro

The sera of allergic persons were obtained from the company SCIOTEC. With the serum of the patient 199439, who is allergic against grasses, birch trees and house dust mites, a Cohn-fractionation was conducted.

From Allergopharma Vertriebsges.mbH., Vienna, the Prick-/Scratch testing solutions (3.0 mL) were received. Following samples were dotted into micro wells:

1) 708 dermatophagoides farinae (130 µg/mL) (dust mite, Ed.)
2) 725 dermatophagoides pteronyssinus (standardised concerning biological activity) (house dust mite, Ed.)
3) 044 fungi I (380 µg protein/mL)
4) 106 mugwort (standardised concerning biological activity)
5) 116 ash tree (320 µg/mL)
6) 108 birch tree (standardised concerning biological activity)
7) 154 short ragweed (320 µg/ml)
8) 006 grasses (standardised concerning biological activity)
9) 015 grasses/cereals (630 µg/ml)

Following samples were additionally dotted:

10) HSA (1 mg/mL)
11) BSA (1 mg/mL)
12) PLGA (2 mg/mL PLGA)

Following fractions of the Cohn-fractionation of the serum 199439 were labelled to GCC:

A) cyro precipitate (blood coagulation factors)
B) 8% ethanol-precipitation (fraction *I*)
C) 25% ethanol supernatant (albumins)
D) 25% ethanol-precipitation (immunoglobulins)
E) GCC (negative control)

At the samples marked with protG (=protein G), the GCC were first labelled with protein G and thereafter with the fractions.

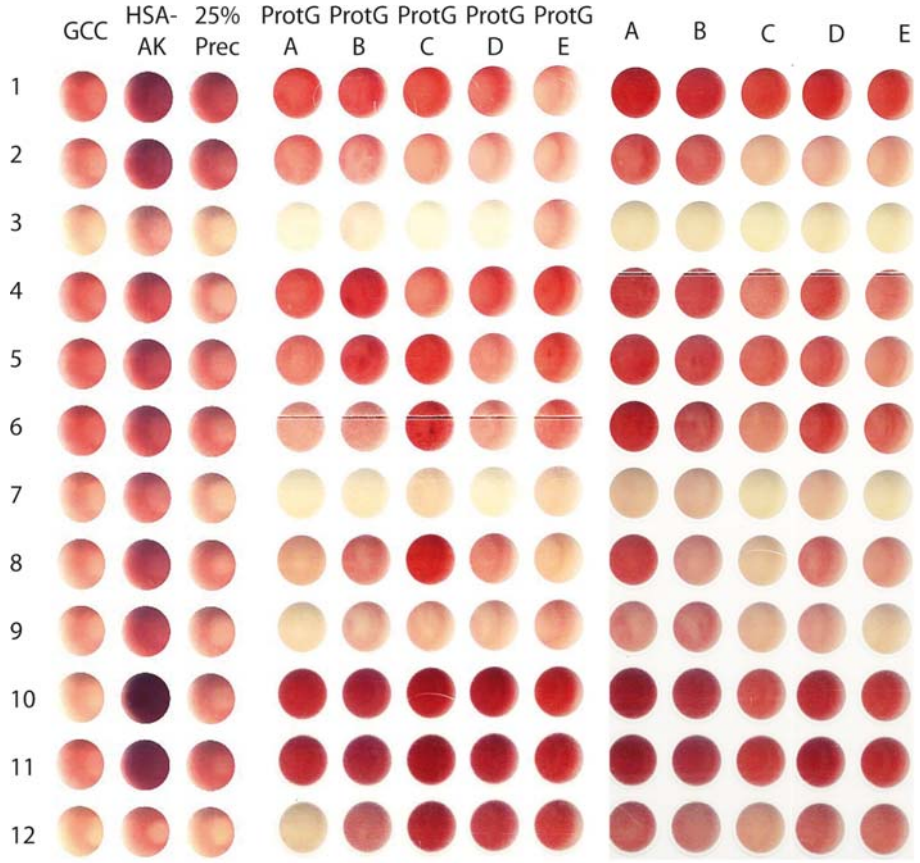


fig. 72 Allergy test *in vitro* with GCC

For interpretation of an *in vitro* allergy test, it is important to know the possible cross sensitivity of an allergen. An overview about the allergens used in the allergy test and their cross reactions is given in (tab. 5) (without demand of completeness).

Allergen	Cross sensitivity to
dermatophagoides (mites)	crustaceans, sea food
fungi	saccharomyces cerevisiae, penicillin
mugwort	birch tree, ragweed
ash tree	olive, lilac
birch tree	**ash tree, mugwort**
short ragweed	banana, melon
grasses	**cereals,** legumes
cereals	grasses

tab. 5 cross sensitivities

The GCC solution (1st row and sample E) provide as negative control. HSA-antibody labelled GCC (2nd row) was used as positive control reacting with the dotted HSA (10) and shows a cross reactivity to the dotted BSA (11). The immunoglobulins after Cohn-fractionation should be in the sample D (25% ethanol-precipitation) and the albumins in sample C.

The patient is allergic to house dust mites (2) (cross sensitivity to other mites (1)), birch tree (6) (cross sensitivity to ash tree (5) and mugwort (4)) and grasses (8) (cross sensitivity to cereales (9).

Signals, which agree with the allergy of the patients are visible at sample C with protein G and at sample D without protein G.

The Cohn-fractionation was conducted with only 1 mL of serum, whereby the main problem was the age of this serum (older than 10 years) and the half-life of IgE (tab. 3). So much better the result of Cohn-fractionation in combination with Gold Cluster Labelling Test.

To affirm the results, the experiment has to be repeated with a fresh blood withdrawal and the results have to be compared with other allergy tests, mainly a skin prick test, to diagnose cross sensitivities.

List of Tables

Table of Figures

Abbreviations

A	absorbance
Ab	antibody
Ag	antigen
ALP	alkaline phosphatase
APC	antigen presenting cell
CBB	Coomassie Brilliant Blue
CLISA	Cluster Linked Immunosorbent Assay
e.g.	exempli gratia
DES	drug-eluting stents
ELISA	Enzyme-Linked ImmunoSorbent Assay
FIA	fluoro immunoassay
FG	gelatine from cold water fish skin
GCC	gold cluster colloid
H52, H53	rabbits immunised with PLGA-NP
HRP	horseradish peroxidase
IA	immunoassay
Ig	immunoglobulin
NP	nanoparticle
o/n	over night
PAGE	Polyacrylamide Gel Electrophoresis
PEG	polyethylene glycol
PLGA	poly(lactic-*co*-glycolic acid)
RIA	radio immunoassay
rt	room temperature
TCR	T-cell receptor
w/o/w	water-in-oil-in water

Chemicals

***A**cetic acid,* CH_3COOH, MW 60.05, Merck

Acetone, $(CH_3)_2CO$, MW 58.08, J.T.Baker

Acrylamide/Bis, 30% acrylamide, 0.8% Bis (N,N-methylene bisacrylamide), Sigma

ALP, Alkaline phosphatase from Bovine Calf Intestine, 0.02 mL, 22 mg protein/mL, 5590 units/mg protein, Sigma

Anti-human-IgG-Peroxidase, SB 2D40-05, #C248-X549J (provided by SCIOTEC)

Anti-rabbit-IgG-HRP (from unknown origin)

APS, ammoniumpersulfate, MW 228.2, $(NH4)_2S_2O_8$, Serva

APTS, 3-aminopropyltriethoxysilane, $C_9H_{23}NO_3Si$, MW 221.37, Fluka

***B**CIP,* 5-bromo-4-chloro-3-indolyl phosphate, MW 433.62, Pierce

Bio-Rad Protein Assay, dye reagent concentrate, Bio-Rad Laboratories GmbH

Bisamino-PEG, O,O´-bis(3-aminopropyl)polyethylene glycol, MW ~1500, Fluka

Bromophenolblue, indicator pH 3.0-4.6, $C_{19}H_{10}Br_4O_5S$, MW 669.99, Merck

BS^3, bis(sulfosuccinimidyl)suberate, MW 572.43, Pierce

BSA, albumin from bovine serum, Cohn *V* Fraktion, Sigma

***C**BB-R250,* Coomassie Brilliant Blue R-250, Serva

Chloranil, p-Chloranil, $C_6Cl_4O_2$, MW 245.88, purum ≥ 97.0%, Fluka

Citric acid-1-hydrat, $C_6H_8O_7 \cdot H_2O$, MW 192.12, Riedel de Haën

Cystamine dihydrochloride, $C_4H_{12}N_2S_2 \cdot 2HCl$, MW 225.20, Sigma-Aldrich

Cysteamine hydrochloride, $C_2H_7NS \cdot Cl$, MW 113.61, Fluka

***D**isodiumhydrogenphosphate,* Na_2HPO_4, MW 141.96, Riedel de Haën

1,4-Dioxane, $C_4H_8O_2$, MW 88.11, Sigma

DL-α-Lipoic acid, $C_8H_{14}O_2S_2$, MW 206.33, Fluka

DTT, 1,4-dithio-D,L-threitol, $C_4H_{10}O_2S_2$, MW 154.25, Fluka

***E**DC*, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, MW 191.7, Sigma

Ellman's Reagent, 5,5´-dithio-bis-(2-nitrobenzoic acid), DTNB, MW 396.4, Pierce

Ethanol abs., C_2H_5OH, MW 46.07, Merck

Ethyl acetate, $C_4H_8O_2$, MW 88.10, Merck

***F**ish gelatine,* gelatine from cold water fish skin, Sigma

***G**lutaraldehyde,* $C_5H_8O_2$, MW 100.12, d_4^{20} 1.063, Fluka

Glycerin, $C_3H_8O_3$, MW 92.09, Riedel de Haën

Glycine, $C_2H_5NO_2$, MW 75.07, Gerbu

Gold(III)chloride trihydrate, $HAuCl_4 \cdot 3H_2O$, MW 393.83, Sigma-Aldrich

H*SA,* 96-99%, albumin from human serum, Sigma

Hycdrochloric acid, HCl*,* 37-38%, MW 36.46, J.T.Baker

Hydrogen peroxide, H_2O_2, MW 34.02, Merck

I*sopropanol*, $CH_3CHOHCH_3$, MW 60.10, J.T.Baker

L*DH,* lactate dehydrogenase, from pig's heart, 5 mg/ml, Roche

L-LDH, from bovine muscle, 12.6mg protein/ml (Biuret), 643units/mg protein, Sigma

M*agnesium chloride,* $MgCl_2 \cdot 6H_2O$, MW 203.30, Sigma-Aldrich

2-Mercaptoethanol, C_2H_6OS, MW 78.13, Fluka

Methanol, CH_3OH, MW 32.04, J.T.Baker

N*-Acetyl-L-cysteine,* $C_5H_9NO_3S$, MW 163.19, Sigma

NADH, β-nicotinamide adenine dinucleotide, reduced form, disodium Salt, $C_{21}H_{27}N_7O_{14}P_2Na_2$, MW 709.4, Sigma

NBT, nitroblue tetrazolium, $C_{40}H_{30}N_{10}O_6Cl_2$, MW 817.60, Pierce

Nitric acid, HNO_3, MW 63.01, Merck

O*PA,* O-phtaldialdehyde, $C_6H_4(CHO)_2$, MW 134.14, Fluka

P*ageRuler*^{TM}*,* Prestained Protein Ladder, #SM0671, Fermentas

PLGA, poly(DL-lactide-co-glycolide) (50:50), MW 07462, Fluka

Pluronic F-68, Sigma

PonceauS, $C_{22}H_{12}N_4Na_4O_{13}S_4$, usb

Protein G´, from Streptococcus sp., recombinant, expressed in E.coli, Sigma

Pyruvic Acid, $C_3H_4O_3Na$, Sodium Salt, MW 110.0, Sigma

S*DS,* Sodium dodecylsulfate, $C_{12}H_{25}O_4S \cdot Na$, MW 288.4, Serva

Sodium borohydride, $NaBH_4$, MW 37.83, Aldrich

Sodium carbonate-decahydrate, $Na_2CO_3 \cdot 10H_2O$, MW 286.14, Riedel de Haën

Sodium chloride, NaCl, MW 58.44, Riedel de Haën

Sodium dihydrogenphosphate, NaH_2PO_4, MW 119.98, Riedel de Haën

Sodium hydrogencarbonate, $NaHCO_3$, MW 84.0, Riedel de Haën

Sodium hydroxide, NaOH, Merck

Sodium phosphate, Na_3PO_4, MW 163.94, Riedel de Haën

Sodium thiocynate, NaSCN, MW 81.07, Fluka

Succinic anhydride, C_4HO_3, MW 100.07, Merck

Sulfo-NHS, N-hydroxysulfosuccinimide, $C_4H_4NO_6SNa$, MW 217.14, Pierce

Sulfo-SMCC, succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate, MW 334.33, Pierce

Sulfo-SMPB , Sulfosuccinimidyl-4-(*p*-maleimidophenyl)butyrate, $C_{18}H_{15}N_2O_9SNa$, MW 458.36, Pierce

Sulfuric acid, H_2SO_4, MW 98.08, Merck

T*EMED*, N,N,N′,N′-tetramethylethylenediamine, $C_6H_{16}N_2$, MW 116.20,

TMB, 3,3',5,5'-tetramethylbenzidine, $C_{16}H_{20}N_2$, MW 240.35, SCIOTEC

TNBS, trinitrobenzene sulfonic acid, 5%(w/v), $C_6H_3N_3O_9S$, MW 293.17, Sigma

Toluene, C_6H_5CH3, MW 92,14, Baker

Tris, Tris(hydroxymethyl)aminomethane, $C_4H_{11}NO_3$, MW 121.14, AppliChem

Trisodiumcitrate-dihydrate, $C_6H_5O_7Na_3 \cdot 2H_2O$, MW 294.10, Riedel de Haën

TritonX100, 4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol, Fluka

Tween20, $C_{58}H_{114}O_{26}$, MW 1227.72, Sigma

Curriculum Vitae

Name:	Helmut Hubert Hinterwirth
Geburtsdatum:	20.11.1981
Geburtsort:	A - 4400 Steyr (OÖ)
Staatsangehörigkeit:	Österreich
Wohnort:	Severinusstrasse 7/9
	A – 4470 Enns

Schulbildung:

1988 - 1992	Volksschule Enns
1992 - 2001	Don-Bosco-Gymnasium, D-59329 Diestedde
2001	Matura

2001 - 2002	Zivildienst beim Österreichischen Roten Kreuz, Dienststelle Enns
seit 2002	ehrenamtliches Mitglied beim Roten Kreuz, Dienststelle Enns

Studium:

2002 - 2007	Grundstudium Chemie (1.Abschnitt) an der Universität Wien
2007 - 2009	Chemie vertiefend (2.Abschnitt) an der Universität Wien
2008 - 2009	Tutorium
2008 – 2009	Diplomarbeit

Attachment: A Novel Dot-Blotting Method for Immunoassays

universität wien

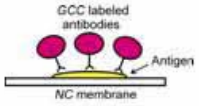
MFPL Max F. Perutz Laboratories

A novel dot-blotting method for immunoassays

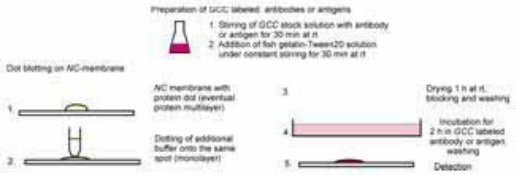
Haifa Al-Dubai*, Helmut Hinterwirth and Fritz Pittner
Max F. Perutz Laboratories, Department of Biochemistry , University of Vienna

A novel micro method is presented to quantify antigens using nitrocellulose (NC) membranes as a support for dot-blotting antigens and employing gold-cluster colloid (GCC) labeled antibodies.

Principle:



Method:



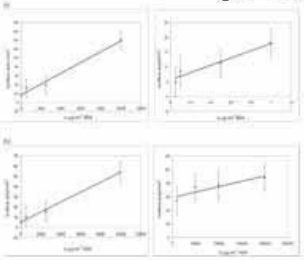
The membrane has to be blocked with a protein lacking free –SH groups as e. g. fish gelatine to avoid binding of GCC onto free NC. After application of the gold colloid cluster labeled antibodies the signal is visually readable and can be compared to a color scale prepared from a dilution series of known sample concentrations. The color reaction product is stable for a very long time and does not fade.

Results:

a) BSA samples (concentrations in the range between 2E5 to 0.001 µg/cm²) 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 2E5 to 0.025 µg/cm²) 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.

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 (β-galactosidase antibody)

a) B-lactoglobuline (BLG) samples (concentrations in the range between 1 E5 to 1 µg/cm²) dotted onto NC-membrane without dotting of additional buffer onto the same spot.
b) BLG samples dotted onto NC-membrane and dotting additional buffer onto the same spot, to minimize multilayers of protein.
c) BLG samples reacted with GCC labeled polyclonal BSA antibodies (negative control)



Calculation of antigen concentration from surface area of spot after addition of 5 mm³ 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.
BSA samples in various concentrations (left: 5 to 1 E-4 µg/cm² and right: 0.05 to 1 µg/cm²)
HSA samples in various concentrations (left: 5 to 1 E-4 µg/cm² and right: 1 E-4-2 E5 µg/cm²)

Conclusions:

The sensitivity of the method is comparable to that of ELISA if not better. Beyond that only very small amounts of antibody are needed. This method is an alternative to the use of expensive, enzyme-conjugated antibodies for a number of applications, such as testing purified sera of allergic persons with respect to different antigens or tracking of antibodies during purification or hapten inhibition tests.

References

[1] Gebbers J.; *GMS German Medical Science* **5** (2007), ISSN 1612-3174

[2] Kothwala D. et al.; *Trends in Biomaterials & Artificials Organs* **22** (2008) 83-88

[3] Park K., Acharya G.; *Advanced Drug Delivery Reviews* **58** (2006) 387-401

[4] Black J.; *J Biomed Mater Res* **16** (1982) 159-167

[5] Williams D. in *Definitions in Biomaterials,* (1987), Elsevier, New York

[6] Williams D. in *The William´s Dictionary of Biomaterials* (1999), Liverpool University Press, UK

[7] Williams D.; *Medical Device Technology* **14** (2003) 10-13

[8] Sevastianov V.; *Trends in Biomaterials & Artificials Organs* **15** (2002) 20-30

[9] Adams D.; *American Journal of Pathology* **84** (1976) 164-191

[10] Hench L.; Best S. in *Biomaterials Science, 2nd Edition;* (2004) 154 Elsevier, San Diego, California, Ratner B. et al (Ed.)

[11] Athanasiou K et al.; *Biomaterials* **17** (1996) 93-102

[12] Zalfen A. et al.; *Acta Biomaterialia* **4** (2008) 1788-1796

[13] Silber S.; *Zeitschrift für Kardiologie* **93** (2004) 649-663

[14] Ehrlich P.; *Handbuch der pathogenen Mikroorganismen* **3** (1913) 337-374

[15] Petrak K.; *Journal of Biomaterials Science, Polymer Edition* **17** (2006) 1209-1219

[16] Rojanasakul Y.; Azad N.; *American Journal of Drug Delivery* **4** (2006) 79-88

[17] Cheng J. et al.; *Biomaterials* **28** (2007) 869-876

[18] Garnett M.; Kallinteri P.; *Occupational Medicine* **56** (2006) 307-311

[19] Langer K.; *Forschung Frankfurt* **4** (2006) 48-51

[20] Vega-Villa K. et al.; *Advanced Drug Delivery Reviews* **60** (2008) 929-938

[21] Soppimath K.; *Journal of Controlled Release* **70** (2001) 1-20

[22] Davis S. et al.; *Biochemical and Biophysical Research Communications* **177** (1991) 861-866

[23] Anderson J.; Shive M.; *Advanced Drug Delivery Reviews* **28** (1997) 5-24

[24] Tracy M. et al.; *Biomaterials* **20** (1999) 1057-1062

[25] Müller M. et al.; *J Biomed Mater Res* **66A** (2003) 55-61

[26] Neumann J. in *Immunbiologie* (2008), Springer-Verlag Berlin Heidelberg

[27] Roitt I.; Brostoff J.; Male D. in *Kurzes Lehrbuch der Immunologie, 3.Auflage* (1995) 44, Thieme Stuttgart, New York

[28] http://www.emc.maricopa.edu/faculty/farabee/BIOBK/ANTIBODY.gif (29.04.2009)

[29] Bundesamt für Veterinärwesen; *Richtlinie Tierschutz 3.04* (1999) 1-15, Schweizerische Eidgenossenschaft

[30] Lottspeich F.; Engels J. in *Bioanalytik, 2.Auflage* (2006) 132, Elsevier, München

[31] Schomburg D.; Salzmann M.; Stephan D. in *Enzyme Handbook 3* (1991), Springer-Verlag Berlin, Heidelberg

[32] Kim E.; Wyckoff H.; *Journal of Molecular Biology* **218** (1991) 449-464

[33] Schomburg D.; Salzmann M.; Stephan D. in *Enzyme Handbook 7* (1994), Springer-Verlag Berlin, Heidelberg

[34] Mason P. et al.; *Journal of Biology Chemistry* **257** (1982) 3669-3675

[35] Frens G.; *Nature Physical Science* **241** (1973) 20-22

[36] Hermanson G. in *Bioconjugate Techniques, 2nd Edition*; (2008) 925, Elsevier, Illinois, USA

[37] Al-Dubai H.; Pittner G.; Pittner F.; *Monatshefte für Chemie* **139** (2008) 1531-1536

[38] Lottspeich F.; Engels J. in *Bioanalytik, 2.Auflage* (2006) 266, Elsevier, München

[39] Palmer D.; Peters T; *Clinical Chemistry* **15** (1969) 891-901

[40] Gorecki M.; Patchornik A.; *Biochimica Biophysica. Acta* **303** (1973) 36-43

[41] Hermanson G.; Mallia A.; Smith P. in *Immobilized Affinity Ligand Techniques;* (2008) 275-279, Academic Press, San Diego

[42] Pittner F. in *Analytical Biotechnology* (2002) 1-42, Birkhäuser Verlag, Basel, Schalkhammer (Ed.)

[43] Greg T. Hermanson in *Bioconjugate Techniques, 2nd Edition*, (2008) 243, Elsevier, Illinois, USA

[44] Fillafer C.; Wirth M.; Gabor F.; *Langmuir* **23** (2007), 8699-8702

[45] Schomburg D., Stephan D. in *Enzyme Handbooks 9* (1995) 1.1.1.27, Springer-Verlag Berlin Heidelberg

[46] Kopperschläger G.; Kirchberger J.; *Journal of Chromatography B: Biomedical Applications* **684** (1996) 25-49

[47] Ollier S.; Davies R. in *Allergien* (1991), Spektrum der Wissenschaft, Heidelberg

[48] Mari A.; *Clinical and Experimental Allergy* **38** (2008) 1089-1094

[49] Petrides P.; Ansorge S. in *Biochemie & Pathobiochemie, 7.Auflage,* Löffler G., Petrides P. (Ed.) (2003) 1155, Springer-Verlag Berlin Heidelberg

[50] Aalberse R. et al.; *Allergy* **56** (2001) 478-490

[51] Löffler G.; Petrides P. in *Biochemie und Pathochemie, 7.Auflage* (2003) 1110, Springer-Verlag, Berlin Heidelberg

[52] Cohn E.; *Chemical Reviews* **28** (1941) 395-417