universität wien

DIPLOMARBEIT

Evaluierung von Methoden zum Coating von Implantatmaterialien mit derivatisierten Biopolymeren im Hinblick auf den Einsatz im "Drug targeting"

Verfasser

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Meinem Großvater
Lorenz Oberhofer

The man who has fed the chicken every day throughout its life at last wrings its neck instead, showing that more refined views as to the uniformity of nature would have been useful to the chicken. *Bertrand Russell*

Acknowledgments

Given that this page will be the most read of this thesis, I am seeking forgiveness in the case I affront some people by forgetting to mention them.

So first of all I would like to thank my family, especially my mother, who made all this possible. It was not easy for you with a son like me, but you always watched my back and stood behind me. Without your infinite support I never would have got this far. Let me say "Ein recht ein herzliches vergelt's Gott"!
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No Thanks to the Austrian government (along with most other governments in the world) for investing money in fancy military toys instead of supporting its students.

Abstract

Our studies aim to establish an all-purpose system, in which one type of implant can be used for every patient, due to an independent loading concept, whereas drug dose and type are individually adaptable. Biocompatible, biodegradable polymer beads can be charged with a large number of different therapeutics. The implant itself will be camouflaged with a polymer layer, that should be optimised for tissues located in the implant environment. Furthermore this layer will be derivatised chemically to contain specific binding pockets for the drug loaded biodegradable nano particles.
A method for quantification of such binding sites is needed. Different approaches have to be evaluated regarding amount, stability and specificity of the immobilised binding sites.

Zusammenfassung

Das Ziel dieser Untersuchung ist ein "Drug Targeting System" zu entwickeln, bei welchem ein Implantattyp universell einsetzbar ist. Ein unabhängiges Ladekonzept, wobei Dosis und Art der Medikation individuell justierbar sind, soll dem Rechnung tragen. Biokompatible, bioabbaubare Polymerbeads können mit einer großen Anzahl an unterschiedlichen Therapeutika beladen werden. Das Implantat soll mit einer Polymerschicht überzogen werden, welche für das umliegende Gewebe optimiert ist. Weiters soll diese Schicht spezifische Bindestellen für die medikamentbeladenen abbaubaren Nanopartikel besitzen, welche durch chemische Derivatisierung eingebracht werden.
Es soll ein Methode zur Quantifizierung solcher Bindestellen entwickelt werden. Verschiedene Ansätze müssen evaluiert werden im Hinblick auf Anzahl, Stabilität und Spezifität der immobilisierten Bindestellen.

Contents

Chapter 1

Introduction

Once upon a time there was a German chemist, physician, immunologist and serologist named Paul Ehrlich. Over a hundred years ago he founded the concepts of chemotherapy when he declared:

> Eine wesentliche Aufgabe ... wird es sein, Substanzen und chemische Gruppierungen aufzufinden, welche eine besondere Verwandschaft zu bestimmten Organen besitzen (organotrope Stoffe). Von besonderer Wichtigkeit wird es aber sein, solche gewissermaßen als Lastwagen fungierende Substanzen mit chemischen Gruppierungen von pharmakologischer oder toxikologischer Wirkung zu versehen, so daß sie gleichzeitig die ihnen anvertraute Last an die geeigneten Stellen befördern. [1]

In other words this means: A main challenge will be to find substances and chemical groups, which have a particular relation to certain organs. It will be of outstanding importance to supply trucks with pharmacological or toxicological effective substances, so that they can carry their payload to the appropriate location.
Theophrastus Paracelsus already believed that drugs must have barbs (spicula), so that they can attach on destined organs. In terms of chemistry these spicula are chemical groups with affinity to those organs. Ehrlich drew the picture of a poison arrow. The arrowhead (spiculum) is a haptophore group that binds to its target (chemical receptor on the cell surface). Due to the reason that a haptophore does not necessarily have a therapeutic effect it should be combined with a drug molecule, similar to an arrow poison[2].
Now this is exactly what modern drug targeting approaches are about.

1.1 Drug targeting principles

In general there are four categories of drug targeting principles:

- Direct application of drugs
- Spontaneous drug accumulation in leaky areas
- Physical targeting
- Targeting with high affinity vector molecules

These principles will be discussed in a brief manner. The method of targeting with high affinity vector molecules is the topic of the following sections.

Direct application

Sometimes it is possible to directly administer a drug into an affected area. Williams et al. showed that a single intra-articular injection of a liposomal hormone conjugate is effective in the treatment of arthritis in a rat model[3]. However such approaches will be successful only in a clearly localized area, where the drug is retained for an adequate time. Actually this is not the case in most diseases, so that commonly this method is not usable.

Spontaneous drug accumulation

The most important mechanism of spontaneous drug accumulation is the enhanced permeability and retention effect (EPR-effect)[4]. Due to a lack

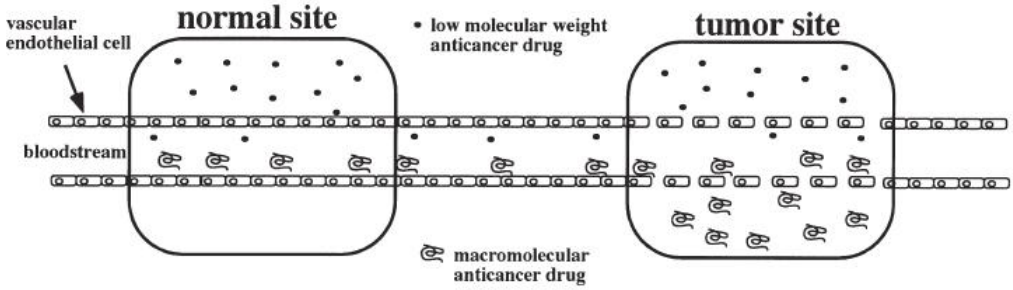

Figure 1.1: The EPR effect[5]

of nutrients and oxygen tumours cannot grow much larger than two mm^3

in size, so they have to secret growth factors for angiogenesis. These capillaries have an abnormal shape. Furthermore solid tumours have an elevated level of vascular permeability factors[6]. Macromolecules and liposomes can permeate the leaky vasculature and with a molecular weight over 50 Kilo Dalton (kD), they are retained there for a longer period than in normal tissue[7].

Physical targeting

Physical parameters such as temperature or pH can be used for drug targeting. Inflamed tissue or tumours for example show a higher temperature, due to an increased blood stream in that region. Weinstein et al. developed drug loaded thermosensitive liposomes. The released drug accumulated several times faster in tumours. The effect could be enforced by external heating of the targeted area[8].
Another attractive example for physical targeting is drug conjugation with magnetic nano particles. They are retained in the targeted area with a magnetic field. Munnier et al. combined doxorubicin (anthracycline antibiotic used in cancer chemotherapy) with Super Paramagnetic Iron Oxide Nano Particles (SPIONs). They could show that the released doxorubicin exhibits a cytotoxic activity at least as high as that of a pure drug solution, with the additional advantage of local accumulation by an externally applied magnetic field[9]. Recently a SPION aerosol was established and tested in vivo (mouse). After inhalation an eight fold increase in SPION concentration was observed in the lobe subjected to an external magnetic field as compared to the opposite lobe. By extending the distance between the thorax wall and the magnet from one to two mm, the relative SPION accumulation decreased from eight to 2.5[10]. The fact that the magnetic field decreases in distance might limit the benefits of this concept in humans simply because of the bigger organ sizes of humans compared to those of mice.

Targeting with high affinity vector molecules

This is the most promising effort to overcome the issues of drug therapies. The concept is already realised in some of the most powerful toxins known today, e.g. botulinum toxin, diphtheria toxin. Ehrlich proposed the mechanism of action referring to the diphtheria toxin:

> We must, therefore, thus represent the action of the poison that with the help of the haptophore groups, the toxin molecule becomes 'anchored' to the cell, and that it comes in this way within the sphere of action of the toxophore group.[11]

Different haptophores are going to be discussed in the following sections.

1.2 Monoclonal antibodies

Usually an antibody or Immunoglobulin (Ig) consists of two heavy (H) and two light (L) amino acid chains. In higher vertebrates there are five classes of antibodies, respectively the five different types of H chains.

Table 1.1: Antibody classes (subclasses)

Ig class	H chain	Structure
IgA_1 ; IgA_2	α_1 ; α_2	dimer
IgD	δ	monomer
IgE	ϵ	monomer
IgG_1, IgG_2, IgG_3, IgG_4	γ_1, γ_2, γ_3, γ_4	monomer
IgM	μ	pentamer

The majority of antibodies used in therapy are of the IgG class, in which two identical H chains combined with two identical L chains (of the κ or λ class) form a IgG with an Y like shape. The H and L chains are sub classified in constant (C) and variable (V) regions. The amino terminal ends of the variable regions of the heavy and light chain (V_H and V_L) form the antigen binding pocket, whereas the constant regions of the H chain (C_H) dictate the biological function of the antibody.

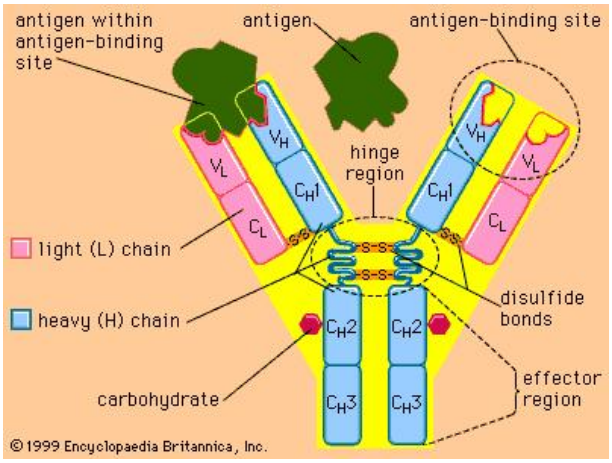

Figure 1.2: Schematic view of an IgG antibody[1]

Furthermore there are three stretches of approximately ten amino acids in length within the V_H and V_L chains with an unusually high variability. They are termed the complementary determing residues that make contact with the antigen determinant[12]. The rest of the variable fragments

[1]From Encyclopaedia Britannica http://www.britannica.com/EBchecked/topic-art/27988/107006/The-structure-of-an-antibody-molecule-represents-the-dramatic-rearrangements

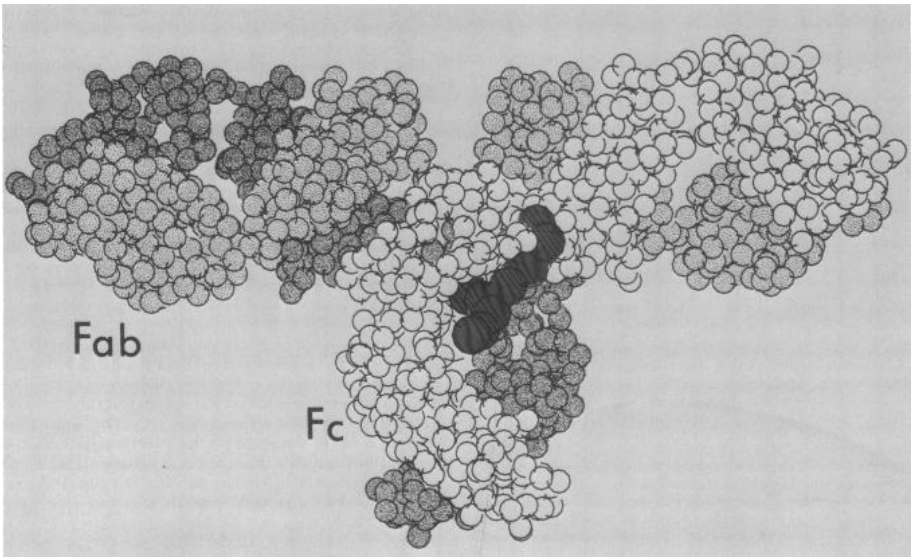

Figure 1.3: Space-filling view of a human IgG1 molecule. One H chain is in white and the other is dark gray; the two L chains are lightly shaded. The black spheres represent the complex carbohydrate[15].

are relatively constant and are building the framework regions.
The three dimensional structure of a whole antibody molecule was elucidated in 1967 by x-ray diffraction[13]. The theoretical model of a key (antigen) that fits exactly in just one lock (antibody) was used to explain the specificity of antibodies. But when the antibody binds the antigen structural rearrangements can occur in both of them. The interaction therefore has more the character of a handshake than that of the "lock and key" model[14].

In the beginning the whole antisera from immunised animals were used. Thereby different B-cells are producing different antibodies against different epitopes of an antigen. But with the development of monoclonal antibodys (mAbs) in 1975 by Köhler and Milstein[2] the revolution had begun. A mouse myeloma cell line was fused to spleen cells of an immunised mouse according to the method of Harris and Watkins using a Sendai virus strain[16]. The myeloma cells had a specific resistance and were not able to grow on selective media. Furthermore they could not secrete full antibody chains, whereas the spleen cells where able to grow on the selective media and produced healthy antibodies towards the antigen used for immunisa-

[2]Nobel Price award in Physiology or Medicine in 1984 along with N.K. Jerne: "For theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of monoclonal antibodies"

tion. Hence they were able to select only for fused cells (hybridoma cells) with the potential of immortality (from the myeloma cell) and production of antigen specific antibodies (from the spleen cells)[17].
The secreted antibodies derive from a single B-cell and are therefore termed monoclonal antibodies. Now these mAbs are more similar to a homing missile than to a simple arrow head and are approaching Ehrlich's vision of the magic bullet.

1.3 Production of monoclonal antibodies

For a long time the standard method for mAb production consisted of an in vivo immunisation and an in vivo production. At the end of the immunisation period the animal is sacrificed and antibody producing cells are harvested from the spleen. Now these cells are fused to myeloma cells and selected for specificity. Then the desired mAbs are produced by ascites induction, i.e. priming of the animal (with pristan or IFA) followed by intraperitonal injection of the hybridoma cells. After one to three weeks the ascites fluid is removed, containing a mAb concentration up to 28 mg/ml[18].
The first in vitro immunisation was performed by Hengartner et al. in 1978[19]. Nowadays kits are available for this procedure. The advantages to in vivo immunisations are:

- Fast production of mAbs, i.e. a few days compared to weeks with the in vivo method.
- Very small amount of antigen is needed.
- The immune response is not subject to the usual control mechanisms which apply to self antigens and weak antigens[20].

The most important fact is that one is not legally obligated to produce mAbs from an animal origin because human lymphocytes might be used as well in the process[21].
The in vitro production of mAbs requires some skills in cell culture. The hybridoma cells can be cultivated in a Tissue culture flask (TCF), permeable gasbag or in a more elaborate approach by using a Hollow fibre cartridge (HFC). In the first two production methods the cells have to be adapted to growing in serum free media to avoid contamination with bovine antibodies, whereas in the HFC approach the cells are separated from media by a semi permeable membrane with a defined M_r cut off.

Table 1.2: Comparison of different in vitro mAb production methods[22]

Method	Disadvantages	Advantages
TCF	Cells must be adapted to serum free media, occupies the most incubator space, large volumes of media must be processed	technically easy
Gas permeable bags	Cells must be adapted to serum free media, large volumes of media must be processed	Technically easy, occupies less space then TCF, may require less media then TCF
Hollow fibre cartridge	Large initial investment, technically more challenging, mechanical disruption or contamination, large amounts of media expended	Large yield in a small volume, culture media may contain high concentrations of foetal calf serum

1.4 Modification of monoclonal antibodies

Due to their murine origin, mAbs showed a high immunogenicity in clinical treatment[23]. In the mid eighties the first chimeric antibodies were generated with recombinant DNA technologies to overcome these issues[24–26]. They consisted of the murine F_{ab} part that was conjugated to a human F_c antibody fragment. Lo Buglio et al. showed that these chimeric mAbs have an increased circulation time in humans, i.e. six times as long as murine Immunoglobulin G (IgG) but only one-fifth as long as human IgG[27].
The next step towards a more human mAb was performed by Riechmann et al. in 1988. The idea was not to conjugate the whole F_{ab} part to a human F_c fragment, but to transplant only the antigen binding sites (i.e. the hyper variable or complementary determing regions from rodent origin in this case). Given that the sequences of the hyper variable regions do not contain characteristic rodent or human motifs, these "reshaped" mAbs should be indistinguishable from human antibodies[28].

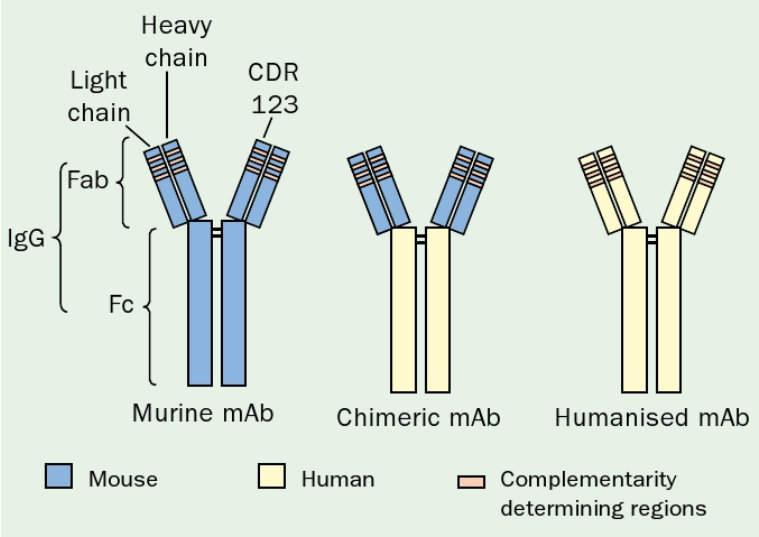

Figure 1.4: Structure of a mAb and modifications of a murine mAb [29]

As a last conclusion this trend in antibody modification led to the generation of fully human mAbs. Different approaches reach the goal:

- In vitro immunisation of human lymphocytes (mentioned earlier in this section)
- mAb production in transgenic mice
- mAb production using phage display technologies

The mAb production with human lymphocytes has proven to be very difficult. The fusion of these cells to murine myeloma cell lines resulted in preferential loss of human chromosomes. Moreover the hybridoma cells are unstable [30].
Lonberg et al. created transgenic mice, which are able to express human antibodies [31]. They selected hybridoma cells secreting human mAbs with binding constants in the range of corresponding murine mAbs already used in clinical trials. In 2007 the first human mAb (Panitumumab[3]) produced with such transgenic mice (XenoMouse system[4]) was approved for the clinical treatment of advanced colorectal cancer [32].

[3] Human anti-EGFR mAb

[4] Transgenic mice with inactivated mouse antibody production machinery replaced by the human Immunoglobulin heavy and light chain loci

1.4.1 Antibody digestion and creation of scFv

If the antibodies are used only for targeting reasons the effector functions of the constant regions are not needed. Antibodies can be engineered by protein digestion. For example a digestion with papain produces two Fab' fragments and one Fc fragment, whereas the digestion with pepsin leads to one $F(ab')_2$ fragment and numerous Fc sub fragments.
In a more elaborate approach a single chain Fv fragment (scFv) can be produced with cloning techniques. A scFv consists of the V_L and the V_H fragments linked by a polypeptide chain that connects the carboxy-terminal residue of the V_L domain with that of the amino terminus of the V_H domain[33]. The genes encoding the domains of interest are cloned in a vector and subsequently expressed in E.coli. The resulting scFv are the smallest antibody fragments (M_r = 26 kD) that still possess the complete antigen binding pocket with a similar specificity and affinity as the natural fragment from which they derive[34].

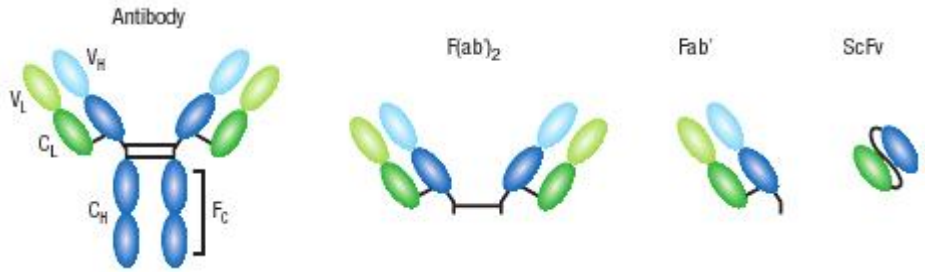

Figure 1.5: Antibody fragments produced with protein digestion or molecular biology techniques[35]

1.4.2 Phage display technology

Phage display is a very powerful genetic tool. The idea is to clone a DNA fragment into a phage coat protein. Now the phage expresses the protein on its coat surface corresponding to the genetic sequence inside[36]. Mc Cafferty et al. succeeded in expressing a scFv on the surface of a filamentous fd phage[37]. The phages binding to the antigen were isolated by a simple affinity chromatography.
Very large libraries can be generated by randomly combining different heavy and light chain variable antibody fragments[38,39]. In a screen assay an immobilised antigen is incubated with such a library. Phages with affinity to the antigen will bind on the surface, whereas the rest is washed

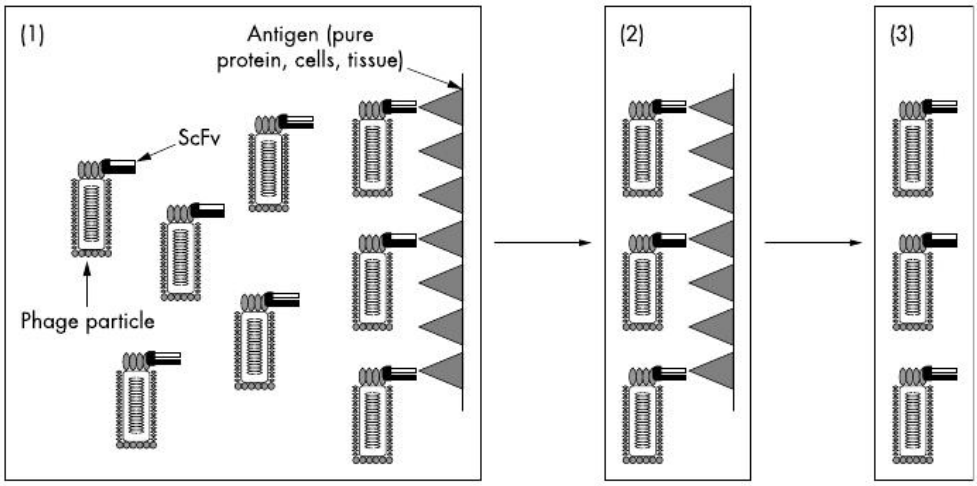

Figure 1.6: Selecting antibodies from phage libraries[40]. (1) Phages are incubated with the antigen of interest. (2) Phages that express scFv fragments that do not bind to the antigen are washed away. (3) Phages that express scFv fragments that bind to the antigen are isolated, expanded, purified, and used in further applications.

away. Then the bound phages are amplified by infection of E.coli from whom the scFvs can be isolated for further usage.

1.5 Monoclonal antibodies in clinical trials

In 1986 the first mAb (Muromonab OKT-3 [5])[41] was approved by the Food and Drug Administration (FDA) to treat acute renal rejection in patients who are resistant to the standard steroidal therapy[6]. A decade later mAbs found their way into cancer chemotherapy. Rituximab[7] was the first member for the treatment of B-cell non Hodgkin's lymphoma[43]. Today there are about 3600 ongoing or completed clinical trials including mAbs, which is about 5% of all registered trials[8]. These data clearly show how emerging mAb-therapy has become. Most mAbs are used in cancer therapy, but also the treatments of autoimmune diseases, transplant rejection, viral and even bacterial infections, are under investigation.

[5] murine monoclonal IgG2a antibody that binds to the CD3 complex on T cells, thus blocking their function

[6] 1993 a second indication for Muromonab was approved for the treatment of acute rejection in heart and liver transplants (http://fda.gov)

[7] chimeric mAb against CD20 with a human F_c part that can mediate tumour lysis using human complement or ADCC effector cells[42].

[8] http://clinicaltrials.gov with 67000 trials registered in 160 countries

Table 1.3: Monoclonal antibodies in clinical trials

Intervention	Targeting antibody	Disease
Gavilimomab	Murine anti-CD147 mAb	Acute graft versus host disease
3F8-mAb, etoposide, isotretionin	Murine anti-GD2 mAb	Neuroblastoma
Mik-β-1	Murine anti-IL-2R β mAb	Chronic lymphocytic leukaemia
Edrecolomab	Murine anti-CSGP 17-1A mAb	Stage II colon cancer
CNTO 328	Chimeric anti-IL-6 mAb	Metastatic prostate cancer
Cetuximab, Cisplatin	Chimeric anti-EGFR mAb	Metastatic or recurrent head and neck cancer
Infliximab	Chimeric anti-TNF α mAb	Crohn's disease
RAV12	Chimeric anti-RAAG12 mAb	Metastatic pancreatic cancer
Fontolizumab	Humanised anti-IFNγ mAb	Crohn's disease, Colitis, Intestinal disease
CAL	Humanised anti-PTHrP mAb	Breast cancer metastatic to bone
HuJ591	Humanised anti-PSMA mAb	Prostate cancer
ING-1(heMAb)	Humanised anti-Ep-CAM mAb	Advanced adenocarcinoma
Trastuzumab, Paclitaxel	Humanised anti-HER2 mAb	Recurrent or metastatic breast cancer
Lintuzumab, cytarabine, etoposide, mitoxantrone	Humanised anti-CD33 mAb	Acute myelogenous leukaemia
HuM291	Humanised anti-CD3 mAb	Advanced or recurrent lymphoma
Hu3S193	Humanised anti-Lewis-Y mAb	Advanced colorectal carcinoma

Continued on next page

Table 1.3 – continued from previous page

Name	Targeting antibody	Disease
HuHMFG1	Humanised anti-MUC-1 mAb	Locally advanced or metastatic breast cancer
MRA	Humanised anti-IL-6R mAb	Systemic Lupus Erythematosus
F105	Human anti-gp-120 mAb	HIV infections
CAT-192	Human anti-TGF β1 mAb	Systemic sclerosis, scleroderma
Cixutumumab (IMC-A12)	Human anti-IGF-IR mAb	Metastatic soft tissue sarcoma
Adalimumab	Human anti-TNF α mAb	Rheumatoid Arthritis, Crohn's disease
Raxibacumab	Human anti-BAPA mAb	Anthrax (tested in healthy subjects)
Sevirumab	Human anti-CMV mAb	Cytomegalovirus retinitis, HIV infections

Although some therapies are very promising, it is important to be very careful, especially in phase I trials (i.e. first test in human subjects).
On March 13 2006 a tragic accident happened at the Northwick Park Hospital in London. Six test persons received an intravenous injection of TGN1412 antibody[9]. Two hours after the injection all six volunteers suffered from a cytokine storm leading to headache, backache, nausea, a drop in blood pressure and finally to multiple organ failure[44].
TGN1412 was developed to treat leukaemia and autoimmune diseases such as rheumatoid arthritis. It acts as a superagonist that induces the activation and expansion of immunosuppressive regulatory T cells. Usually two signals are needed to get this response, but TGN1412 can trigger it alone. TeGenero performed in vitro and in vivo test with Cynomolgus Macaques, due to a 100% homology of the CD28 T cell receptor of the monkeys and humans. No side effects were observed and the drug was considered safe.
Two possibilities could explain the different in vivo responses in humans

[9]Humanised monoclonal antibody against the CD28 T cell receptor; developed by TeGenero Immuno Therapeutics

and non human primates. The first would be that the magnitude of the TGN1412 response elicited in monkey T cells is much lower than that in human T cells (quantitative model). Alternatively, TGN1412 stimulation of monkey T cells might not activate the same intracellular signalling pathways as it does in human cells (qualitative model) and, therefore, might not induce cytokine synthesis[45]. In addition Stebbings et al. showed that the monkeys peripheral blood mono nuclear cells do not produce cytokines in vitro[46].
Much more precaution is now taken in clinical trials involving antibodies and especially immunomodulatory substances, since this day in 2006, where six healthy test persons almost died.

1.6 More arrow heads or targeting moieties

Monoclonal antibodies are by far the most popular molecules for targeting approaches. Nevertheless there is a huge pool of other entities who might be even more effective in solving the issues of drug therapies. Some of them are discussed in a briefly manner in this section.

1.6.1 Aptamers

Aptamers are small DNA or RNA oligonucleotides who exhibit ligand binding characteristics. They can be isolated from a large pool of random sequences somehow similar to phage display technology.

The procedure was developed independently by Ellington et al. and Tuerk et al.[48,49] :

- In a first step hundred random nucleotides flanked by defined regions for primer binding are made by solid phase synthesis, yielding approximately 10^{15} individual sequences.
- The sequences are amplified by Polymerase chain reaction (PCR) resulting in a final diversity of 10^{13} sequences.
- Transcription with RNA polymerase.
- Polyacrylamide gel electrophoresis to ensure size homogeneity of the RNA molecules.
- Affinity chromatography using the target of desire as column material.

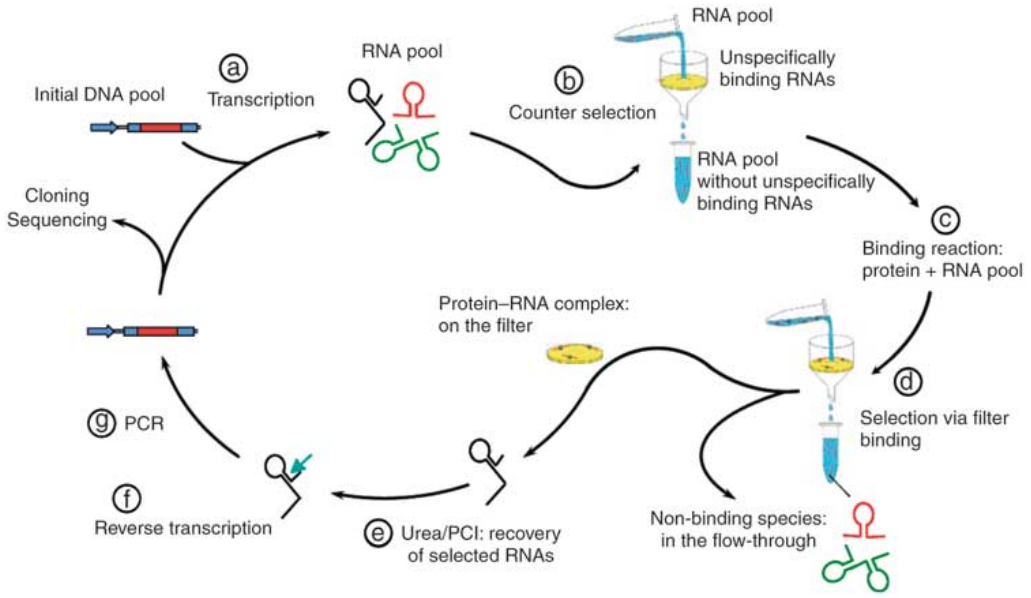

Figure 1.7: Generation of aptamers with SELEX[47]

- Reverse transcription to gain the complementary DNA's.
- Next round of PCR amplification

With every repeated cycle the aptamers with affinity to the column are enriched. For example Ellington and Szostak reported that less than 1% of the applied RNA bound to the column in the first two cycles, but in the fourth round over 50% of the applied RNA was bound.
Now these aptamers have some clear advantages over mAbs including:

- Fast in vitro production through the systemic evolution of ligands by exponential enrichment (SELEX) process as described above.
- Smaller size of approximately 15 kD
- Lesser immunogenicity
- Because of the in vitro production they can be generated regardless of the immunogenicity or toxicity of the antigen.

In 1998 Ruckman et al. published a highly sophisticated, nuclease resistant and pegylated aptamer against the VEGF[50]. It took only six years for this aptamer, later named pegaptanib (brand name Macugen), to achieve FDA approvement for the treatment of neovascular (wet) age-related macular degeneration[51].
For a recent review in the field of aptamers please see Yang et al[52].

1.6.2 Oligopeptides

Oligopeptides (OPs) might be another promising candidate for drug targeting. OPs consist of very short amino acid chains. In 1984 Geysen et al. tried to find the immunologically relevant epitope of the coat protein of foot-and-mouth disease virus. They synthesised every possible hexapeptide combination from the whole coat protein, immobilised them and scanned with ELISA. They found out which part (the epitope) of the coat protein was responsible for antibody interaction with a resolution of seven amino acids in length. Further amino acid variation of the found hexapeptide led to the identification of the two most important amino acids of that epitope[53]. Maybe with this experiment the idea to create OP libraries and examine their potential binding to a target was born.
Short time later the first libraries were generated either by fusing OPs

- to phages[54–56]
- to a DNA binding domain[57]
- or to polymer beads[58,59]

The process for isolation is similar to that used in phage display technology (see page 10), only that this time not a whole scFv is fused to the coat protein of the phage but only a short sequence of about six to fifteen amino acids. Then again with every panning round followed by amplification the OPs with affinity to the target can be isolated.
Usually the selection of useful OPs is done in vitro by screening against purified immobilised targets. But there are some publications that describe an in vivo screening[60,61]. For example Pasqualini et al. showed that a cyclic nonapeptide displayed on a phage with affinity to αv integrins was accumulated very selective in the tumour vasculature after i.v. injection in mice[62].
Recently Krag et al. expanded this trend in in vivo screenings to humans with stage IV cancer. They screened two phage displayed OP libraries along with a scFv library. In the end they were able to identify one targeting candidate from the OP libraries and six from the scFv library, verifying the results in an immunostaining experiment. Furthermore no severe side effects related to the phage injection could be observed during the study as well as no immune response against the phages did develop during the first week after administration[63]. The earlier mentioned OP against the αv integrins[10] showed some encouraging results in phase II clinical

[10]Now in a pentameric cyclic form (L-arg-gly-L-asp-D-phe-L-val) named Cilengitide

trials. It was well tolerated exhibiting modest anti tumour activity[64]. Now Cilengitide is going into phase III clinical trial in combination with standard therapy for subjects with newly diagnosed glioblastoma multiforme[11]

[11]The most common and most aggressive brain tumour

Chapter 2

Research Objectives

In this work we are developing a new drug targeting system, that might solve some of the problems such approaches are still confronted with. Usually implants are not tolerated by the immune system. Some approaches exist in which a drug is included in the implant to suppress the immune response. But the drug in this implant type is bleeding out after a short period, hence losing its beneficials. Therefore we are trying to establish a reloadable implant surface. Our idea is to mark the target in the patient (e.g. implant, tumour) with specific binding sites for polymer based drug vehicles. The polymer beads can bind to the target and release the drug. The degradation of the polymer should free the binding sites for a further loading step.
Implant materials (e.g., 316-L stainless steel, ZrO2) are coated with biocompatible polymers. Furthermore this layer will be derivatised chemically to contain specific binding pockets for drug loaded biodegradable nano particles.

Aims of the thesis

- Development of a method for quantification of the biorecognitive sites.
- Testing varied methods for chemical antibody processing.
- Evaluation of different bioconjugate techniques, regarding stability and site direction of the binding pockets.

Chapter 3

Materials

Table 3.1: Reagents

Empirical Formula	Name	Provider
CH_3COOH	Acetic acid, glacial	J.T. Baker (6052)
Al_2O_3	Aluminium oxide 90 neutral	Merck (1.01077.1000)
$C_9H_{23}NO_3Si$	(3- Aminopropyl) triethoxysilane	Fluka (09324)
$C_{16}H_{28}N_4O_4S$	Biocytin	Sigma (B-1758)
$C_{10}H_{15}N_2O_3S$	d-Biotin	Sigma (B-4639)
	Bisamino PEG	Fluka (14529)
$C_6Cl_4O_2$	Chloranil	Fluka (23290)
$C_9H_{21}ClO_3Si$	(3- Chloropropyl) triethoxysilane	Sigma (569615-100ML)
$C_6H_8O_7$	Citric acid	Sigma (C0759-500G)
$C_3H_7NO_2S$	L- cysteine	Sigma (C7352-100G)
$Na_2HPO_4 \cdot H_2O$	Disodium hydrogen phosphate	Merck (1.06580.1000)
$C_8H_{17}N_3 \cdot HCl$	EDC	Sigma (E7750-25G)
$C_{10}H_{14}N_2Na_2O_8$	EDTA Disodium salt	Appli. Chem (A2937,1000)
$C_{14}H_8N_2O_8S_2$	Ellmann's reagent	Pierce (22582)
$C_5H_8O_2$	Glutaraldehyde Solution	Fluka (49626)

Continued on next page

Table 3.1 – continued from previous page

Empirical Formula	Name	Provider
HCl	Hydrochloric acid, 37-38%	J.T. Baker (6081)
H_2O_2	Hydrogen peroxide, 30%	Merck (1.07209.0250)
$C_6H_{13}NO_2$	L- Leucine	Sigma (L8000-25G)
$C_8H_{14}O_2S_2$	Lipoic acid	Sigma (T5625-1G)
$MgCl_2 \cdot 6\ H_2O$	Magnesium chloride hexahydrate	Riedel de Haen (31413)
C_2H_7NS	2- Mercaptoethylamine	Sigma (M-9768)
$CH_3(OCH_2CH_2)_nOH$	Methoxy PEG	Aldrich (20.250-9)
$NiSO_4 \cdot 6\ H_2O$	Nickel sulphate hexahydrate	Appli. Chem (A0827,0100)
$C_9H_6O_4$	Ninhydrin Spray Solution	Merck (1.06705.0100)
$C_6H_4NO_2PO_4H$	p- Nitrophenyl phosphate	Koch Light Ltd. (93092)
	PLGA	Aldrich (531154-1G)
KCN	Potassium cyanide	Merck (4967)
	Protein Assay	Bio-Rad (500-0006)
CH_3COONa	Sodium acetate	Riedel de Haen (25023)
$NaBH_4$	Sodium borohydride	Aldrich (21,346-2)
$Na_2CO_3 \cdot 10\ H_2O$	Sodium carbonate decahydrate	Riedel de Haen (31431)
$C_6H_5Na_3O_7 \cdot 2\ H_2O$	Sodium citrate tribasic	Riedel de Haen (32320)
$NaH_2PO_4 \cdot H_2O$	Sodium dihydrogen phosphate	Merck (1.06346.1000)
NaCl	Sodium chloride	Riedel de Haen (31434)
$NaHCO_3$	Sodium hydrogen carbonate	Riedel de Haen (31437)
NaOH	Sodium hydroxide	Riedel de Haen (30620)
$NaIO_4$	Sodium periodate	Aldrich (31,144-8)

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Table 3.1 – continued from previous page

Empirical Formula	Name	Provider
$C_4H_4O_3$	Succinic anhydride	Merck (8.00683.0500)
$C_{16}H_{15}N_2O_9SNa$	sulfo- SMCC	Pierce (22322)
$C_4H_4NO_6SNa$	sulfo- NHS	Pierce (24510)
	TMB61 HRP substrate	Sciotec
$C_{58}H_{114}O_{26}$	Tween 20	Sigma (P9416-100ML)

Chapter 4

Methods

The methods used in this thesis will be described in a general manner.

4.1 Chromatography

4.1.1 Gel Filtration

Gel filtration or size exclusion chromatography is a fast and simple method to separate proteins or other macromolecules based on their size (hydrodynamic volume). The stationary phase in a gel filtration system consists of a porous polymer matrix with a well defined pore size. Small molecules can enter the pores causing retention of the latter, whereas molecules larger then the pore size elute faster, due to the shorter way they have to travel through the column.
There is no interaction as usual in chromatography between the stationary and the mobile phase. Therefore a wide range of mobile phase systems can be applied without affecting the separation process. In most cases an aqueous buffer system, suiting the proteins to be purified, will work well.
Protocol:

1. Suspend a Sephadex™ column material or the equivalent in the desired buffer that is used in the chromatography. Equilibrate for at least two hours under vacuum to degas. Use a magnetic stirrer at low rpm to mix gently.

2. Pack a column with the suspension. For desalting purposes the column size should be at least five times the applied sample volume. Avoid the introduction of air bubbles.

3. Wash with buffer used in the chromatography step. Ten times the column size is appropriate.

4. Apply the sample in as small a volume as possible. 1 $^{ml}/_{min}$ is the flow rate that will work in most cases.

5. Collect fractions of the desired volume and monitor for protein at 260 nm, or take an aliquot of each to perform a Bradford Assay.

4.1.2 Nickel-Chelate Affinity Chromatography

Nickel chelate affinity chromatography is a powerful purification technique. Proteins and other macromolecules are able to form complexes with metal ions. The column material consists of an immobilized chelating agent, able to complex metal ions. Weak associated coordination sites of the ion then can interact with specific proteins running through the column. Elution can be performed by

- lowering the pH.
- raising the ionic strength.
- adding a chelating agent such as EDTA, that will compete with the protein metal complex.

This chromatography system will bind more or less only IgG class immunoglobulins, while other proteins will not be retarded. So this method can be used to extract the IgG fraction of antisera or to purify antibody-enzyme conjugates. Eluting conditions are rather mild, so activity loss can be reduced to a minimum. IgGs lacking the Fc part of the antibody do not interact with Nickel ions and thus can not be purified with this technique. The following protocol describes how a Ni^{2+}- chelate affinity chromatography is done:

1. Pack a column with an appropriate volume of an iminodiacetic acid support. 1 ml can bind up to 50 mg antibodies, however not more than 10 – 20 mg should be applied per ml gel.

2. Prepare a 50 $^{mg}/_{ml}$ Ni^{2+}- solution in H_2O. Apply 1 ml of this solution per ml of gel to the column.

3. Wash the column with 5 gel-bed volumes of H_2O to remove excess Ni^{2+}.

4. Equilibrate with 5 gel-bed volumes of binding buffer: 10 mM sodium phosphate, 150 mM NaCl, pH 7. The high ionic strength of the buffer will minimize non specific ionic interactions between the positively charged support and negatively charged compounds in the sample.

5. Dissolve the sample into binding buffer and apply this solution to the column. Collect fractions of the desired volume and monitor for protein at 260 nm, or take an aliquot of each to perform a Bradford Assay.

6. Wash with 150 mM saline solution until no more protein can be detected. Elute the bound sample using

   - 100 mM sodium acetate, 0.5 M NaCl, pH 5
   - binding buffer containing 20 mM EDTA

   as the elution buffer.

7. Perform a gel filtration to transfer the sample in a suitable storage buffer.

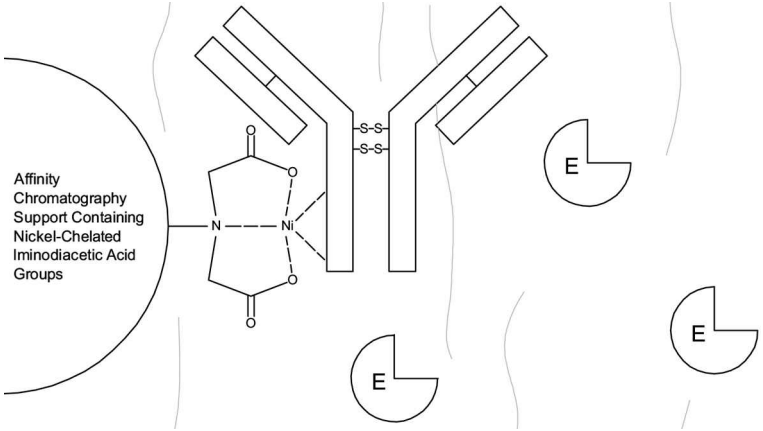


Figure 4.1: Ni^{2+}- chelated iminodiacetic acid groups can be used to purify antibodies. The Ni^{2+}- chelate binds to the Fc region so that antibodies are retained, whereas other enzymes can pass through the column [65].

4.2 Conjugation And Immobilization

4.2.1 Glutaraldehyde conjugation

Glutaraldehyde is one of the oldest and at the same time most popular homobifunctional cross-linker in use. It was introduced in 1962 by Sabatini et al. as a fixative for electron microscopy[66]. The reactive aldehyde groups are able to form Schiff bases with primary amines. This reversible bond can be reduced with $NaBH_4$ to the secondary amine which is stable.

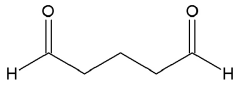


Figure 4.2: Glutaraldehyde MW 100.11 g/mol

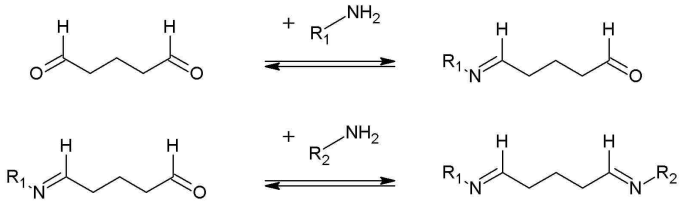


Figure 4.3: Schiff base linkage of primary amines with glutaraldehyde

In aqueous solution glutaraldehyde forms α, β-unsaturated polymers, which can react with primary amines in a Michael-type reaction, resulting in secondary amine linkage[67]. Due to its homobifunctional nature the reproducibility of glutaraldehyde conjugates is low. In solution large conjugates are produced, which will precipitate. But when immobilization on solid supports is performed these problems are not that severe, because the reaction steps of activation and conjugation can be separated and so self polymerization of the bio molecule is not possible. On the carrier surface

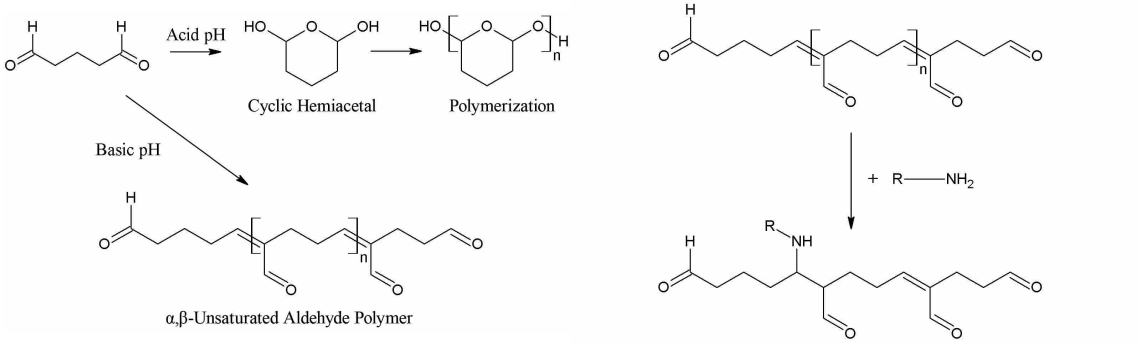


Figure 4.4: Self polymerization of glutaraldehyde and Michael-type addition of primary amines

the bio molecule can be covalently linked by more than one reactive group. A so called multi attachment will be achieved. It is unlikely that all Schiff base linkages will be cleaved at the same time, so that no further reduction step is needed to gain stable immobilization.

A typical conjugation protocol involving glutaraldehyde:

1. Suspend an amino carrier in a 2.5% solution of glutaraldehyde in 0.05 M phosphate buffer, pH 7. Shake gently for one hour.
2. Wash exhaustively with ice water.
3. Add the amine containing bio component in as small a volume as possible.
4. React for 2-4 hours at room temperature.
5. Wash with phosphate buffer and store in 0.05 M saline solution at 4°C.

4.2.2 EDC

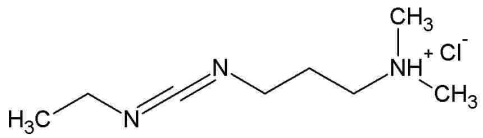


Figure 4.5: EDC 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride MW 191.7 g/mol

EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride) as introduced in conjugation chemistry by Newman et al., is the carbodiimide of choice for the use in bio conjugate procedures. Usually biological substances are water soluble and so is EDC. There is no need to use complicate buffer systems or organic solvents. Reaction by-products are also soluble in water, hence the product can be purified by a fast and simple gel filtration.

EDC reacts with carboxylate groups to a highly reactive O-acylisourea. This intermediate then reacts with primary amines or other nucleophiles to form an amide bond. The O-acylisourea also reacts with sulfhydryl groups, although these conjugates are not as stable as those with primary amines. Oligomerisation will occur if the molecule to be activated contains both, a carboxylate and an amine group. Keep attention not to use EDC in such systems.
The reaction should be performed in an aqueous buffer system, preferably at pH 5. However even at pH 10 positive results are obtainable. Be careful not to use any buffers, that may contain carboxylates or primary amines.

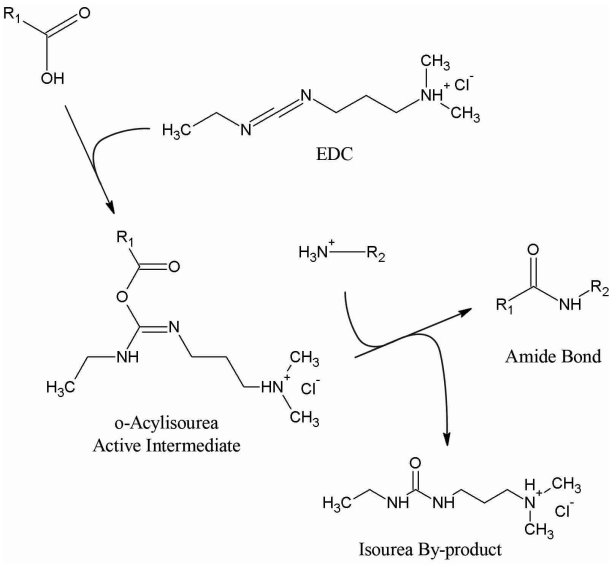


Figure 4.6: EDC reaction scheme

An EDC mediated conjugation might be the method of choice for coupling antibodies to an implant surface, because the resulting amide bond linkage will not be as immunogenic as other cross linkers are. On the other hand an antibody molecule possesses several primary amines, so that no specific site directed linkage is no more possible.

Protocol for EDC conjugation reaction:

1. Dissolve the carboxylate containing reagent in 0.1 M sodium phosphate, pH 5. 0.1 M NaCl may be added if desired.
2. Add EDC in at least a 10-fold molar excess of EDC to carboxylate groups. Activate for one hour at room temperature.
3. Purify the product from excess EDC by gel filtration. If using a solid support simply wash with buffer.
4. Add the amine containing species to the activated carboxylates and allow to react for two hours at room temperature.
5. Perform another gel filtration to purify or in the case of solid supports just wash with buffer.

4.2.3 EDC + s-NHS

By adding s-NHS to a EDC mediated coupling reaction, the likely hydrolysable O-acylisourea intermediate formed by EDC activation is stabilised to a s-NHS ester. The new intermediate is stable long enough in order to perform two step coupling protocols, hence avoiding self polymerisation problems associated with one step protocols.

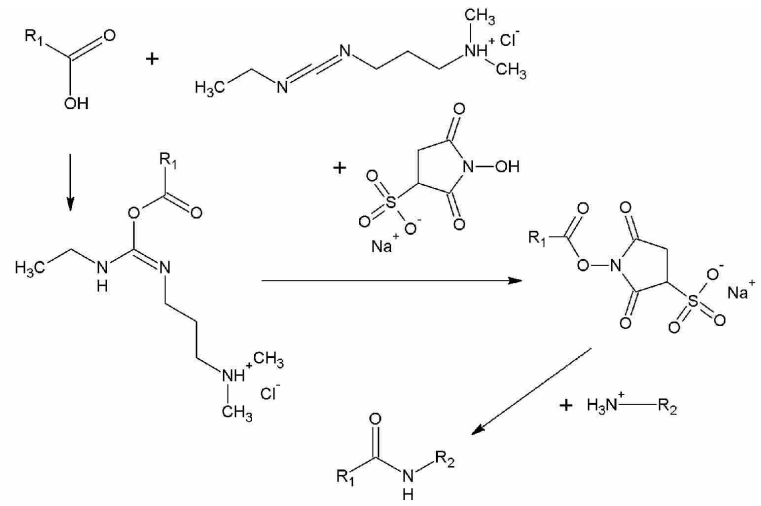


Figure 4.7: EDC + s-NHS mediated conjugation

4.2.4 sulfo-SMCC

Sulfo-SMCC or Sulfosuccinimidyl-4-(N-maleimido-methyl)-cyclohexane-1-carboxylate is a heterobifunctional cross linker which can be used to conjugate antibodies to a primary amine containing species. The NHS-ester end of the cross linker reacts with primary amines, whereas the maleimide group is reactive towards sulfhydryls. The activation and the conjugation step can be separated, resulting in a very efficient coupling reaction. Furthermore antibodies possess sulfhydryls only in the hinge region of the molecule, thus preventing the binding sites to be blocked in the conjugation step.

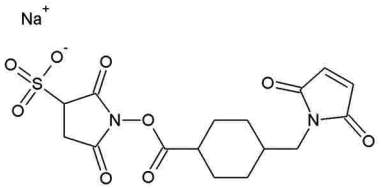


Figure 4.8: Sulfo-SMCC MW 436.37 g/mol

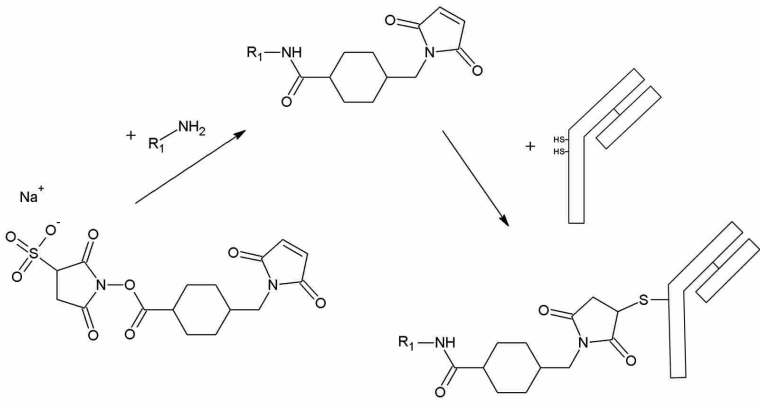


Figure 4.9: Activation of primary amines followed by coupling with sulfhydryl containing antibody fragment

At pH 7 the reaction of the maleimide group with sulfhydryls proceeds a 1000 times faster than its reaction with amines. Due to this reason a given amine containing species can be activated and stored for a quite long time before the conjugation with sulfhydryls is done. Sulfo-SMCC is widely in use today for the creation of sophisticated bio conjugates, e.g. antibodies labeled with nucleotides for cancer therapy[68] or for the incorporation of antigens in virus like particles[69].
A general conjugation protocol using sulfo-SMCC looks as the following:

1. Dissolve the amine containing species in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 .

2. Add sulfo-SMCC in a 40 to 80 molar excess to this solution. Mix to dissolve and allow to react for one hour at room temperature.

3. Purify the activated sample from any excess sulfo-SMCC by gel filtration using Sephadex™ G25, or in the case of solid supports wash with buffer. At this point the activated sample may be freeze dried and stored for a few weeks, without losing maleimide activity.

4. Dissolve the sulfhydryl containing species in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 . Add the maleimide activated reactant at the desired molar ratio. React for at least half an hour at 37°C or one hour at room temperature. The reaction can be done also over night at 4°C.

5. Wash the immobilized product with buffer or use a gel filtration column with an appropriate cut off to purify soluble conjugates.

4.2.5 Immobilization of lipoic acid as a reducing agent

Disulfide bridges in bio molecules can be reduced with mercaptoethanol. A high concentration is needed to drive the reaction to completion due to the low equilibrium constant. A better choice for a reducing agent is dithiothreitol. A sterically favoured dithiolane ring is formed in the reduction reaction. Its still difficult to remove the reduction agent from the sample solution. At least a gel filtration step is needed, while in the meantime re-oxidation of the sulfhydryls may occur.
Gorecki et al. introduced a new insoluble reducing support consisting of immobilized lipoic acid[70]. It can be removed very quickly by centrifugation to overcome the mentioned problems.

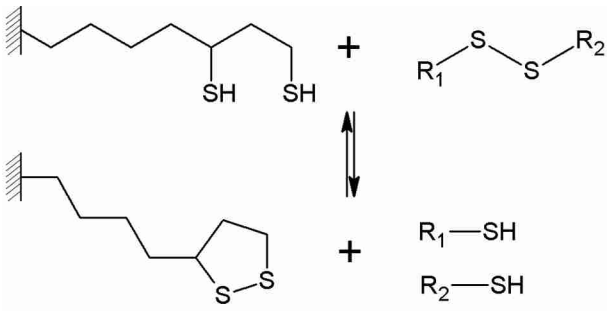


Figure 4.10: Reduction of disulphide with immobilized lipoic acid

This protocol describes the synthesis of such a support:

1. Prepare 15 ml 0.1 M sodium phosphate, pH 6.3 . Add 5 ml dioxane. Dissolve 41.2 mg lipoic acid, 43.4 mg sulfo-NHS and 38.4 mg EDC in this solution and react for 30 minutes at room temperature.

2. Adjust pH to 7.5 with 1 M Na_2HPO_4 and add 0.5 g aminosilanised Swarowsky™ glass powder (or other suitable support materials,i.e. agarose,polyacrylamide . . .). Incubate over night at room temperature.

3. Wash the glass powder in a suction funnel, pore size 4, with:

   - 5 ml DMF, 3X
   - 10 ml EtOH / H_2O 1:1, 2X
   - 10 ml EtOH, 2X

4. Dry over night at 80°C.

5. Prepare 3 ml 50 $^{mg}/_{ml}$ $NaBH_4$ in H_2O. React for 30 minutes at room temperature.

6. Wash the glass powder in a suction filter, pore size 4, with:

   - 10 ml 0.1 M acetic acid, 2X
   - 10 ml H_2O, 2X
   - 10 ml EtOH, 2X

4.3 Analytics

4.3.1 Bradford Protein Assay

1976 M. Bradford introduced a very sensitive dye-binding assay for protein detection[71]. It involves the addition of an acidic dye (Coomassie™Brilliant Blue G-250) to a protein solution and subsequent measurement at 595 nm with a spectrophotometer. The absorbance maximum for an acidic solution of the dye shifts from 465 nm to 595 nm when binding to protein occurs. Beers law can be used for quantification in the linear range of the assay, which reaches from 2 μg/ml and lower up to 120 $\mu g/ml$.

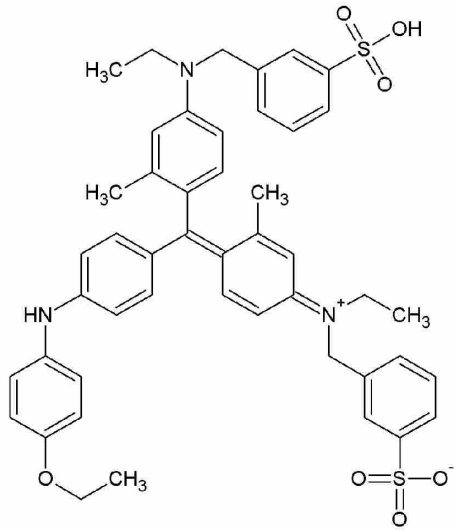


Figure 4.11: Coomassie Brilliant Blue

4.3.2 Kaiser Test

The Ninhydrin reaction as developed by Moore and Stein[72] was adapted for solid phase synthesis by Kaiser et al[73]. Ninhydrin reacts with primary amines on the surface to form Ruhemann's Blue (Figure 4.12). Quantification of the amines is difficult because Ruhemann's blue can be found in solution as well as on the surface. However Sarin et al. claim to overcome this problem by introducing a ion-exchange wash step, so that Ruhemann's Blue will not be able any more to adsorb onto the solid support.

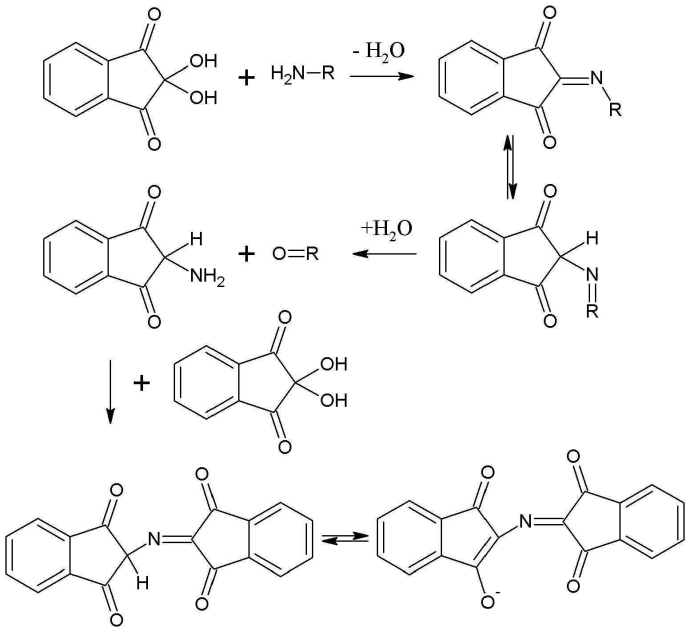


Figure 4.12: Reaction-scheme of the ninhydrin reaction

The protocol for the Kaiser test is adapted from Sarin et al[74].

1. Dissolve 8 g phenol in 2 ml EtOH.
2. Prepare a 10 mM solution of KCN in 0.2 ml H_2O. Fill up to 10 ml with pyridine.
3. Dissolve 50 mg ninhydrin in 950 mg EtOH.
4. Add 0.5 ml of each solution to a test tube containing the sample.
5. Boil for 5 min in H_2O.

The solution turns blue by the presence of primary amines.

4.3.3 Enzyme assay

Enzyme assay is a standard procedure in bio analytics. A broad range of different tests are well elaborated. In general a substrate development is monitored over the time. Following Michaelis and Menten in an enzymatic

reaction a first build enzyme - substrate complex dissociates to form free enzyme and product.

$$[\mathrm{E}] + [\mathrm{S}] \underset{\mathrm{k_{-1}}}{\overset{\mathrm{k_1}}{\rightleftharpoons}} [\mathrm{ES}] \xrightarrow{\mathrm{k_2}} [\mathrm{E}] + [\mathrm{P}]$$

In an initial rate experiment substrate is added in large excess so that

$$\frac{d[S]}{dt} \approx 0$$

and

$$[E]_{total} = [ES] + [E] \approx [ES].$$

The turnover number can be written as

$$V_{cat} = k_2 * [ES].$$

Eventually with the known catalytic constant(dissociation constant of the enzyme substrate complex) k_2 of a given enzyme or by a simple comparison with a calibration curve using enzyme standards, we can quantify the amount of active enzyme in a sample. By creating the above mentioned conditions and measuring substrate development only for a short time, these experiments are fast and quite free from complications, resulting in a high reproducibility.
In this thesis calf intestinal alkaline phosphatase is the common enzyme for initial rate experiments. AP can cleave phosphomonoesters from substrates, i.e. para- Nitro Phenyl Phosphate (p-NPP), which develops a strong yellow color that can be detected at 405 nm.

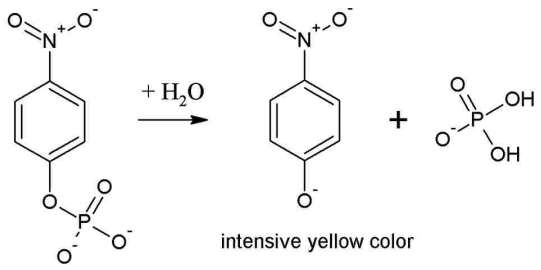


Figure 4.13: Alkaline Phosphatase catalysed hydrolysis of p-NPP

The test is performed in $10 \cdot 10 \cdot 45$ mm disposable macro cuvettes.

1. Fill with 4 ml 100 mM TBS, pH = 9.5, containing 100 mM NaCl and 100 mM $MgCl_2$.
2. Add 10 μl p- NPP stock solution (50 $^{mg}/_{ml}$ in H_2O) and monitor the extinction over the time at 405 nm in a spectrophotometer.

Chapter 5

Results and Discussion

The practical work of this thesis consists of the development and evaluation of different techniques for a drug targeting system. My supervisor Mag. Haifa al Dubai was trying to establish an elaborate type of stent implant. The implant material should be camouflaged with a biocompatible polymer layer, followed by conjugation of specific binding pockets for biodegradable nano particles to the layer. At this time the antibody based binding sites for the polymer beads were not available, so the synthesis procedure for the drug targeting system was tested using stuff we already had in our laboratory.
First of all a quantitative method to detect functional groups, which can be used for coupling on a given surface, is needed. Next different implant materials were coated covalently with Polyethylene glycol (PEG), followed by quantification of PEG chains. Afterwards I tried to gain some exercise in antibody chemistry, including several chemical and enzymatic modification methods as well as with coupling techniques on surfaces.

5.1 Quantification of functional groups on surfaces

In general our implant materials are derivatised chemically to contain free amino groups for coupling steps. The implant materials consist of 316-L surgical stainless steel and ZrO_2 ceramics. Furthermore microscopical cover glasses are used because of their availability, cheapness and low background signal in spectrometrical analysis. Amino groups are introduced by silanisation with 3-Aminopropyl-triethoxysilane (see protocol A.1). The surface needs to contain free hydroxyl groups in order to react with the silanisation agent. Cover glasses and ZrO_2 already have hydroxyl

groups, whereas 316-L has to be etched to generate some (see protocol A.2). There exists a widely used analytical method for amino group quantification as described in section 4.3.2. Our special problem was the very low number of amino groups on the surfaces, far below the detection limit of the Kaiser test. After some discussions we decided to perform an enzymatic assay. The free amino groups are activated with a cross linker followed by conjugation to an enzyme. Now in an initial rate experiment the enzymatic reaction can be monitored over the time (for more details as well as theoretical background see section 4.3.3). The slope is proportional to the concentration of the enzyme, which should be somewhat equal to the number of amino groups. Eventually the test is not about amino group quantification but for the quantification of functional groups that are also available for ligand coupling.

The quantification tests are performed in $10 \cdot 10 \cdot 45$ mm disposable macro cuvettes, containing 4 ml 100 mM TBS, pH = 9.5, with 100 mM NaCl and 100 mM $MgCl_2$. The reaction is started by addition of 10 μl p- NPP stock solution (50 $^{mg}/_{ml}$ in H_2O). The extinction is monitored over the time at 405 nm in a spectrophotometer. A Pasteur pipette is used to mix the cuvette content between the measure points.

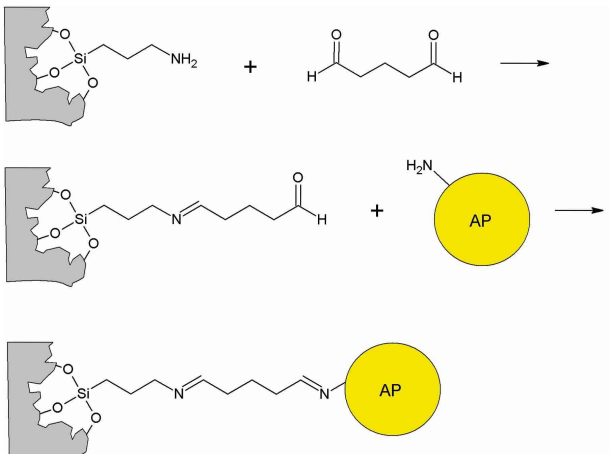


Figure 5.1: AP immobilisation with GA

All used protocols as well as the detailed experimental data can be found in the appendix section. The amount of immobilised enzyme is calculated using a standard calibration function (protocol A.6, data B.7 to B.10) giving the following equation:

$$c\,[^{ng}/_{ml}] = \frac{k - 0.011}{0.247} \qquad \text{and} \qquad m\,[\text{ng}] = c\,[^{ng}/_{ml}] \cdot V\,[\text{ml}]\,,$$

whereat c is the concentration of AP, k is the slope in the initial rate experiment, m is the mass of AP and V is the buffer volume in the cuvette (4 ml). The results of the quantification experiments are summarised in the following table. Different immobilisation setups are organised in blocks within the table.

Table 5.1: Quantification of immobilised AP

Sample Description	Protocol	Data	Slope	Quantity
AP on glass with GA	A.4	B.1	$6.93 \cdot 10^{-4}$	11 ng
Sample B.1 repeated mes. after 3 days storage in PBS	A.4	B.6	$6.11 \cdot 10^{-4}$	10 ng
AP on 0.5 M HCl-etched 316-L with GA	A.5	B.2	$5.96 \cdot 10^{-4}$	10 ng
AP on 6 M HCl-etched 316-L with GA	A.5	B.3	$3.85 \cdot 10^{-4}$	6 ng
AP on 3% HNO_3-etched 316-L with GA	A.5	B.4	$5.44 \cdot 10^{-4}$	9 ng
AP on not etched 316-L with GA	A.5	B.5	$7.74 \cdot 10^{-5}$	1 ng
AP on ZrO_2 with GA	A.12	B.18	$2.14 \cdot 10^{-4}$	3 ng
AP on 3% HNO_3-etched 316-L with EDC pH 5	A.9	B.24	$8.6 \cdot 10^{-4}$	14 ng
AP on PEGylated (with p-chloranil) 0.5 M HCl-etched 316-L with p-chloranil	A.10	B.11	$1.27 \cdot 10^{-4}$	2 ng
AP on PEGylated (with EDC, pH 5) 0.5 M HCl-etched 316-L with GA	A.7, A.8	B.12	$2.44 \cdot 10^{-4}$	4 ng
AP on PEGylated (with EDC, pH 5) 6 M HCl-etched 316-L with GA	A.7, A.8	B.13	$2.38 \cdot 10^{-4}$	4 ng
AP on PEGylated (with EDC, pH 5) 3% HNO_3-etched 316-L with GA	A.7, A.8	B.14	$2.38 \cdot 10^{-4}$	4 ng
AP on PEGylated (with EDC, pH 10) 0.5 M HCl-etched 316-L with GA	A.7, A.8	B.15	$2.35 \cdot 10^{-4}$	4 ng
AP on PEGylated (with EDC, pH 10) 6 M HCl-etched 316-L with GA	A.7, A.8	B.16	$2.31 \cdot 10^{-4}$	4 ng

Continued on next page

Table 5.1 – continued from previous page

Sample Description	Protocol	Data	Slope	Quantity
AP on PEGylated (with EDC, pH 10) 3% HNO_3-etched 316-L with GA	A.7, A.8	B.17	$2.32 \cdot 10^{-4}$	4 ng
Blank sample: AP on not silanised PEGylated (with EDC, pH 10) 0.5 M HCl-etched 316-L with GA	A.7, A.8	B.19	$4.16 \cdot 10^{-6}$	0 ng
AP on PEGylated (with EDC, pH 5) 3% HNO_3-etched 316-L with EDC pH 5	A.7, A.9	B.20	$3.53 \cdot 10^{-4}$	6 ng
AP on PEGylated (with EDC, pH 10) 3% HNO_3-etched 316-L with EDC pH 10	A.7, A.9	B.21	$1.97 \cdot 10^{-4}$	3 ng
AP on PEGylated (with EDC, pH 5) 0.5 M HCl-etched 316-L with EDC pH 5	A.7, A.9	B.22	$5.11 \cdot 10^{-4}$	8 ng
AP on PEGylated (with EDC, pH 10) 0.5 M HCl-etched 316-L with EDC pH 10	A.7, A.9	B.23	$2.51 \cdot 10^{-4}$	4 ng
Biotinylated AP (A.15) on avidin coated cover glass (A.13)	A.16	B.25	$2.51 \cdot 10^{-3}$	40 ng
Biotinylated AP (A.15) on biotinylated cover glass sample 1 (A.15)	A.17	B.26	$2.51 \cdot 10^{-4}$	4 ng
Biotinylated AP (A.15) on biotinylated cover glass sample 2 (A.15)	A.17	B.27	$3.61 \cdot 10^{-4}$	6 ng

The first block shows the results of a direct immobilisation of AP on solid supports. Different etching agents can be compared. Etching with 0.5

M HCl gives the best results similar to 3% HNO_3. With 6 M HCl, the most concentrated acid, only half of the amount of AP could be detected as compared to the other etching agents. Not silanised supports do not give a signal (data not shown). Unetched 316-L stainless steel gives a signal strength that is one order of magnitude below that one of etched steel, obviously demonstrating how important the etching step is. The ZrO_2 immobilisate also shows very promising results. The amount of AP (3 ng) might seem low, but when including the surface of the ZrO_2 tab ($\approx 0.3\,cm^2$) in the calculation, it exceeds all other immobilisates (surface $\approx 1.5\,cm^2$). The immobilised amount of AP on glass is equal to that on 316-L stainless steel. Due to this fact later on all immobilisation experiments are carried out on glass.
Second block includes a single experiment whereat AP was linked directly to a silanised 316-L foil using the EDC method (see section 4.2.2). This is the best support. However because of the time consuming EDC immobilisation the experiment was not verified and further investigation is needed.
Third block shows a single support using p-chloranil to conjugate AP on a PEGylated surface. The quantity of AP is rather low. Moreover p-chloranil seems not to be an unproblematic chemical when used in living organisms. No more experiments are performed using p-chloranil as the crosslinker.

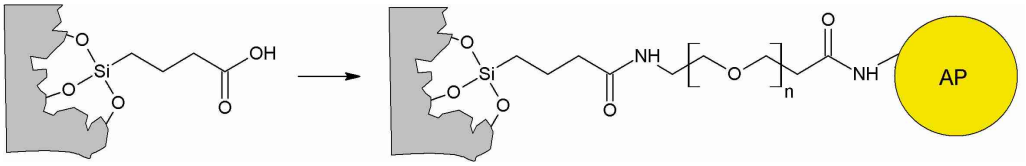


Figure 5.2: PEG linked to surface using EDC followed by AP immobilisation with EDC

The following block lists the results of 316-L foils that are camouflaged with PEG (coupled with EDC). The PEG chains contain a terminal amino group, which is used to couple AP using GA. After the PEG linking no more differences could be observed between the different etching methods. Two different EDC protocols are used for PEG immobilisation (one working at pH 5 and one with a pH of 10), giving the same results. When compared to the direct GA linkage of AP without PEG, only half of the amount could be immobilised with a PEG chain layer in between the support surface and the AP.
In the fifth block the results of PEGylated surfaces (using EDC) and subsequent AP coupling with EDC are listed. Now a clear difference between pH 5 and pH 10 was observed. The pH 5 protocol allows to link approx-

imately the double quantity of AP to the support as compared to pH 10, almost reaching the same amounts that have been detected without an intermediate PEG layer.
Last block shows some avidin biotin experiments. By incubating an avidin coated cover glass (using GA) with biotinylated AP an amount of 40 ng is detected, which is one order of magnitude above all other samples. Theoretically avidin has four biotin binding sites. That could explain the strong signal enhancement. The other setup consists of a biotinylated surface incubated with avidin, followed by incubation with biotinylated AP. The amount of AP detected falls in the range of the common setups, wherein AP is directly conjugated to a surface. An explanation for the different outcomes might be a sterical hindrance of avidin when interacting with the short biotin chains linked to a surface.

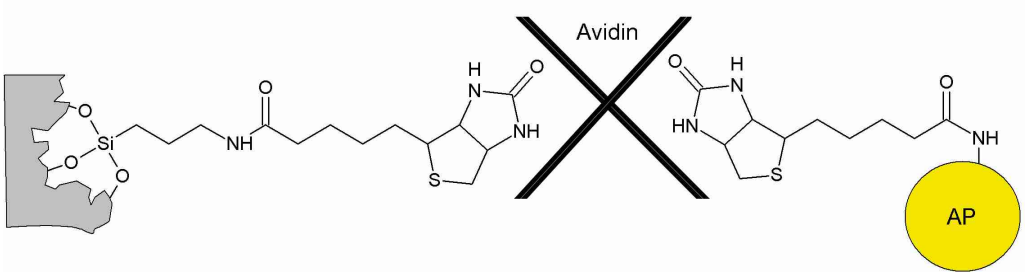


Figure 5.3: Bridged Avidin Biotin immobilisation

Recap

After the disappointing outcomes of the Kaiser Test the new developed enzymatic method shows very promising results. The detection limit of this method is far below 1 ng of immobilised enzyme per cm^2 of solid support surface. Results are of high reproducibility. The highest density of immobilised enzyme is obtained with the EDC conjugation method at pH 5, followed by the GA method. The microscopical cover glasses are going to be the solid support of choice for further tests. After etching 316-L stainless steel, preferably with a 0.5 M solution of HCl, the immobilisate density reaches the same values as compared to cover glasses.

5.2 Quantification of antibody based targeting moieties on surfaces

As already mentioned the specific antibody against the polymer beads was not available at the time when I started my diploma thesis work. Therefore some old antibodies from the fridge were used as model antibodies, to gain some expertise in antibody chemistry, including:

- Monoclonal mouse anti Hemagglutinin antibody (mαHA)[1]
- Monoclonal biotinylated donkey anti goat antibody (dαg)
- Anti Horseradish Peroxidase antibody from unknown origin (αHRP)
- Polyclonal goat anti mouse antibody HRP conjugate (gαm-HRP)
- Polyclonal rabbit anti mouse antibody HRP conjugate(rαm-HRP)

Before antibody processing the crude antibody solutions are purified in a Nickel-Chelate affinity chromatography step (see section 4.1.2). All immobilisation experiments are carried out using microscopical cover glasses as the solid support.

5.2.1 dαg Pretests

In this first antibody experiment the goal was to be able to detect antibodies on a surface. The biotinylated antibody is immobilised using the GA method (protocol A.21), or the sulfo-SMCC method (A.22). The sulfo-SMCC procedure (see section 4.2.4) is used for direct coupling of the dαg antibody on blank surfaces as well as PEGylated surfaces (A.23). For detection an avidin-AP conjugate is created (see A.14). The avidin moiety should interact with the biotin tag of the immobilised antibody, whereas the AP can be detected as usual. Unfortunately this setup does not show a positive outcome.
The next idea was to detect the dαg antibody using a secondary gαm-HRP conjugate. Usually the secondary antibody binds the primary, but in this setup the primary antibody (αg) binds the secondary which is from goat origin. TMB is used as the substrate.

[1] Provided by Manfred Koranda

Test setup:

- Place the cover glass in a macro cuvette
- Add 4 ml 0.1 M PBS pH 7
- Add 20 μl TMB in EtOH
- Add 40 μl H_2O_2

After 20 seconds the solution begins to turn blue indicating the presence of HRP. All three different test samples, i.e. dαg on glass with GA, dαg on glass with sulfo-SMCC and dαg on PEGylated glass with sulfo-SMCC, developed the blue color. It takes the blank samples more time to develop the blue colour (after two minutes), but this outcome is considered not to be good enough for a quantitative test. Some different blocking techniques have to be evaluated to gain a lower background signal for the blanks.

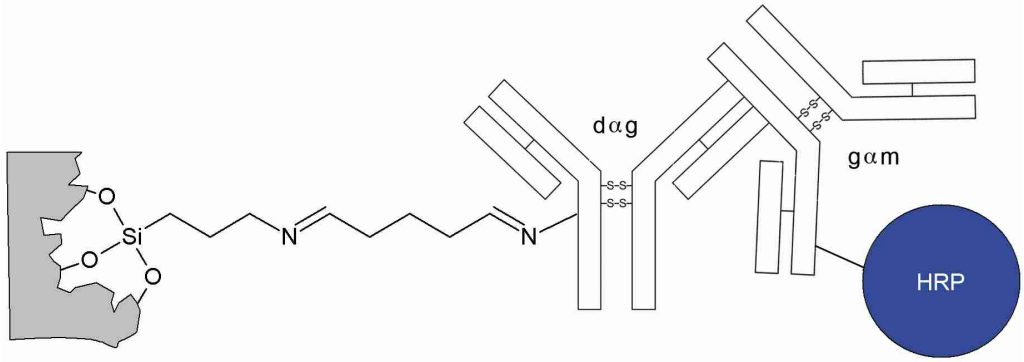


Figure 5.4: dαg antibody immobilised with GA; The dαg antibody recognises a 2nd antibody HRP conjugate from goat origin

5.2.2 mαHA Pretests

The experiments from the previous section are repeated using a slightly different setup. A first immobilised antibody, i.e. mαHA, is detected by a 2nd antibody HRP conjugate (rαm-HRP). Now this is the typical ELISA procedure. The mαHA antibody is linked to the surface using GA or s-SMCC (protocols A.19 and A.20). After a washing step any bound rαm-HRP is detected by adding HRP substrate as described in the previous section. The cover glass surfaces turned blue immediately, already indicating that the blue dye used for the test is not soluble in water. But for a quantitative

method in a spectrophotometer a soluble dye is needed in order to gain a steady signal. The poor solubility of HRP dyes along with the strong background of the blank samples are considered the biggest issues at this time.

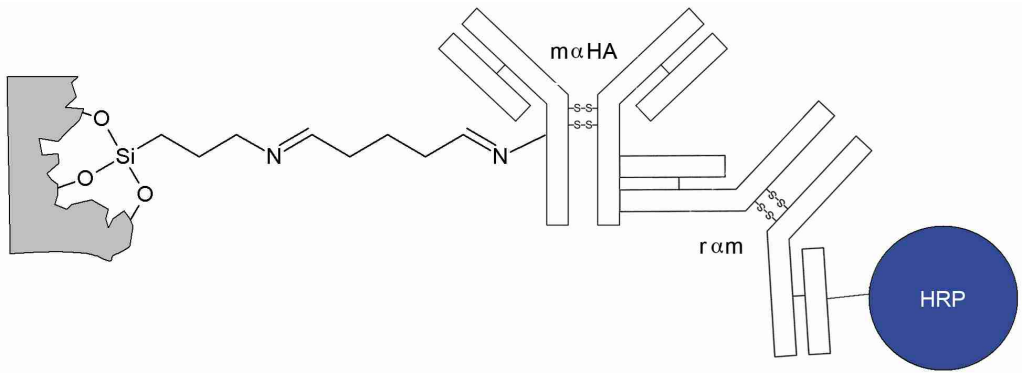


Figure 5.5: mαHA antibody immobilised with GA; The mαHA antibody is recognised by a 2nd rαm-HRP in an ELISA like setup

5.2.3 αHRP immobilisation

Until now every setup utilised two antibodies, one immobilised on the surface and one for detection of the first one. Now a new model is created in which only one antibody is immobilised. This antibody immediately recognises a dye developing enzyme that can be used for quantification. An αHRP antibody is meeting this demands.
The αHRP antibody is linked with GA or s-SMCC (protocols A.25 and A.26) to the silanised cover glasses. HRP can be used for detection of any bound αHRP antibody. The detection protocol now includes a block wash step with an added surfactant and BSA as a blocking agent.

- Wash the cover glass antibody support with 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 containing 1 ‰ Triton X-100 and 26 $^{mg}/_{ml}$ BSA for one hour.
- Add 2 μl of a 1.5 $^{mg}/_{ml}$ HRP solution.
- Incubate for one hour
- Wash with 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 containing 1 ‰ Triton X-100 for one hour.

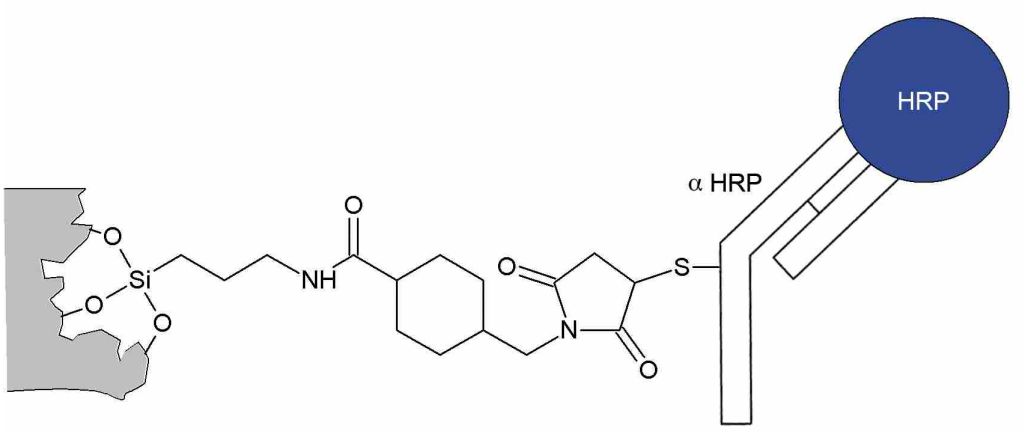


Figure 5.6: αHRP antibody immobilised with s-SMCC; The αHRP antibody binds HRP to yield a blue color

- Wash with 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 .
- Add 4 ml 0.1 M PBS, pH 7.
- Add 20 μl TMB in EtOH
- Add 40 μl H_2O_2

The two αHRP supports (GA and s-SMCC immobilisation) as well as a positive control, wherein HRP is directly linked to the surface, turned blue after thirty seconds. Finally the blank sample does not show any color development after one hour of incubation time.

5.2.4 Evaluation of HRP substrates

After including a blocking step in the quantification protocol the background of blank samples was no longer a problem. So now we have a perfect model system beside the fact that the used HRP dye is not water soluble. When trying to quantify a immobilisate in the spectrophotometer the signal does not increase steadily. When looking at the cover glasses it could be seen that the TMB dye adsorbed on the surface falsifying the measurements.
Hence different HRP substrates are tested, hoping to find a water soluble one.

The used substrates include:

- catechol
- resorcinol
- guaiacol (used as a 10% solution in DMSO)
- ascorbic acid

Wave length scans of the different substrates are performed in their reduced and oxidised forms. Only catechol and guaiacol show different spectra in the oxidised form as compared to the reduced one. Catechol has an absorption maximum at 421 nm, whereas that one of guaiacol can be found at 477.5 nm. Comparing these maxima with that of a pure HRP solution (402 nm), guaiacol seems to be the substrate of choice. The peak of catechol is too near to the HRP peak, thus lowering the detection limit. Next guaiacol solutions are tested with different concentrations of dissolved HRP. The results are promising, showing a steady signal over three orders of magnitude of HRP concentration.

Table 5.2: Quantification of dissolved HRP with guaiacol

HRP concentration	**slope**
70.6 nM	$4.6 \cdot 10^{-3}$
7.1 nM	$3.5 \cdot 10^{-4}$
0.71 nM	$3.2 \cdot 10^{-5}$

All these tests were performed with dissolved HRP. Now the guaiacol substrate is tested with immobilised HRP and once again the oxidised guaiacol adsorbed on the cover glass. In the end no suitable HRP substrate, which is soluble in water and does not adsorb on glass surfaces, could be found.

5.2.5 A HRP-AP chimaera

Having in mind the idea of Ehrlich, that a haptophore does not necessarily have a therapeutic effect and should therefore be combined with a drug molecule similar to an arrow poison, another setup is developed. The

haptophore moiety consists of the HRP enzyme, that can attach to the surface bound αHRP antibody. But as seen before the analytical (instead of the therapeutic) effect of HRP is quite frustrating. So why not combine it with an enzyme that has great potential in analytics, for example AP, which has already prevailed in previous tests.
HRP has some sugar residues that can be oxidised to aldehydes using $NaIO_4$. These aldehyde groups can react with amino residues of AP giving a Schiff base linkage, which is reduced to the secondary amine using $NaBH_4$ (for further details see protocols A.27 and A.29).
The resulting HRP-AP chimaera can be quantified easily using the same setup as described in section 5.1.

5.2.6 Quantification of GA immobilised antibodies

Now the antibody immobilisates are quantified using the new AP-HRP conjugate. All immobilisates are prepared as described in protocol A.25, wherein the αHRP antibodies are coupled to the amino silanised cover glasses with the GA method (section 4.2.1).

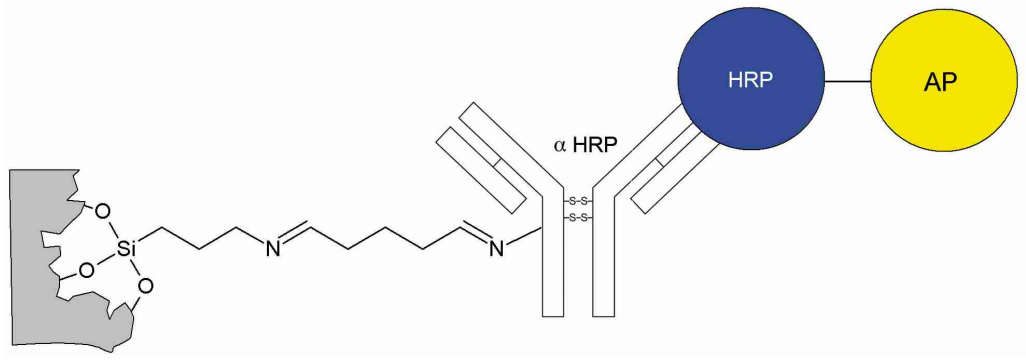


Figure 5.7: αHRP antibody immobilised with GA; The αHRP antibody binds the HRP-AP conjugate to yield a yellow color

The block-wash step from section 5.2.3 is adapted to the AP-HRP conjugate:

- Wash the cover glass antibody support with 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 containing 1 ‰ Triton X-100 and 26 $^{mg}/_{ml}$ BSA for one hour.
- Add 2 μl of a 1.5 $^{mg}/_{ml}$ AP-HRP solution.
- Incubate for one hour

- Wash with 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 containing 1 ‰ Triton X-100 for thirty minutes.
- Wash with 100 mM TBS, pH = 9.5, with 100 mM NaCl and 100 mM $MgCl_2$ for 10 minutes.
- Add 4 ml 100 mM TBS, pH = 9.5, with 100 mM NaCl and 100 mM $MgCl_2$ along with the support in a $10 \cdot 10 \cdot 45$ mm disposable macro cuvette
- Add 10 μl p- NPP stock solution (50 $^{mg}/_{ml}$ in H_2O) to start the reaction.
- Monitor extinction over time at 405 nm in a spectrophotometer, mixing the cuvette content with a pasteur pipette between measure points.

The following table lists the results from these first quantitative tests. The calculated amounts are not that accurate, because the activity of the new HRP-AP might not be comparable to that one of the pure AP. However the results should give the range of immobilised antibodies to have somewhat an idea of how much is immobilisable.

Table 5.3: Quantification of GA immobilised αHRP antibodies (part A)

Sample Description	**Data**	**Slope**	**Quantity**
αHRP on cover glass with GA, incubated with AP; blank 1	B.28	$9.41 \cdot 10^{-6}$	≈ 0 ng
BSA on cover glass with GA, incubated with AP-HRP; blank 2	B.29	$-2.98 \cdot 10^{-6}$	≈ 0 ng
αHRP on cover glass with GA, incubated with AP-HRP; sample 1	B.30	$9.33 \cdot 10^{-5}$	≈ 1.3 ng
αHRP on cover glass with GA, incubated with AP-HRP; sample 2	B.31	$1.43 \cdot 10^{-4}$	≈ 2.1 ng
αHRP on cover glass with GA, incubated with AP-HRP; sample 3	B.32	$1.65 \cdot 10^{-4}$	≈ 2.5 ng
αHRP on cover glass with GA, incubated with AP-HRP; sample 4	B.33	$1.73 \cdot 10^{-4}$	≈ 2.6 ng

Continued on next page

Table 5.3 – continued from previous page

Sample Description	Data	Slope	Quantity
sample 1; repeated measurement[2]	B.34	$6.11 \cdot 10^{-4}$	$\approx 0.8\,\text{ng}$
sample 2; repeated measurement	B.35	$7.38 \cdot 10^{-5}$	$\approx 1\,\text{ng}$
sample 3; repeated measurement	B.36	$8.07 \cdot 10^{-5}$	$\approx 1.1\,\text{ng}$
sample 4; repeated measurement	B.37	$5.41 \cdot 10^{-5}$	$\approx 0.7\,\text{ng}$

The amount of immobilised antibodies lies somewhere in the range of 2 ng per cover glass. Blank 1 shows no cross reactivity of pure AP with the immobilised αHRP antibody. Blank 2 shows that now unspecific adsorption on the cover glass takes place. After ten days of storage at 4°C only half of the initially coupled antibodies are detected. The reasons might be the labile Schiff Base linkage of the antibodies, as well as activity loss of AP-HRP when stored in a pH=7.2 buffer.

[2] After ten days storage in PBS pH=7.2 at 4°C

2nd round of quantification of GA immobilised antibodies

Another four samples are prepared as described above. Results are listed in the following table:

Table 5.4: Quantification of GA immobilised αHRP antibodies (part B)

Sample Description	Data	Slope	Quantity
αHRP on cover glass with GA, incubated with AP-HRP; sample 1	B.38	$1.52 \cdot 10^{-4}$	≈ 2.3 ng
αHRP on cover glass with GA, incubated with AP-HRP; sample 2	B.39	$9.07 \cdot 10^{-5}$	≈ 1.3 ng
αHRP on cover glass with GA, incubated with AP-HRP; sample 3	B.40	$2.58 \cdot 10^{-4}$	≈ 4 ng
αHRP on cover glass with GA, incubated with AP-HRP; sample 4	B.41	$2.28 \cdot 10^{-4}$	≈ 3.5 ng
αHRP on cover glass with GA, incubated with AP; blank 1	B.42	$1.77 \cdot 10^{-5}$	≈ 0.1 ng
αHRP on cover glass with GA, incubated with AP; blank 2	B.43	$1.68 \cdot 10^{-5}$	≈ 0.1 ng
sample 1; repeated measurement[3]	B.44	$9.55 \cdot 10^{-5}$	≈ 1.4 ng
sample 2; repeated measurement[4]	-xxx-	-xxx-	-xxx-
sample 3; repeated measurement	B.45	$9.21 \cdot 10^{-5}$	≈ 1.3 ng
sample 4; repeated measurement	B.46	$1.95 \cdot 10^{-4}$	≈ 3 ng

In this experiment series the amount of immobilised antibody varied from 1.3 to 4 ng. Blanks showed no cross reactivity with pure AP. Some problems

[3] After two days storage in PBS pH=7.2 at 4°C

[4] Sample 2 was destroyed falling on the floor

arose during sample storage. Sample 2 was completely destroyed, whereas sample 1 and 3 broke in pieces. The results from the repeated measurement are therefore not comparable. However it is quite apparent that storage of the GA conjugates results in severe activity loss even in a short storage period of two days.
Looking at the whole picture of all GA immobilisates, this technique results in low stability conjugates with a low reproducibility. Hence the method will not meet the demands required to design auspicious medical devices.

5.2.7 Quantification of s-SMCC immobilised antibodies

Antibody immobilisates are quantified using the new AP-HRP conjugate. All immobilisates are prepared as described in protocol A.26, wherein the αHRP antibody fragments are coupled to the amino silanised cover glasses with the s-SMCC method (section 4.2.4). The test is performed as described in the previous section.

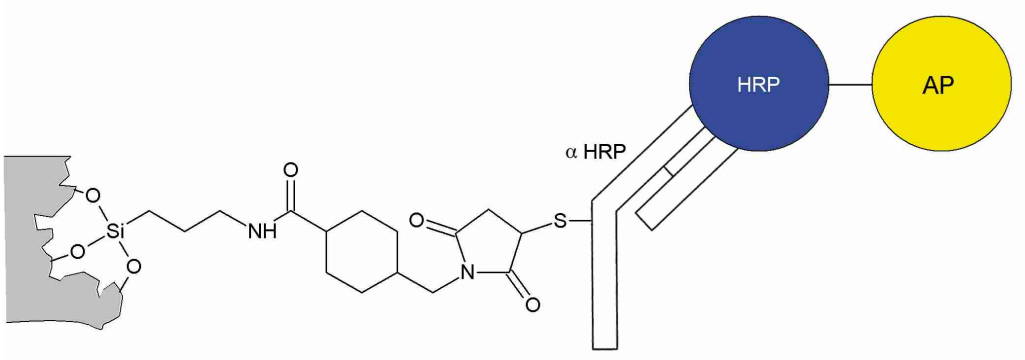


Figure 5.8: αHRP antibody fragment immobilised with s-SMCC; The αHRP antibody binds the HRP-AP conjugate to yield a yellow color

The following table summarises the results of s-SMCC conjugates:

Table 5.5: Quantification of s-SMCC immobilised αHRP antibody fragments

Sample Description	Data	Slope	Quantity
αHRP fragment on cover glass with s-SMCC, incubated with AP-HRP; sample 1	B.47	$3.05 \cdot 10^{-4}$	≈ 4.8 ng

Continued on next page

Table 5.5 – continued from previous page

Sample Description	Data	Slope	Quantity
αHRP fragment on cover glass with s-SMCC, incubated with AP-HRP; sample 2	B.48	$2.66 \cdot 10^{-4}$	$\approx 4.1\,\mathrm{ng}$
αHRP fragment on cover glass with s-SMCC, incubated with AP-HRP; sample 3	B.49	$3.76 \cdot 10^{-5}$	$\approx 0.4\,\mathrm{ng}$
αHRP fragment on cover glass with s-SMCC, incubated with AP-HRP; sample 4	B.50	$2.46 \cdot 10^{-4}$	$\approx 3.8\,\mathrm{ng}$
αHRP fragment on cover glass with s-SMCC, incubated with AP-HRP; sample 5	B.51	$1.54 \cdot 10^{-4}$	$\approx 2.3\,\mathrm{ng}$
αHRP fragment on cover glass with s-SMCC, incubated with AP-HRP; sample 6	B.52	$1.65 \cdot 10^{-4}$	$\approx 2.5\,\mathrm{ng}$
αHRP fragment on cover glass with s-SMCC, incubated with AP; blank	B.53	$-2.94 \cdot 10^{-6}$	$\approx 0\,\mathrm{ng}$
sample 1; repeated measurement [5]	B.54	$9.37 \cdot 10^{-4}$	$\approx 1.3\,\mathrm{ng}$
sample 2; repeated measurement	B.55	$1.27 \cdot 10^{-4}$	$\approx 1.9\,\mathrm{ng}$
sample 3; repeated measurement	B.56	$1.88 \cdot 10^{-5}$	$\approx 0.1\,\mathrm{ng}$
sample 4; repeated measurement	B.57	$1.18 \cdot 10^{-4}$	$\approx 1.7\,\mathrm{ng}$
sample 5; repeated measurement	B.58	$1.27 \cdot 10^{-4}$	$\approx 1.9\,\mathrm{ng}$
sample 6; repeated measurement	B.59	$1.28 \cdot 10^{-4}$	$\approx 1.9\,\mathrm{ng}$
sample 1; repeated measurement [6]	B.60	$1.24 \cdot 10^{-7}$	$\approx 0\,\mathrm{ng}$

Continued on next page

[5] After four days storage in PBS pH=7.2 at 4°C
[6] After 23 days storage in PBS pH=7.2 at 4°C

Table 5.5 – continued from previous page

Sample Description	Data	Slope	Quantity
sample 2; repeated measurement	B.61	$3.85 \cdot 10^{-5}$	≈ 0.5 ng
sample 3; repeated measurement	B.62	$-5.79 \cdot 10^{-5}$	≈ 0 ng
sample 4; repeated measurement	B.63	$2.64 \cdot 10^{-5}$	≈ 0.2 ng
sample 5; repeated measurement	B.64	$5.24 \cdot 10^{-5}$	≈ 0.7 ng
sample 6; repeated measurement	B.65	$4.09 \cdot 10^{-5}$	≈ 0.5 ng

For a better view on the degradation of these samples have a look at the following table.

Table 5.6: Degradation of s-SMCC supports

Sample	After 1 Day (ng)	After 4 Days (ng)	After 23 days (ng)
1	4.8	1.3	0
2	4.1	1.9	0.5
3	0.4	0.1	0
4	3.8	1.7	0.2
5	2.3	1.9	0.7
6	2.5	1.9	0.5

The amount of coupled antibody seems quite high in the first round of testing. Sample 3 is a failure and is not going to be included in the discussion. After a initial bleeding out phase all samples show the same amount of immobilised antibody after 4 days storage. This means that then no more unspecifically or labile bound antibody is present on the support. The result is very accurate with amounts varying from 1.7 to 1.9 ng in four samples.

The long term stability (23 days) of this method is not bad either. Storage conditions were not ideal and in the end infections could be seen in the storage flasks. Nevertheless a small rest of activity could be detected. The reason for the decrease is probably not the lability of the antibody support but that of the AP itself.
In general the s-SMCC coupling is the method of choice comparing it to the GA technique due to:

- Higher density of immobilised binding sites
- Better stability of the support
- Site directed coupling of the targeting moieties

For further long term stability tests a variation of the method is needed, in which the analyte (in our case AP) can be reloaded in every round of testing.

5.3 Gimmicks

After having figured out the enormous potential of heterogeneous reactions, some solid supports useful for different reaction steps were synthesised.
Normally a time consuming chromatography step is needed when processing proteins (antibodies) chemically. Within chromatography unreacted reagents as well as by products and so on are removed. This is necessary because sometimes these reagents will interfere in following reaction steps. For example: When coupling antibodies through the sulfhydryl residues in the hinge region, they are reduced with a sulfhydryl containing agent. This reactant is added in large excess to drive the reduction to completion. Then in a later coupling step, targeting the sulfhydryls, the crosslinker with affinity to the latter will never find a reduced antibody in the huge sulfhydryl pool of the reducing agent.
To avoid the chromatography step an immobilisation of lipoic acid on glass powder was performed (for details see protocol A.33). The powder is simply added to the antibodies and removed by filtration or centrifugation, with the antibodies ready to use.
The lipoic acid - glass powder support shows a specific activity of 3.17 $^{mmol}/_{g}$ when compared to pure lipoic acid in an Ellmann's Assay[7]. This means that

[7]The disulfide bond in Ellmann's reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid), is cleaved by present sulfhydryls and the products are detected photometrically

when one would need an amount of 3.17 mmol of lipoic acid for reduction, 1 g of glass powder support has to be used. Because of the heterogeneous nature of this reducing device, the reduction step is no more an equilibrium reaction, thus much less reactant is needed as compared to soluble ones, e.g. β-mercaptoethanol.
Another support consists of immobilised pepsin on alumina (protocol A.32. This is useful for antibody cleavage[8]. The support was tested in a digestion assay using Coomassie™Brilliant Blue G-250 stained BSA. The absorption of the stained BSA decreases when digested by pepsin. After comparing the results to pure pepsin standards an amount of 0.5 μg per mg of alumina was found.
Given that the drug "trucks" in our system consist of PLGA nano beads, some detection tools were synthesised including:

- Immobilised PLGA on glass powder using the s-NHS + EDC technique (protocol A.35), as a affinity chromatography support for purification of potential PLGA antibodies.
- An AP-PLGA conjugate (protocol A.36) as a detection tool for PLGA antibodies, somewhat similar to 2nd antibody-enzyme conjugates used in ELISA staining, with a PLGA chain as targeting moiety.

At this time I was at the end of my practical work. Therefore no characterisation experiments were performed with these last two supports. But like the rest of the gimmicks they might be useful for my supervisor and potential successors.

[8]Pepsin digestion leads to one F(ab')$_2$ antibody fragment.

Chapter 6

Conclusion and Future Perspectives

The within this thesis developed detection method, using AP, has proven to be very sensitive with a detection limit below 1 ng. On average an amount of approximately 1 ng AP per cm^2 of support surface was found. Considering a typical drug dosage of 0.1 $^{mg}/_{kg}$ a support surface of $\approx 100\,cm^2$ is needed in order for the drug to have an effect in a clearly localised area of 10 ml in volume. This might be a hindrance to the potential applicability of such a system. However these calculations are a rough estimation and have to be evaluated within in vivo tests anyway.
For future perspectives I recommend to include the methods presented in the introduction chapter in the production process of a drug targeting system. In my opinion the most powerful type of system consists of:

- An in vivo scanning for potential targets using scFv, aptamer or OP libraries
- Isolation of the promising candidates
- Conjugation to drug loaded degradable polymer beads

Maybe one day it is possible to perform a biopsy of an affected area, use the specimen to scan for targeting ligands and couple positives to drugs. That would be a highly individual therapy, wherein every patient has his own personal drug targeting system.

Appendix A

Protocols

This section gives a short description of the experimental work in chronological order. Consider the fact that most experiments where pretests and the used conditions may be far from optimum. The results of possible product characterisations are listed in section B.

A.1 Silanisation of surfaces

The only important aspect for a successful reaction is that the sample possesses active hydroxy groups, which can react with the silanisation agent. Cover glasses and ZrO_2 samples used in this work already contain those groups, whereas steel samples have to be etched to introduce them. The following method is used in all of the silanisation steps in this thesis: Activate the sample for one hour in a 5% solution of aminosilane in 95% EtOH at room temperature. Wash with EtOH and dry over night at 110°C.

A.2 Etching of 316-L stainless steel

A mild etching procedure is needed to introduce active hydroxy groups for further silanisation. Cut a 316-L foil in small pieces of approximately 1 cm. 0.5 M HCl, 6 M HCl and 3% HNO_3 are the etching agents of choice. Etch for 15 min at room temperature. A fast spot test using a filter paper treated with 5% NaSCN can be useful to detect iron ions in the acids, thus indicating successful etching.
Store in H_2O for at least two days to ensure hydroxy group formation.

A.3 Kaiser test for amino group determination

Dissolve 8 g phenol in 2 ml EtOH. Prepare a 10 mM solution of KCN in 0.2 ml H_2O. Fill up to 10 ml with pyridine. Dissolve 50 mg ninhydrin in 950 mg EtOH. Add 0.5 ml of each solution to a test tube containing the sample. Boil for 5 min in H_2O.

A.4 Alkaline phosphatase on glass with glutaraldehyde

Fill an eprouvette containing aminosilanised cover glasses with 3 ml of a 2.5% glutaraldehyde solution in 0.05 M PBS, pH 7. Shake gently for one hour. Wash with large amounts of ice water. Dissolve 40 μl of alkaline phosphatase solution in 300 μl of 0.05 M PBS pH 7. Add 100 μl of this solution to each of the cover glasses placed on a microscope slide. Incubate over night in a wet box at room temperature. Wash them exhaustively with PBS. Store in 0.05 M saline solution at 4°C.

A.5 Alkaline phosphatase on 316-L foil with glutaraldehyde

Prepare a 2.5% glutaraldehyde solution in 0.05 M PBS, pH 7. Add 4 ml to the aminosilanised 316-L foil in a test tube. Shake for one hour. Wash with ice/H_2O. Dilute alkaline phosphatase to a final concentration of 22 μg/ml in 0.05 M PBS, pH 7. Add 200 μl of this solution to each of the 316-L foils. Incubate over night in a wet box at room temperature. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.6 Calibration function alkaline phosphatase

Prepare a stock solution of alkaline phosphatase at a concentration of 4.5 μg/ml in 0.1 M TBS containing 0.15 M NaCl and 5 mM $MgCl_2$, pH 9.5. Add different aliquots from 0.5 μl to 4 μl in a disposal cuvette and fill up to 4 ml with TBS. Measure absorption in a spectrophotometer against a blank at 405 nm.

A.7 PEGylation of 316-L using EDC

A terminal carboxylate group is needed to perform EDC mediated coupling. Dissolve 1 g succinic anhydride in 75 ml 0.5 M phosphate buffer, pH 6.1. Add 25 ml of this solution to the aminosilanised 316-L foils in a test tube. Shake over night at room temperature. The amino groups are transformed into carboxylates, which can be activated with EDC. Two different buffer systems are tested. Prepare 30 ml of 0.1 M citrate buffer (pH 5) and carbonate buffer (pH 10). Add 50 mg EDC and 0.4 g bis-AminoPEG to each. Incubate each 316-L foil in 10 ml of the different buffers over night at room temperature. Wash with PBS.

A.8 AP on PEGylated 316-L with glutaraldehyde

After the PEG coupling (Protocol A.7) AP can be attached to the terminal amino group of the PEG chains. Each sample is activated for one hour in 1.5 ml 2.5% glutaraldehyde solution in 0.05 M PBS, pH 7. Wash with ice/H_2O. Add 0.2 ml PBS containing 11 $\mu g/ml$ alkaline phosphatase to each sample. Incubate over night at room temperature. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.9 AP on PEGylated 316-L with EDC

The PEGylated 316-L prepared in experiment A.7 are activated once again with 2.5 ml phosphate buffer (0.5 M, pH 6.1) containing 0.22 g succinic anhydride over night at room temperature to transform the terminal amino group of the PEG chain into a carboxylate. Wash with PBS. Split the samples. Activate half of them in 0.1 M citrate buffer (pH 5) and the other ones in 0.1 M carbonate buffer (pH 10). Add 1 ml of the chosen buffer with 5 mg EDC to each sample. Incubate for 90 min. Prepare a solution of AP in citrate/ carbonate buffer (see protocol A.7) at a concentration of 11 $\mu g/ml$. Drop 0.4 ml to each sample and react over night at room temperature. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.10 PEGylation and coupling of AP on 316-L with p-chloranil

Prepare a 1% solution of p-chloranil in toluene. Add the aminosilanised 316-L foils and react for 30 min at room temperature. Wash with toluene, acetone and H_2O. Dissolve 0.1 g bis-AminoPEG in 5 ml H_2O. Incubate the activated samples for 90 min in this solution. Wash with H_2O.
The immobilized PEG chains are activated once again with 1% p-chloranil in toluene for 30 min at room temperature. Wash with toluene, acetone and H_2O. Prepare a solution of AP in 0.05 M PBS at a concentration of 7 $\mu g/ml$. Add 0.2 ml to each sample and allow to react over night at room temperature. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.11 PEGylation of glass using glutaraldehyde

Activate five cover glasses in 16 ml of a 2.5% glutaraldehyde solution in 0.05 M PBS pH 7 for one hour at room temperature. Wash with ice/H_2O. Incubate in 16 ml 0.05 M PBS containing 0.5 g bis-AminoPEG over night at room temperature. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.12 AP on ZrO_2 with glutaraldehyde

A silanised ZrO_2 tab is activated for 1 hour with a 2.5% glutaraldehyde solution in 0.05 M PBS, pH 7. Wash with ice/H_2O. Add AP in 0.05 M PBS at a concentration of 11 μl to the sample. Allow to react over night at room temperature. Store in 0.05 M saline solution at 4°C.

A.13 Immobilization of proteins on glass with glutaraldehyde

Activate aminosilanised cover glasses in a 2.5% glutaraldehyde solution in 0.05 M PBS at pH 7 for one hour at room temperature. Prepare the following protein solutions:

- 0.5 μl biotinylated donkey anti goat antibody in 0.2 ml 0.05 M PBS, pH 7.

- 0.1 mg HSA in 0.2 ml 0.05 M PBS, pH 7.
- 0.1 mg avidin in 0.2 ml 0.05 M PBS, pH 7.
- 0.1 mg streptavidin in 0.2 ml 0.05 M PBS, pH 7.

Wash the cover glasses with ice/H_2O and incubate with the different solutions over night at room temperature. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.14 AP-Avidin conjugate

Prepare 0.25 ml of a solution containing 1 mg/ml avidin and 4 $^{mg}/_{ml}$ AP in 0.2 M PBS with 0.15 M NaCl, pH 7.4. Add 2.5 μl of 25% glutaraldehyde in H_2O to the solution, mix and react for one hour at room temperature. To quench any excess aldehyde groups add 10 μl of 0.2 M lysine in 0.5 M carbonate buffer, pH 9.5. Block for two hours at room temperature.
The conjugate is purified by gel filtration. Fill a 3 ml disposal column with Sephadex™ G-25 in PBS to a final bed volume of approximately 2 ml. Chromatography is performed with a peristaltic pump at a flow rate of 1 $^{ml}/_{min}$ using PBS at pH 7 as elution buffer. Dextran blue elutes at 2.4 ml, whereas dnp-glycine appears at 3.5 ml. Wash the column with 20 ml elution buffer before applying the sample solution. Collect 0.5 ml fractions. The fractions are tested for AP activity. Take a 10 μl aliquot of each fraction and dilute it to 100 μl with 100 mM TBS, pH = 9.5, containing 100 mM NaCl and 100 mM $MgCl_2$. Add 1 μl of a 5 $^{mg}/_{ml}$ p- NPP stock solution. Fractions containing AP-avidin conjugate will turn yellow immediately. Pool the active fractions and store at 4°C.

A.15 Biotinylation of glass and AP

Suspend 100 mg biotin in 0.5 ml H_2O. Add 1 M NaOH until the suspension turns clear. The total volume increases to 3 ml. Dissolve 80 mg EDC in 0.5 ml in H_2O. Mix the two solutions and shake for two hours at room temperature.
In the coupling step biotin is reacted in a ten fold excess. Take 0.55 ml of AP stock solution (9 $^{mg}/_{ml}$, $3.51 * 10^{-5}$ mol) and add 3 ml of the activated biotin ($1.17 * 10^{-4}$ $^{mol}/_{ml}$, $3.51 * 10^{-4}$ mol). Split the remaining 0.5 ml biotin on two aminosilanised cover glasses. Incubate both approaches over night at room temperature.

Wash the cover glasses with PBS and store in 0.05 M saline solution at 4°C. The AP conjugate is purified by gel filtration with the column prepared in experiment A.14. Use PBS pH 7 as elution buffer at a flow rate of 1 $^{ml}/_{min}$. Apply 300 μl aliquots and collect 300 μl fractions. Monitor for AP activity (see experiment A.14). Pool the fractions showing AP activity and store at 4°C.

A.16 Biotinylated AP on avidin coated glass

Add 100 μl biotinylated AP (see experiment A.15) to a cover glass with immobilized avidin (experiment A.13). Wash twice with 0.05 M PBS and then with 100 mM TBS, pH = 9.5, containing 100 mM NaCl and 100 mM $MgCl_2$.

A.17 Biotinylated AP on biotinylated glass

Incubate the biotinylated cover glasses with 100 μl 0.5 $^{mg}/_{ml}$ avidin in 0.05 M PBS, pH 7. Wash with PBS. Dilute 100 μl biotinylated AP to 200 μl with PBS. Add 100 μl to each cover glass. Incubate for 30 min at room temperature. Wash with PBS and then with 100 mM TBS, pH = 9.5, containing 100 mM NaCl and 100 mM $MgCl_2$.

A.18 Ni^{2+} chelate affinity chromatography of mαHA antibody

Fill a 3 ml disposal column with iminodiacetic acid beads (Pierce) to a final bed volume of 1 ml. Attach a peristaltic pump. Wash with 10 ml H_2O at 1 $^{ml}/_{min}$. Load the iminodiacetic acid support with 0.5 ml of 0.5 M $NiSO_4 \cdot 6\,H_2O$ in H_2O at a flow rate of 0.5 $^{ml}/_{min}$. Wash with 15 ml of 0.01 M PBS containing 0.15 M NaCl at pH 7 (binding buffer). Apply 2 ml *mαHA* serum at a flow rate of 0.3 $^{ml}/_{min}$. Wash with 20 ml binding buffer at 1 $^{ml}/_{min}$. Elute with 0.1 M NaOAc containing 0.5 M NaCl at pH 5. Collect 250 μl fractions. Take a 5 μl aliquot of each and add 10 μl of Bio-Rad™ protein assay diluted 1:4 in H_2O. Fractions containing antibody will develop a strong blue color. Neutralize by adding 2.5 μl 1 M NaOH to each fraction and store at 4°C.

A.19 mαHA on glass with glutaraldehyde

Activate the aminosilanised cover glasses in a 2.5% glutaraldehyde solution in 0.05 M phosphate buffer with 0.15 M NaCl, pH 7. for one hour at room temperature. Wash with ice/H_2O. Add 100 μl purified antibody (see experiment A.18 to each cover glass and incubate for two hours at room temperature. Wash three times 15 min with PBS.
A secondary antibody is used to check for successful coupling. Add 2 μl 2nd rαm-HRP (rabbit anti mouse - HRP conjugate) in 100 μl PBS to each cover slip. Incubate for 30 min at room temperature. Wash three times 15 min with PBS. Incubate the cover glasses in 0.05 M PBS containing 40 μl H_2O_2 and 4 μl 1 mM TMB in EtOH. HRP can oxidize TMB to yield a blue dye.

A.20 mαHA on glass with sulfo-SMCC

Dilute 100 μl Ni^{2+}-chelate purified mαHA antibody (from experiment A.18) in 100 μl sodium phosphate containing 0.15 M NaCl, 10 mM EDTA, pH 7.2. Add 1.5 mg 2-mercaptoethylamine and mix to dissolve. React for 90 min at 37°C.
The aminosilanised cover glasses are activated in 0.1 M phosphate buffer containing 0.15 M NaCl, 1 $^{mg}/_{ml}$ sulfo-SMCC, pH 7.2. Allow to react for 30 min at room temperature. Wash with PBS and store wet until the antibodies are ready for coupling.
The reduced *mαHA* antibody is purified from excess mercaptoethylamine by gel filtration over Sephadex™ G25 using the column from exp. A.14. Elute with 0.1 M PBS containing 0.15 M NaCl, 0.01 M EDTA, pH 7.2 at a flow rate of 1 $^{ml}/_{min}$. Collect 250 μl fractions and monitor for the antibody using Bio-Rad™ protein assay as described in experiment A.18. For a successful coupling step it is extremely important that mercaptoethylamine is removed quantitatively. Pool the antibody containing fractions, dilute to the desired volume with PBS and add the solution immediately to the previously activated cover slips. The reduced sulfhydryl groups of the antibody fragments can be oxidized easily, thus longer storage is not recommended. Incubate over night at 4°C. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.21 dαg on glass with glutaraldehyde

The aminosilanised cover glasses are activated in a 2.5 % glutaraldehyde solution in 0.1 M sodium phosphate with 0.15 M NaCl, pH 7.2, for one hour at room temperature. Wash with ice/H_2O. Dilute the dαg antibody 1:10 in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 and add a 100 µl aliquot to each activated cover glass. Allow to react over night at 4°C. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.22 dαg on glass with sulfo-SMCC

Dilute 30 µl dαg antibody to 130 µl with PBS containing 0.15 M NaCl, 10 mM EDTA, pH 7.2. Add 1 mg 2-mercaptoethylamine and mix to dissolve. React for 90 min at 37°C.
The aminosilanised cover glasses are activated in 0.1 M phosphate buffer containing 0.15 M NaCl, 1 $^{mg}/_{ml}$ sulfo-SMCC, pH 7.2. Allow to react for 30 min at room temperature. Wash with PBS and store wet until the antibodies are ready for coupling.
The reduced dαg antibody is purified from excess mercaptoethylamine by gel filtration over Sephadex™ G25 using the column from exp. A.14. Elute with 0.1 M PBS containing 0.15 M NaCl, 0.01 M EDTA, pH 7.2 at a flow rate of 1 $^{ml}/_{min}$. Collect 250 µl fractions and monitor for the antibody using Bio-Rad™ protein assay as described in experiment A.18. Pool the antibody containing fractions, dilute to the desired volume with PBS and add the solution immediately to the previously activated cover slips. Incubate over night at 4°C. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.23 dαg on PEGylated glass with sulfo-SMCC

Dilute 5 µl dαg antibody to 55 µl with PBS containing 0.15 M NaCl, 10 mM EDTA, pH 7.2. Add 1 mg 2-mercaptoethylamine and mix to dissolve. React for 90 min at 37°C.
The PEGylated cover glasses (from experiment A.11) are activated in 0.1 M phosphate buffer containing 0.15 M NaCl, 1 $^{mg}/_{ml}$ sulfo-SMCC, pH 7.2. Incubation should be performed in a larger volume than 200 µl, due to the extreme hydrophobicity of the PEGylated glass. Allow to react for 30 min at room temperature. Wash with PBS and store wet until the antibodies are ready for coupling.

The reduced dαg antibody is purified from excess mercaptoethylamine by gel filtration over Sephadex™ G25 using the column from exp. A.14. Elute with 0.1 M PBS containing 0.15 M NaCl, 0.01 M EDTA, pH 7.2 at a flow rate of 1 $^{ml}/_{min}$. Collect 250 μl fractions and monitor for the antibody using Bio-Rad™ protein assay as described in experiment A.18. Pool the antibody containing fractions, dilute to the desired volume with PBS and add the solution immediately to the previously activated cover slips. Incubate over night at 4°C. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.24 HRP on glass with glutaraldehyde

An aminosilanised cover glass is activated in a 2.5 % glutaraldehyde solution, containing 0.1 M sodium phosphate with 0.15 M NaCl, pH 7.2, for one hour at room temperature. Wash with ice/H_2O. Dilute 1 μl of a 1 $^{mg}/_{ml}$ HRP solution to a final volume of 200 μl with 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 and add this mixture to the activated cover glass. Allow to react over night at 4°C. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.25 αHRP on glass with glutaraldehyde

An aminosilanised cover glass is activated in a 2.5 % glutaraldehyde solution, containing 0.1 M sodium phosphate with 0.15 M NaCl, pH 7.2, for one hour at room temperature. Wash with ice/H_2O. Dilute 1 μl of the αHRP solution to a final volume of 200 μl with 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 and add this mixture to the activated cover glass. Allow to react over night at 4°C. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.26 αHRP on glass with sulfo-SMCC

Dilute 1 μl αHRP antibody to 50 μl with PBS containing 0.15 M NaCl, 10 mM EDTA, pH 7.2. Add 1 mg 2-mercaptoethylamine and mix to dissolve. React for 90 min at 37°C.
The aminosilanised cover glasses are activated in 0.1 M phosphate buffer containing 0.15 M NaCl, 1 $^{mg}/_{ml}$ sulfo-SMCC, pH 7.2. Allow to react for

30 min at room temperature. Wash with PBS and store wet until the antibodies are ready for coupling.
The reduced αHRP antibody is purified from excess mercaptoethylamine by gel filtration over Sephadex™ G25 using the column from exp. A.14. Elute with 0.1 M PBS containing 0.15 M NaCl, 0.01 M EDTA, pH 7.2 at a flow rate of 1 $^{ml}/_{min}$. Collect 250 µl fractions and monitor for the antibody using Bio-Rad™ protein assay as described in experiment A.18. Pool the antibody containing fractions, dilute to the desired volume with PBS and add the solution immediately to the previously activated cover slips. Incubate over night at 4°C. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.27 $NaIO_4$ activation of HRP

Dissolve 2 mg HRP in 200 µl 0.01 M sodium phosphate, containing 8.8 mM $NaIO_4$. Protect from light and allow to react for one hour at room temperature.
Purify the activated HRP from excess $NaIO_4$ by gel filtration over Sephadex™ G25 using the column from exp. A.14. The HRP containing fractions are identified by the characteristic brown green color of activated HRP. Pool the fractions containing the enzyme and store at 4°C.

A.28 Biocytin conjugated with $NaIO_4$ activated HRP

Dissolve 0.5 mg biocytin in 0.5 ml eluate from experiment A.27 containing the $NaIO_4$ activated HRP. Adjust the pH to 9 with 20 µl of 1 M sodium carbonate. React for two hours at room temperature.
The resulting Schiff bases are reduced with 5 µl of 5 M $NaBH_4$ in 1 M NaOH for 30 minutes at room temperature. The conjugate is purified from any excess $NaBH_4$ by gel filtration over Sephadex™ G25 using the column from exp. A.14. Collect 250 µl fractions and monitor for the conjugate using Bio-Rad™ protein assay as described in experiment A.18. Pool the fractions containing the enzyme and store at 4°C.

A.29 AP conjugated with $NaIO_4$ activated HRP

Add 100 μl of AP stock solution (9 $^{mg}/_{ml}$) to 300 μl eluate from experiment A.27 containing the $NaIO_4$ activated HRP. Adjust the pH to 9 with 20 μl of 1 M sodium carbonate. React for two hours at room temperature.
The resulting Schiff bases are reduced with 5 μl of 5 M $NaBH_4$ in 1 M NaOH for 30 minutes at room temperature. The conjugate is purified from any excess $NaBH_4$ by gel filtration over Sephadex™ G25 using the column from exp. A.14. Collect 250 μl fractions and monitor for the conjugate using Bio-Rad™ protein assay as described in experiment A.18. Pool the fractions containing the enzyme and store at 4°C.

A.30 Ni^{2+} chelate affinity chromatography of αHRP antibody

The column prepared in experiment A.18 is used. Wash with 15 ml of 0.01 M PBS containing 0.15 M NaCl at pH 7 (binding buffer). Apply 200 μl αHRP antibody at a flow rate of 0.3 $^{ml}/_{min}$. Wash with 20 ml binding buffer at 1 $^{ml}/_{min}$. Elute with 0.1 M NaOAc containing 0.5 M NaCl at pH 5. Collect 250 μl fractions. Take a 5 μl aliquot of each and add 10 μl of Bio-Rad™ protein assay diluted 1:4 in H_2O. Fractions containing antibody will develop a strong blue color. Pool the fractions containing the antibody and neutralize using a Sephadex™ G25 desalting column. Use 0.01 M sodium phosphate containing 0.15 M NaCl at pH 7 as elution buffer. Collect 250 μl fractions and test for protein as described above. Pool the antibody containing fractions and store at 4°C.

A.31 Ni^{2+} chelate pure αHRP on glass with glutaraldehyde

Aminosilanised cover glasses are activated in a 2.5 % glutaraldehyde solution, containing 0.1 M sodium phosphate with 0.15 M NaCl, pH 7.2, for one hour at room temperature. Wash with ice/H_2O. Dilute 75 μl of the Ni^{2+} chelate pure αHRP (from experiment A.30) solution to a final volume of 4.5 ml with 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 and add this mixture to the activated cover glasses. Allow to react for ninety minutes at 37°C. Wash with PBS. Add 5 ml $NaBH_4$ at a concentration of 1 $^{mg}/_{ml}$ to

reduce the resulting Schiff bases. Wash with PBS and store in 0.05 M saline solution at 4°C.

A.32 Pepsin immobilization onto Al_2O_3

Take 1 g Al_2O_3 and wash exhaustively with 250 mM sodium acetate pH 4.5, containing 200 mM NaCl. Dissolve 5.5 mg pepsin in 5 ml 250 mM sodium acetate pH 4.5, containing 200 mM NaCl. Suspend the washed Al_2O_3 in this solution. Incubate over night at room temperature using a magnetic stirrer to mix.
The suspension is washed by centrifugation at 2000 rpm. Decant the supernatant and add a new 10 ml portion 250 mM sodium acetate pH 4.5, containing 200 mM NaCl. Repeat this washing procedure three times. Test each supernatant for protein using Bio-Rad™ protein assay. The washed Al_2O_3 is air dried over night at room temperature. Store at 4°C.

A.33 Lipoic acid immobilization on glass powder

Prepare 15 ml 0.1 M sodium phosphate, pH 6.3 . Add 5 ml dioxane. Dissolve 41.2 mg lipoic acid, 43.4 mg sulfo-NHS and 38.4 mg EDC in this solution. React for 30 minutes at room temperature. Adjust pH to 7.5 with 1 M Na_2HPO_4. Add 0.5 g aminosilanised Swarowsky™ glass powder. Incubate over night at room temperature.
Wash the glass powder in a suction filter, pore size 4, with:

- 5 ml DMF, 3X
- 10 ml EtOH / H_2O 1:1, 2X
- 10 ml EtOH, 2X

Dry over night at 80°C.
Prepare 3 ml 50 $^{mg}/_{ml}$ $NaBH_4$ in H_2O. React for 30 minutes at room temperature. Wash the glass powder in a suction filter, pore size 4, with:

- 10 ml 0.1 M acetic acid, 2X
- 10 ml H_2O, 2X
- 10 ml EtOH, 2X

Dry over night at room temperature.

A.34 Affinity chromatography of antiserum from rabbit 53

Apply 50 μl antiserum from rabbit 53 on a Sephadex™ G25 desalting column (see exp. A.14). Collect 250 μl fractions and monitor for protein using Bio-Rad™ protein assay as described in experiment A.18. Pool the protein containing fractions and apply them on a Ni^{2+} chelate affinity column (see experiment A.18) at a flow rate of 0.3 $^{ml}/_{min}$. Wash with 0.05 M sodium phosphate, pH 7, containing 0.1 M NaCl, until no more protein is eluted (use Bio-Rad™ protein assay for this purpose).
Elute the IgG fraction with 0.05 M sodium phosphate, pH 7, containing 0.1 M NaCl and 0.01 M EDTA. Monitor for protein with Bio-Rad™ protein assay and pool positive fractions. Store at -20°C.

A.35 PLGA on glass powder with EDC

Dissolve 600 mg PLGA in 25 ml DMSO. Prepare 15 mg EDC and 15 mg sulfo-NHS in 1 ml H_2O. Mix the two solutions and react for ten minutes at room temperature. Add 2 g aminosilanised glass powder. Shake the suspension for one hour at room temperature. Wash the glass powder in a suction filter, pore size 4, with:

- 10 ml 0.1 M DMSO, 4X
- 10 ml H_2O/DMSO 1:1 , 2X
- 10 ml H_2O, 4X

Dry over night at room temperature.

A.36 PLGA on AP

Prepare 32 μl PLGA in DMSO at a concentration of 1 $^{mg}/_{ml}$. Add 0.1 mg EDC and activate for 30 minutes at room temperature.
Dilute 50 μl AP stock solution (9 $^{mg}/_{ml}$) to 500 μl with 0.1 M sodium phosphate pH 9. Add the activated PLGA solution and react over night at 4°C. The conjugate is purified over a Sephadex™ G25 desalting column (see exp. A.14). Collect 250 μl fractions and monitor for protein using Bio-Rad™ protein assay as described in experiment A.18. Pool the fractions containing the enzyme and store at 4°C.

A.37 Ni^{2+} chelate pure antiserum from rabbit 53 on glass with glutaraldehyde

Activate aminosilanised cover glasses in a 2.5% glutaraldehyde solution in 0.05 M PBS at pH 7 for one hour at room temperature. Wash with ice/H_2O. Dilute 2.5 μl purified antiserum from experiment A.34 to 200 μl with 0.05 M sodium phosphate pH 7 . Add a 200 μl portion to each cover glass and incubate for one hour at room temperature. Wash with 0.05 M sodium phosphate pH 7 and store at 4°C.

A.38 Ni^{2+} chelate pure antiserum from rabbit 53 on glass powder with sulfo-SMCC

Dilute 250 μl purified antiserum from experiment A.34 to 500 μl with PBS containing 0.15 M NaCl, 10 mM EDTA, pH 7.2. Add 1 mg 2-mercaptoethylamine and mix to dissolve. React for 90 min at 37°C.
The aminosilanised glass powder (0.6 g) is activated in 2 ml 0.1 M phosphate buffer containing 0.15 M NaCl, 1 $^{mg}/_{ml}$ sulfo-SMCC, pH 7.2. Allow to react for 30 min at room temperature. Wash with PBS and store wet until the antibodies are ready for coupling.
The reduced Ni^{2+} chelate pure antibodies are purified from excess mercaptoethylamine by gel filtration over Sephadex™ G25 using the column from exp. A.14. Elute with 0.1 M PBS containing 0.15 M NaCl, 0.01 M EDTA, pH 7.2 at a flow rate of 1 $^{ml}/_{min}$. Collect 250 μl fractions and monitor for the antibody using Bio-Rad™ protein assay as described in experiment A.18. Pool the antibody containing fractions, dilute to the desired volume with PBS and add the solution immediately to the previously activated glass powder. Incubate over night at 4°C. Wash with PBS and store in 0.05 M saline solution at 4°C.

Appendix B

Experimental Data and Regression Lines

Table B.1: EtOH-silanised cover glass; AP immobilized with GA

Time (s)	Extinction
20	0.045
45	0.064
70	0.078
100	0.103
130	0.122
160	0.143
190	0.163
220	0.180
250	0.208

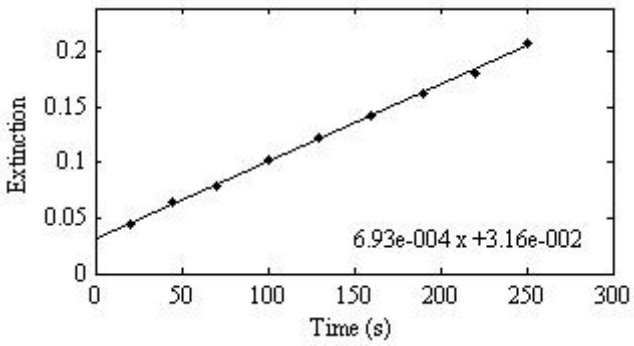


Table B.2: 0.5 M HCl - etched 316-L; AP immobilized with GA

Time (s)	Extinction
60	0.023
100	0.045
150	0.078
185	0.096
240	0.130

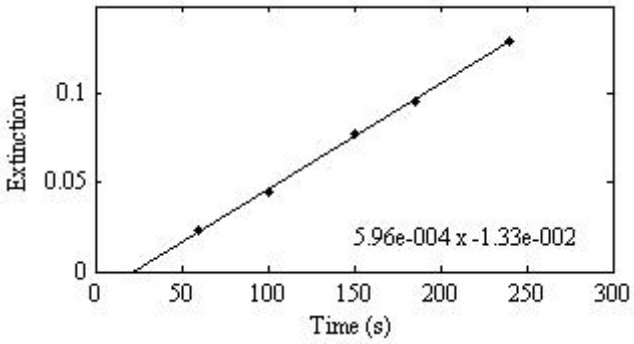


Table B.3: 6 M HCl - etched 316-L; AP immobilized with GA

Time (s)	Extinction
120	0.003
185	0.030
240	0.055
275	0.063
310	0.076

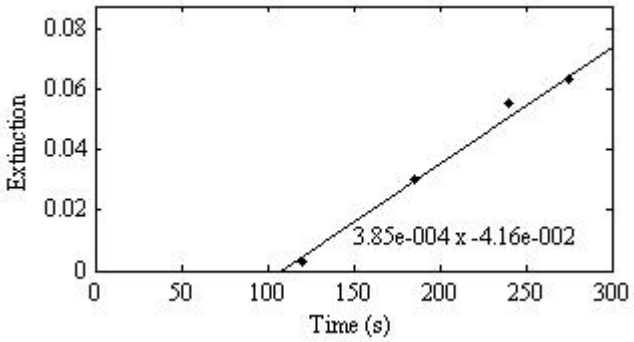


Table B.4: 3 % HNO_3 - etched 316-L; AP immobilized with GA

Time (s)	Extinction
43	0.030
80	0.050
121	0.072
165	0.096
210	0.118
247	0.139
285	0.164

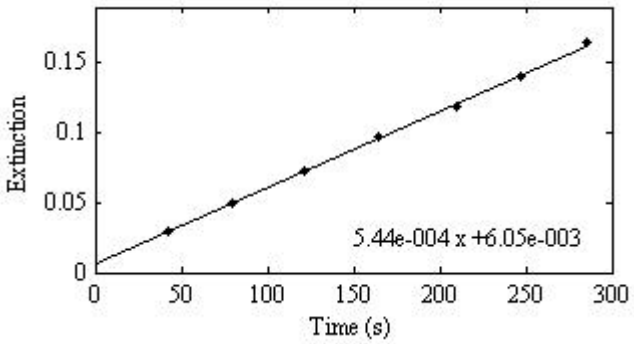


Table B.5: not etched 316-L; AP immobilized with GA

Time (s)	Extinction
45	0.023
85	0.023
125	0.026
155	0.028
195	0.036
240	0.037
275	0.038

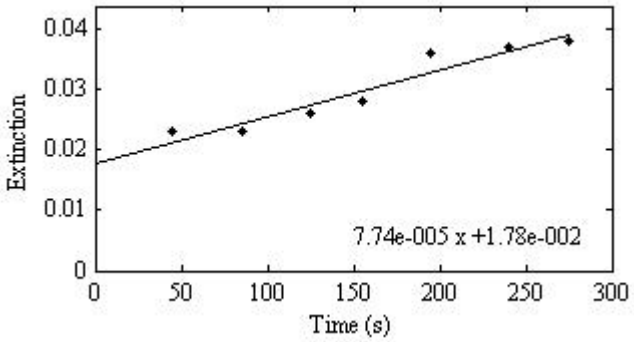


Table B.6: Sample B.1; repeated measurement

Time (s)	Extinction
35	0.059
63	0.067
92	0.084
127	0.119
173	0.140
206	0.156
247	0.185

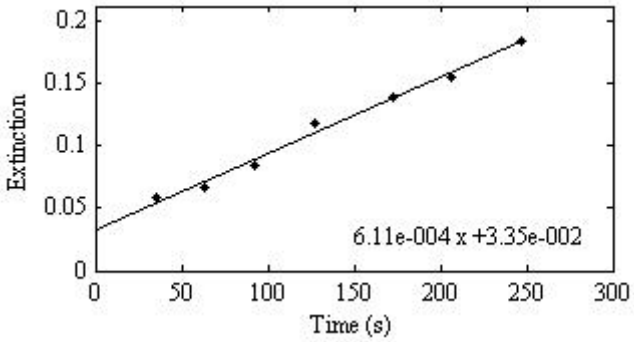


Table B.7: 0.56 ng/ml AP Standard

Time (s)	Extinction
35	0.001
66	0.007
102	0.012
147	0.017
182	0.023
227	0.033

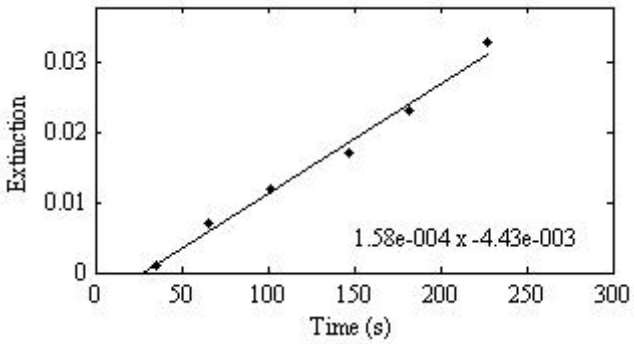


Table B.8: 1.12 ng/ml AP Standard

Time (s)	Extinction
37	0.030
62	0.044
103	0.060
140	0.076
180	0.096
219	0.113
248	0.123

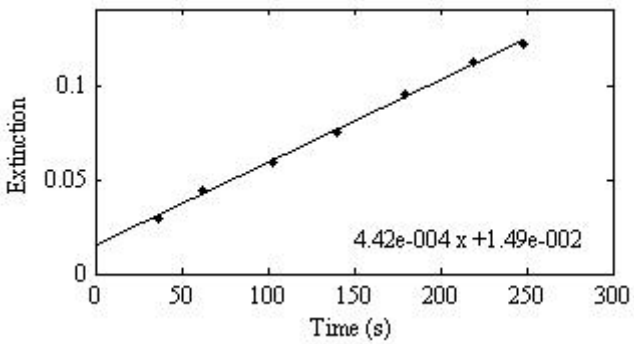


Table B.9: 2.24 ng/ml AP Standard

Time (s)	Extinction
41	0.035
70	0.055
112	0.082
156	0.111
187	0.131
223	0.150
253	0.164

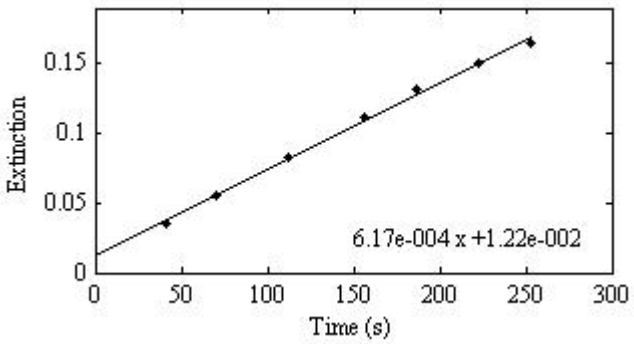


Table B.10: 4.49 ng/ml AP Standard

Time (s)	Extinction
37	0.052
74	0.109
101	0.143
131	0.179
170	0.222
211	0.267
248	0.307

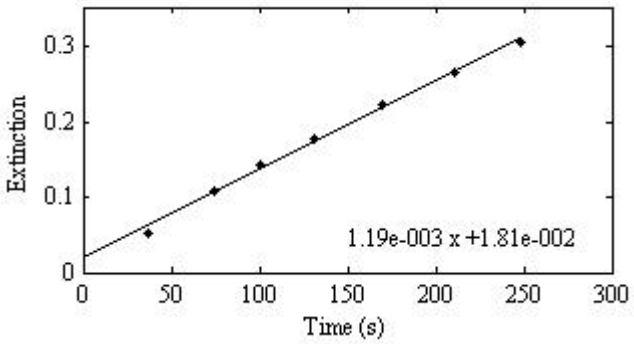


Table B.11: 0.5 M HCl - etched 316-L; PEG immobilized with p-chloranil; AP immobilized with p-chloranil

Time (s)	Extinction
87	0.024
127	0.029
158	0.033
208	0.037
247	0.045
299	0.051
334	0.055

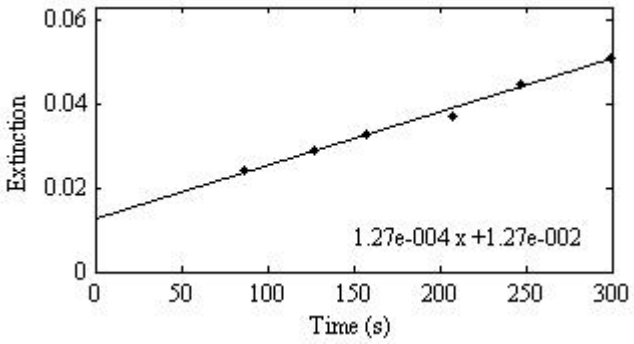


Table B.12: 0.5 M HCl - etched 316-L; PEG immobilized with EDC, pH 5; AP immobilized with GA

Time (s)	Extinction
43	0.024
75	0.031
108	0.039
140	0.047
170	0.055
206	0.063
237	0.071

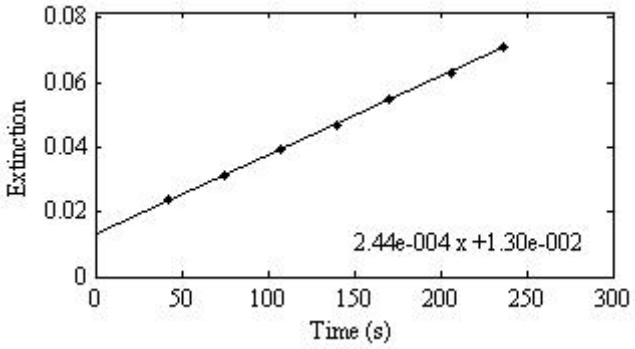


Table B.13: 6 M HCl - etched 316-L; PEG immobilized with EDC, pH 5; AP immobilized with GA

Time (s)	Extinction
41	0.021
80	0.032
111	0.038
155	0.047
185	0.055
227	0.065
253	0.073

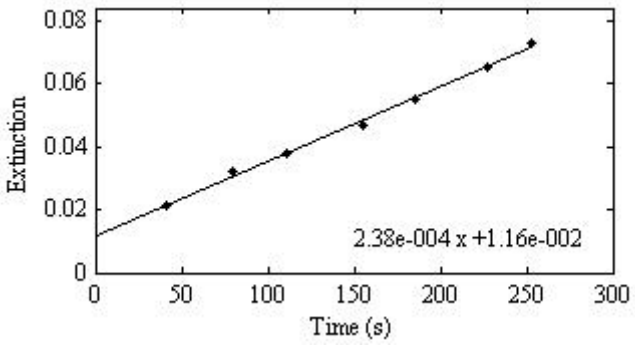


Table B.14: 3 % HNO_3 - etched 316-L; PEG immobilized with EDC, pH 5; AP immobilized with GA

Time (s)	Extinction
32	0.015
71	0.024
117	0.038
169	0.049
209	0.058
273	0.072

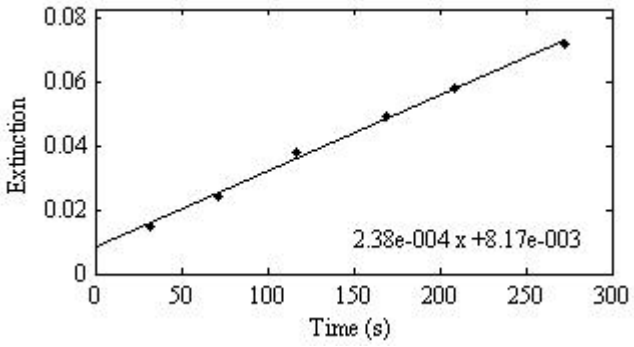


Table B.15: 0.5 M HCl - etched 316-L; PEG immobilized with EDC, pH 10; AP immobilized with GA

Time (s)	Extinction
42	0.025
75	0.03
110	0.037
156	0.048
193	0.059
224	0.067

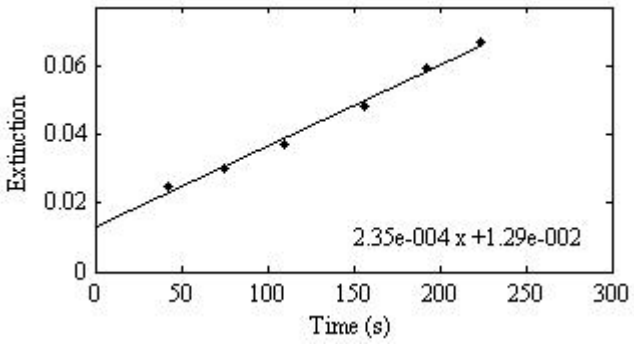


Table B.16: 6 M HCl - etched 316-L; PEG immobilized with EDC, pH 10; AP immobilized with GA

Time (s)	Extinction
34	0.020
69	0.027
100	0.035
144	0.045
180	0.053
219	0.063
250	0.069

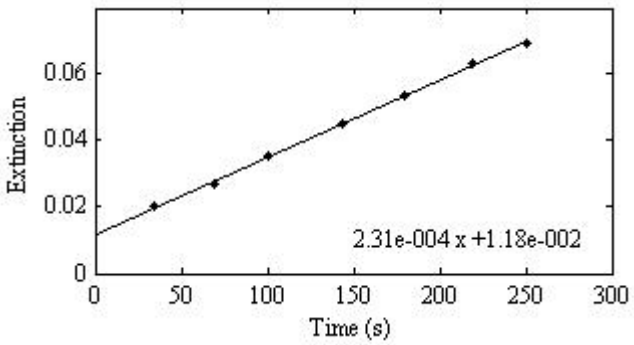


Table B.17: 3 % HNO_3 - etched 316-L; PEG immobilized with EDC, pH 10; AP immobilized with GA

Time (s)	Extinction
41	0.020
78	0.030
119	0.039
156	0.048
186	0.054
218	0.061
247	0.069

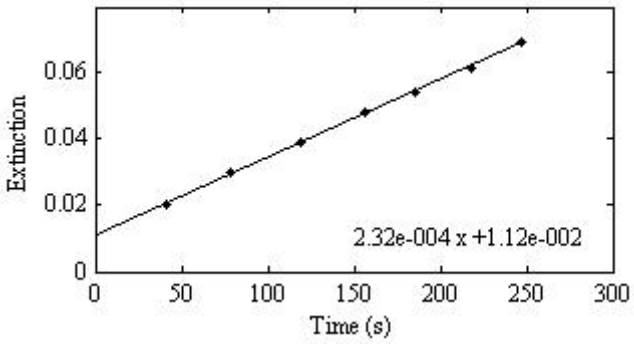


Table B.18: ZrO_2; AP immobilized with GA

Time (s)	Extinction
28	0.020
64	0.029
94	0.034
142	0.044
183	0.053
216	0.061
246	0.067

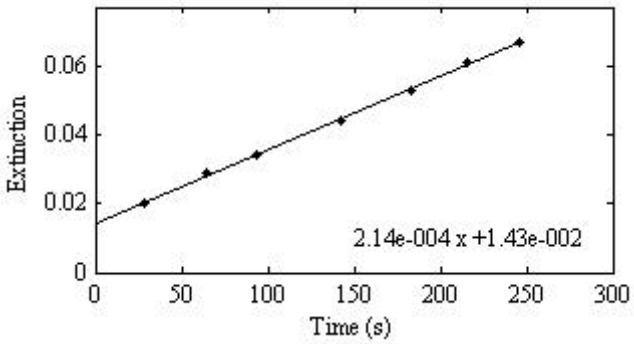


Table B.19: Blank sample; 0.5 M HCl - etched 316-L; not silanised; PEG immobilised with EDC, pH 5; AP immobilised with GA

Time (s)	Extinction
26	0.023
64	0.025
105	0.024
184	0.025
213	0.024

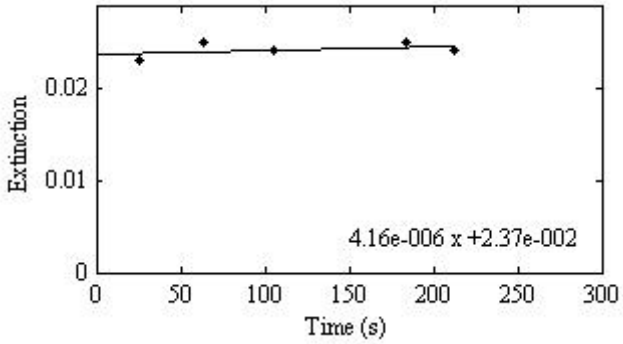


Table B.20: 3 % HNO_3 - etched 316-L; PEG immobilized with EDC, pH 5; AP immobilized with EDC, pH 5

Time (s)	Extinction
34	0.022
68	0.033
101	0.047
134	0.058
181	0.073
226	0.090

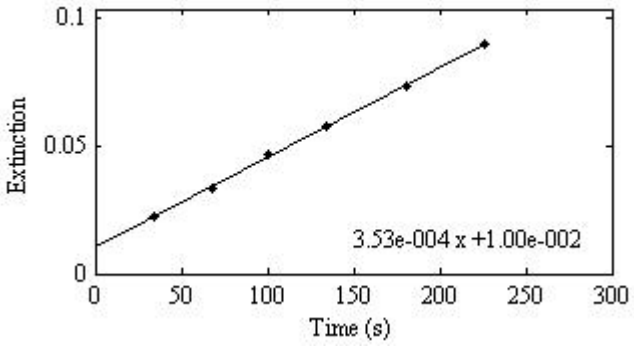


Table B.21: 3 % HNO_3 - etched 316-L; PEG immobilized with EDC, pH 10; AP immobilized with EDC, pH 10

Time (s)	Extinction
32	0.018
76	0.025
115	0.034
159	0.041
200	0.050
240	0.059

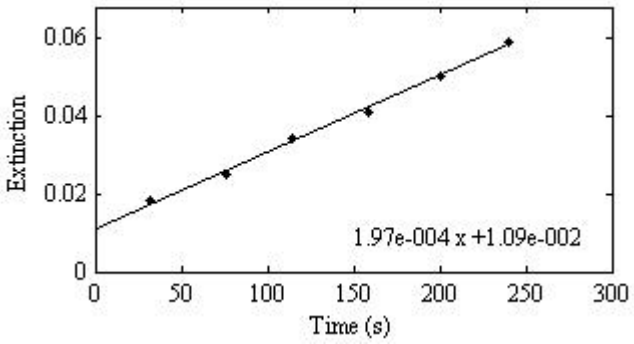


Table B.22: 0.5 M HCl - etched 316-L; PEG immobilized with EDC, pH 5; AP immobilized with EDC, pH 5

Time (s)	Extinction
48	0.032
95	0.060
142	0.086
187	0.104
242	0.126
264	0.150

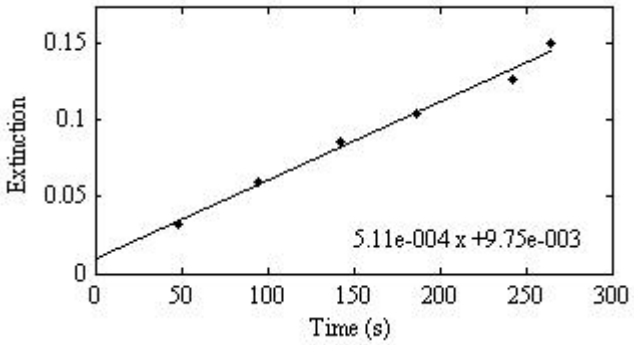


Table B.23: 0.5 M HCl - etched 316-L; PEG immobilized with EDC, pH 10; AP immobilized with EDC, pH 10

Time (s)	Extinction
59	0.022
102	0.032
133	0.044
182	0.052
224	0.063
254	0.072

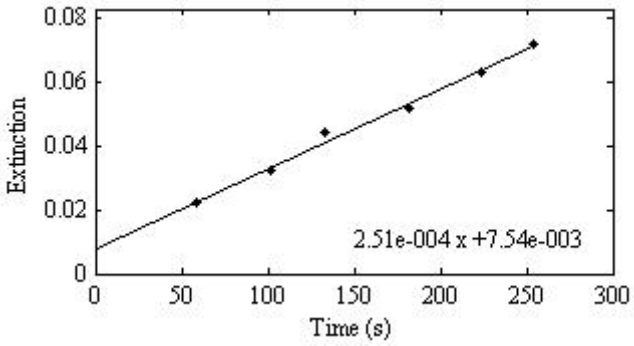


Table B.24: 3 % HNO_3 - etched 316-L; AP immobilized with EDC, pH 5; without immobilized PEG

Time (s)	Extinction
27	0.031
61	0.063
96	0.095
124	0.118
153	0.143
186	0.172
223	0.201
250	0.224

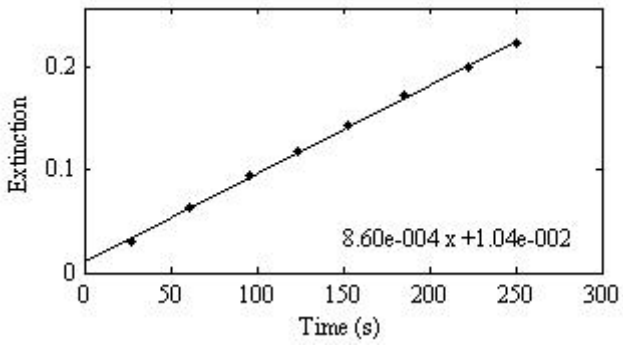


Table B.25: Biotinylated AP on avidin coated cover glass

Time (s)	Extinction
35	0.106
68	0.214
97	0.294
127	0.369
166	0.459
198	0.518

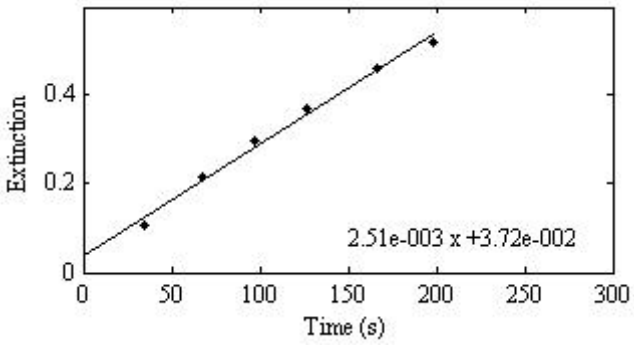


Table B.26: Biotinylated AP on biotinylated cover glass; sample 1

Time (s)	Extinction
33	0.039
62	0.045
91	0.044
136	0.064
186	0.076

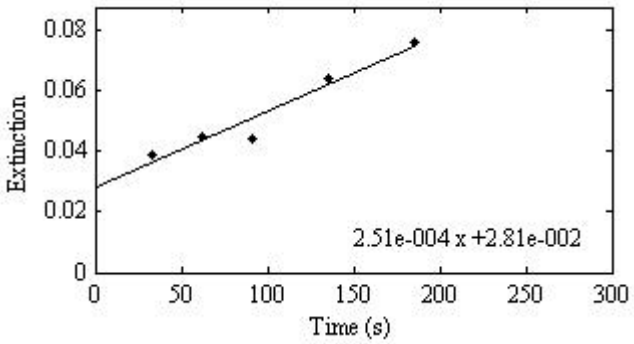


Table B.27: Biotinylated AP on biotinylated cover glass; sample 2

Time (s)	Extinction
30	0.033
72	0.049
103	0.056
140	0.074
172	0.084

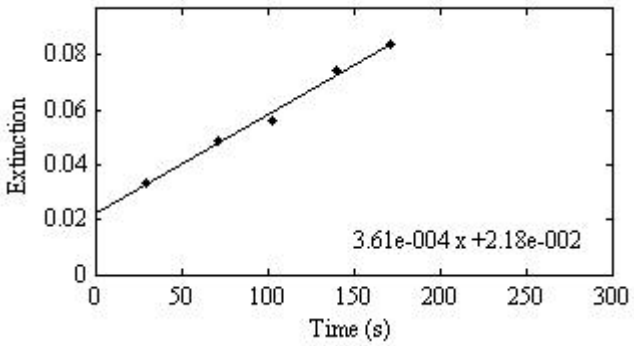


Table B.28: αHRP on cover glass with GA, incubated with AP; blank 1

Time (s)	Extinction
23	0.054
56	0.054
88	0.054
118	0.055
150	0.055

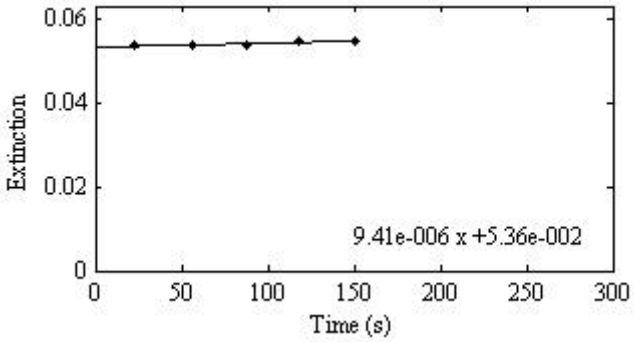


Table B.29: BSA on cover glass with GA, incubated with AP-HRP; blank 2

Time (s)	Extinction
15	0.052
41	0.053
68	0.053
110	0.053
142	0.052
190	0.052

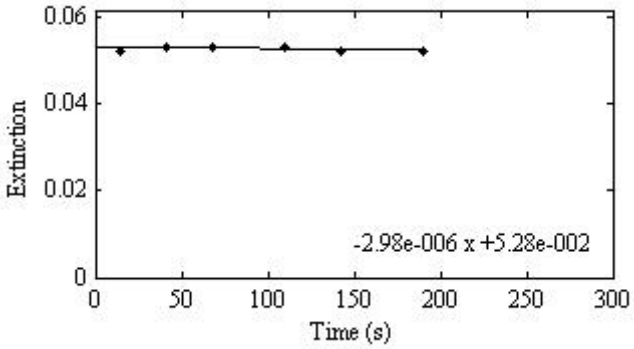


Table B.30: αHRP on cover glass with GA, incubated with AP-HRP; sample 1

Time (s)	Extinction
28	0.057
64	0.058
97	0.061
156	0.066
203	0.072
241	0.076
280	0.079

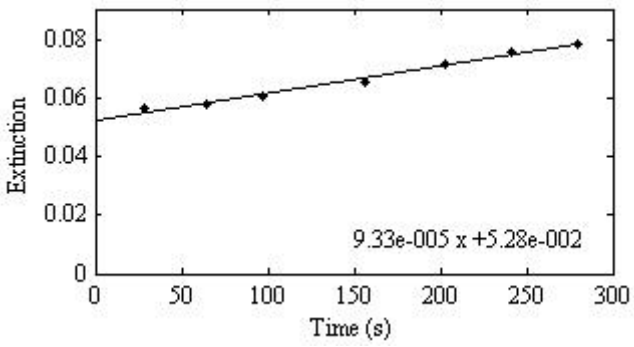


Table B.31: αHRP on cover glass with GA, incubated with AP-HRP; sample 2

Time (s)	Extinction
14	0.060
44	0.064
84	0.066
130	0.073
177	0.081
219	0.091
280	0.096
313	0.101

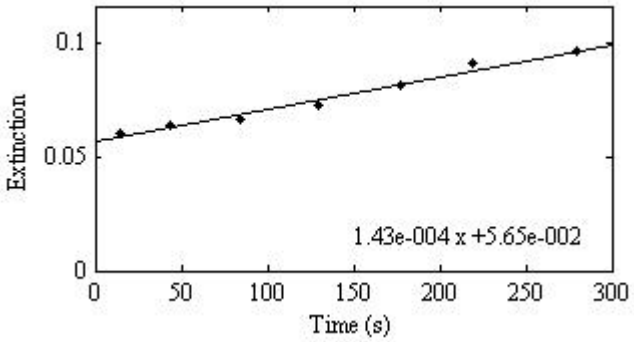


Table B.32: αHRP on cover glass with GA, incubated with AP-HRP; sample 3

Time (s)	Extinction
0	0.109
27	0.113
61	0.120
90	0.122
128	0.130
158	0.133
190	0.141
230	0.148
262	0.151

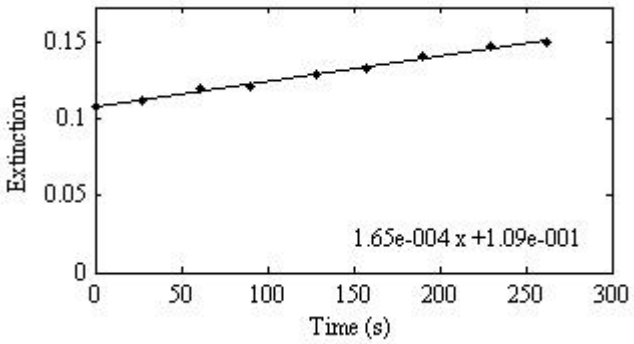


Table B.33: αHRP on cover glass with GA, incubated with AP-HRP; sample 4

Time (s)	Extinction
14	0.055
47	0.062
73	0.067
103	0.072
134	0.080
168	0.083
208	0.089
247	0.096

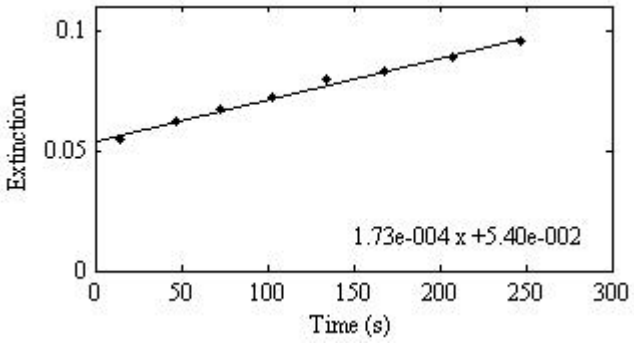


Table B.34: αHRP on cover glass with GA, incubated with AP-HRP; repeated measurement sample 1 (B.30)

Time (s)	Extinction
20	0.028
59	0.029
95	0.030
132	0.033
179	0.035
225	0.038
278	0.043
307	0.045

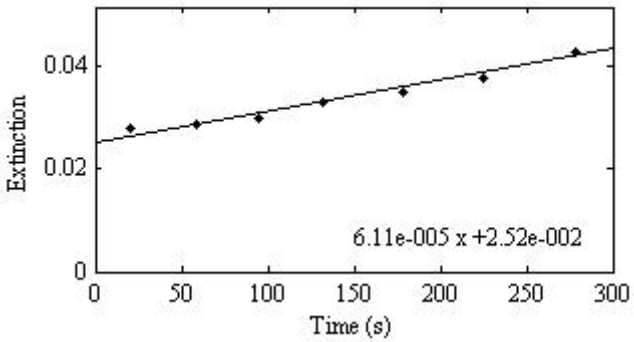


Table B.35: αHRP on cover glass with GA, incubated with AP-HRP; repeated measurement sample 2 (B.31)

Time (s)	Extinction
24	0.028
56	0.035
106	0.036
157	0.039
260	0.050
298	0.051
395	0.056

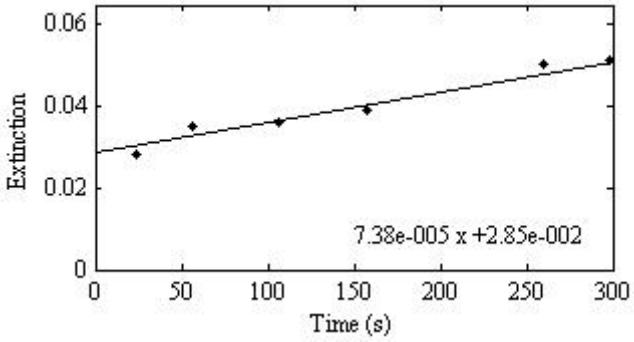


Table B.36: αHRP on cover glass with GA, incubated with AP-HRP; repeated measurement sample 3 (B.32)

Time (s)	Extinction
21	0.031
52	0.033
93	0.037
125	0.040
160	0.047
200	0.046
246	0.048
281	0.052

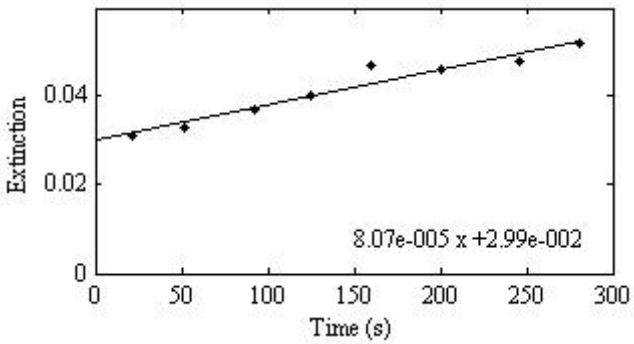


Table B.37: αHRP on cover glass with GA, incubated with AP-HRP; repeated measurement sample 4 (B.33)

Time (s)	Extinction
18	0.027
55	0.027
90	0.029
130	0.031
170	0.032
206	0.034
240	0.035
280	0.041
314	0.043

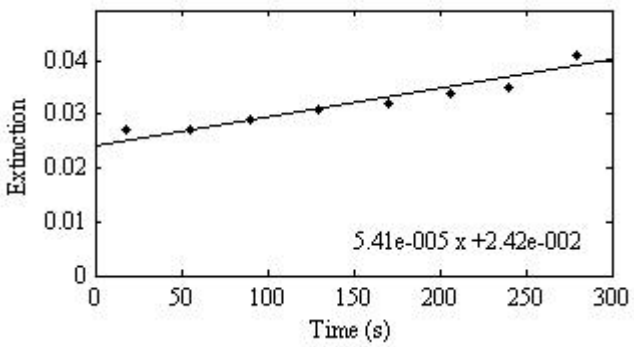


Table B.38: αHRP on cover glass with GA, incubated with AP-HRP; sample 1

Time (s)	Extinction
72	0.031
106	0.037
142	0.043
175	0.048
217	0.051
260	0.063
300	0.065

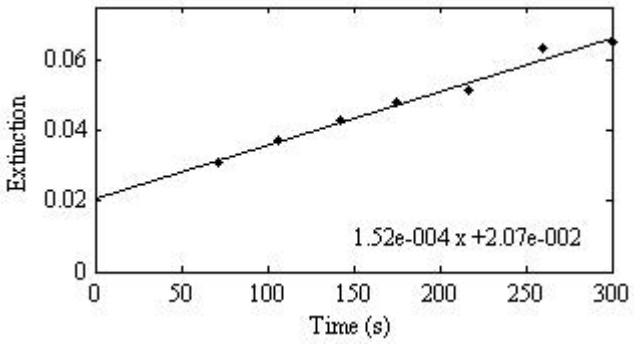


Table B.39: αHRP on cover glass with GA, incubated with AP-HRP; sample 2

Time (s)	Extinction
54	0.033
86	0.041
124	0.044
158	0.045
190	0.048
234	0.052
260	0.054

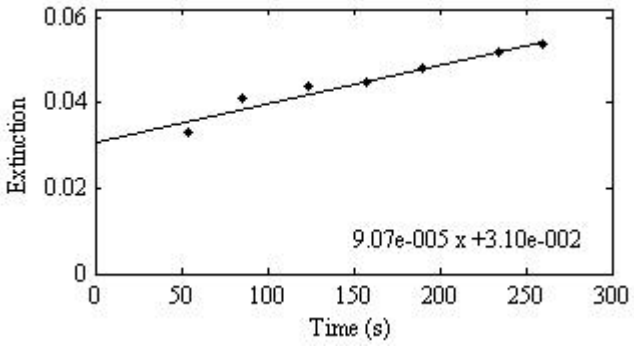


Table B.40: αHRP on cover glass with GA, incubated with AP-HRP; sample 3

Time (s)	Extinction
44	0.044
76	0.047
117	0.060
151	0.066
190	0.077
223	0.089
264	0.100
301	0.106

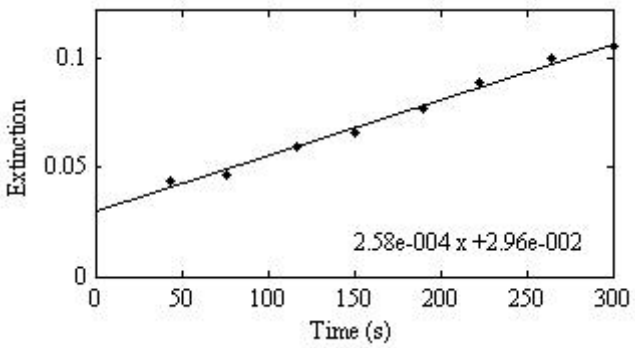


Table B.41: αHRP on cover glass with GA, incubated with AP-HRP; sample 4

Time (s)	Extinction
46	0.044
80	0.048
110	0.052
160	0.067
213	0.077
250	0.090

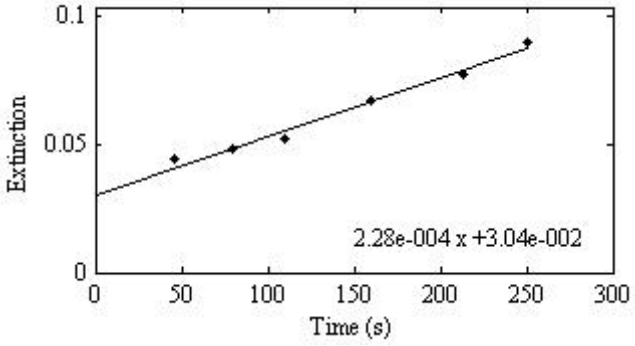


Table B.42: αHRP on cover glass with GA, incubated with AP; blank 1

Time (s)	Extinction
51	0.037
130	0.038
154	0.036
206	0.040
240	0.039
280	0.041

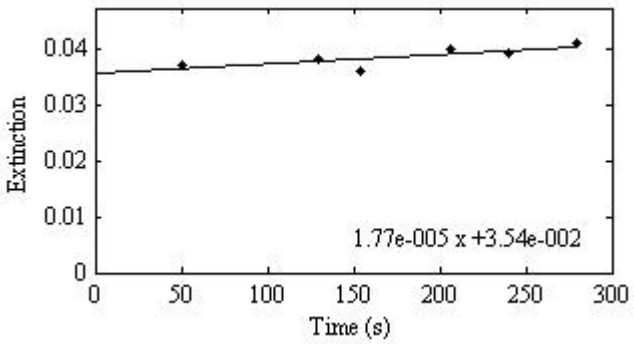


Table B.43: αHRP on cover glass with GA, incubated with AP; blank 2

Time (s)	Extinction
56	0.031
92	0.031
130	0.032
184	0.033

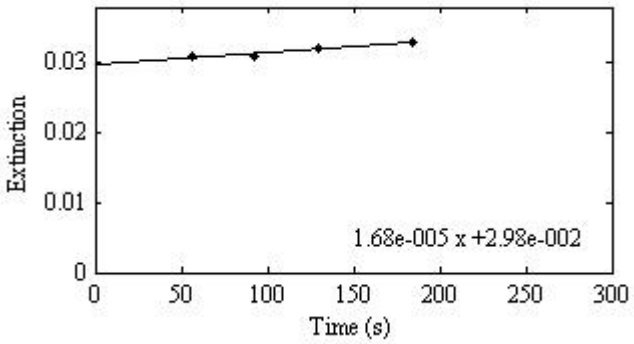


Table B.44: αHRP on cover glass with GA, incubated with AP-HRP; repeated measurement sample 1 (B.38)

Time (s)	Extinction
39	0.060
88	0.063
128	0.067
168	0.071
208	0.076
241	0.079
263	0.080

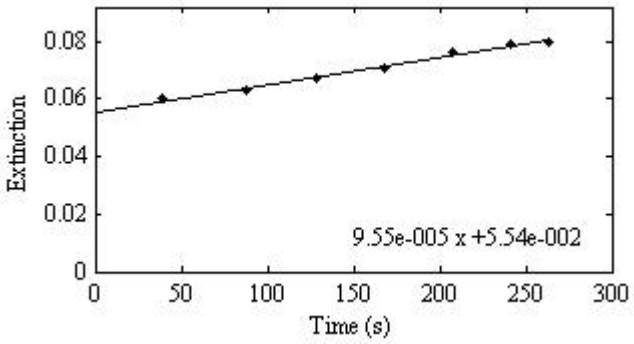


Table B.45: αHRP on cover glass with GA, incubated with AP-HRP; repeated measurement sample 3 (B.40)

Time (s)	Extinction
30	0.042
77	0.052
160	0.056
197	0.059

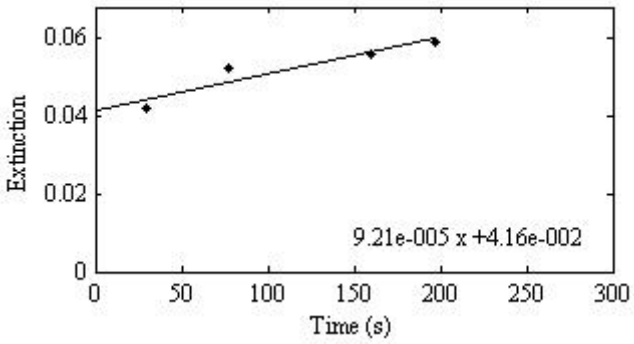


Table B.46: αHRP on cover glass with GA, incubated with AP-HRP; repeated measurement sample 4 (B.41)

Time (s)	Extinction
35	0.049
70	0.055
121	0.065
147	0.073
200	0.080
242	0.090
264	0.093

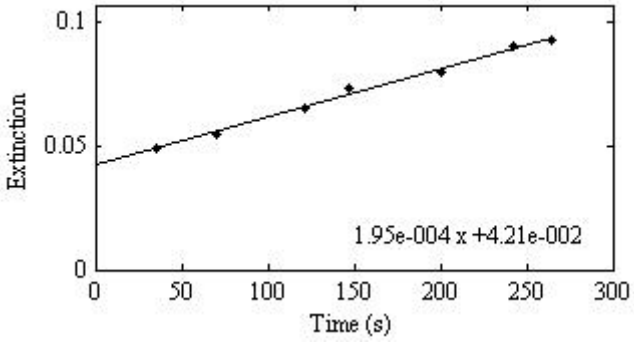


Table B.47: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; sample 1

Time (s)	Extinction
33	0.050
72	0.067
107	0.072
147	0.082
189	0.104
231	0.110

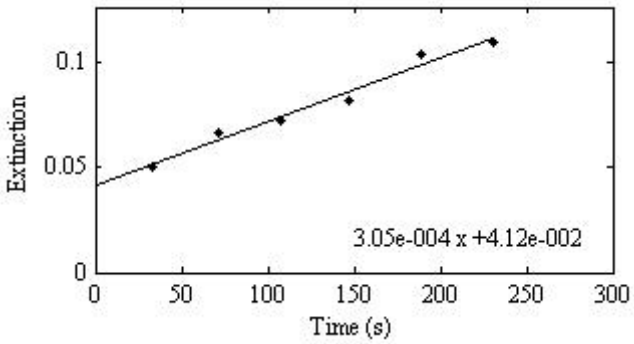


Table B.48: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; sample 2

Time (s)	Extinction
41	0.049
75	0.057
109	0.065
185	0.088
220	0.095
257	0.106

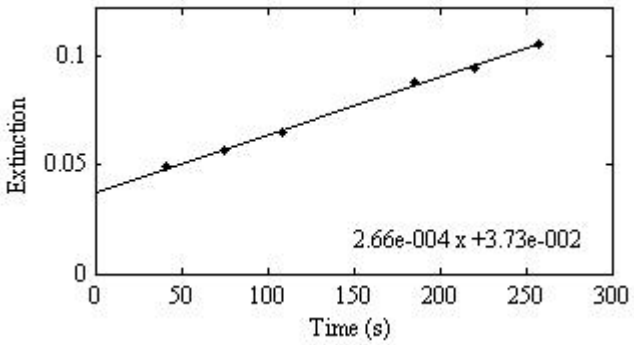


Table B.49: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; sample 3

Time (s)	Extinction
38	0.035
77	0.039
144	0.039
187	0.042
230	0.043

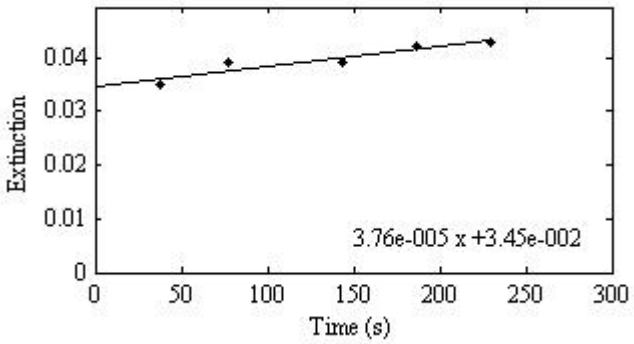


Table B.50: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; sample 4

Time (s)	Extinction
34	0.044
76	0.060
114	0.063
153	0.075
187	0.083
218	0.092
248	0.098

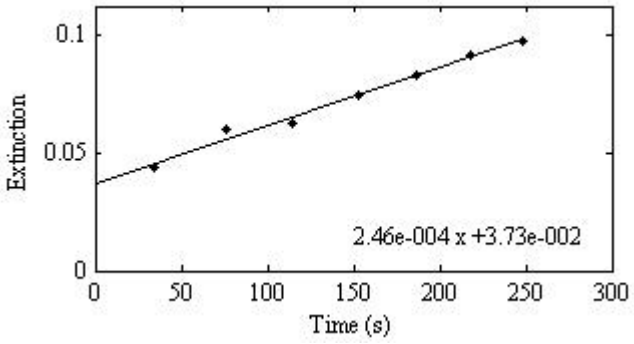


Table B.51: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; sample 5

Time (s)	Extinction
68	0.050
101	0.053
139	0.058
187	0.063
224	0.069
270	0.083

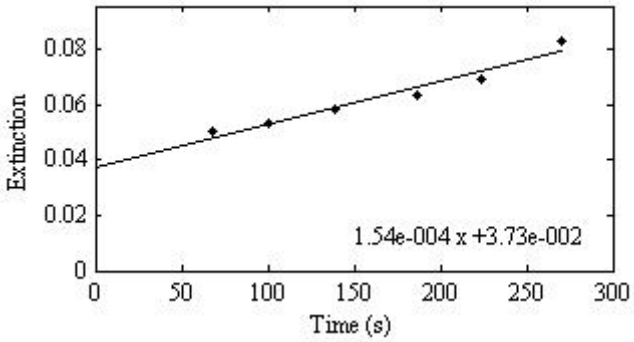


Table B.52: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; sample 6

Time (s)	Extinction
41	0.045
79	0.052
113	0.060
170	0.072
225	0.076
271	0.083

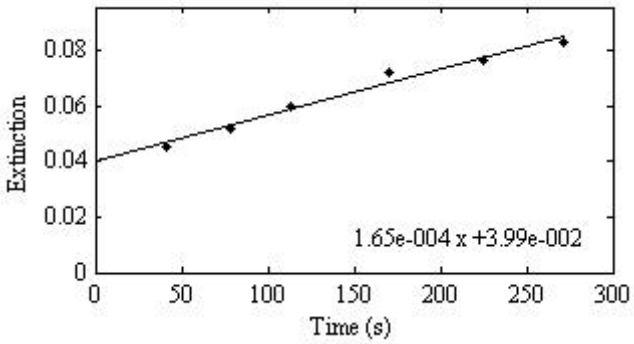


Table B.53: αHRP fragment on glass with sulfo-SMCC, incubated with AP; blank

Time (s)	Extinction
29	0.034
75	0.032
117	0.033
159	0.035
214	0.032

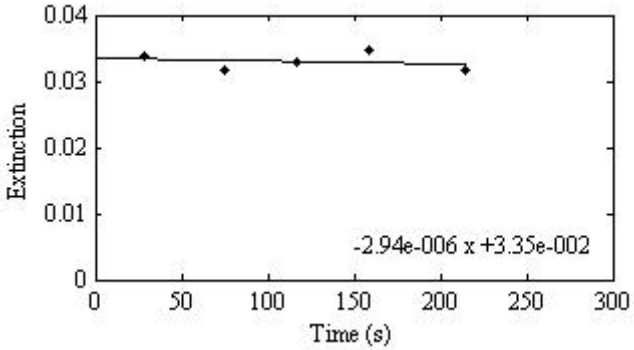


Table B.54: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; repeated measurement sample 1 (B.47)

Time (s)	Extinction
18	0.038
60	0.040
100	0.043
137	0.049
176	0.052

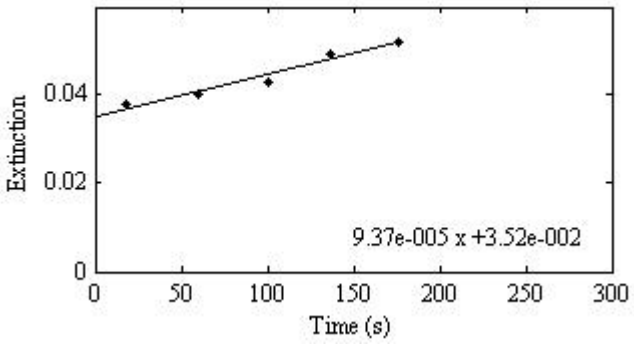


Table B.55: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; repeated measurement sample 2 (B.48)

Time (s)	Extinction
30	0.032
65	0.036
95	0.040
141	0.044
180	0.052
223	0.056

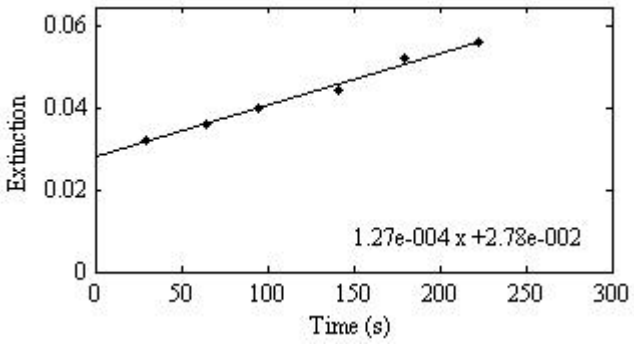


Table B.56: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; repeated measurement sample 3 (B.49)

Time (s)	Extinction
32	0.030
72	0.029
106	0.030
140	0.032

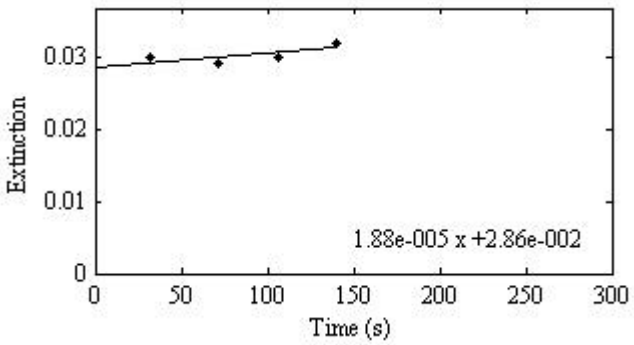


Table B.57: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; repeated measurement sample 4 (B.50)

Time (s)	Extinction
30	0.032
67	0.037
103	0.041
148	0.047
230	0.055
251	0.059

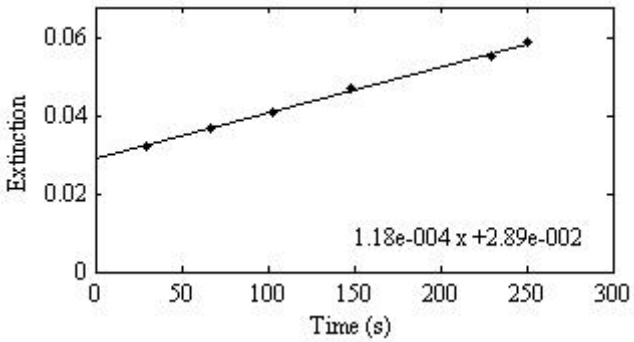


Table B.58: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; repeated measurement sample 5 (B.51)

Time (s)	Extinction
34	0.027
70	0.030
101	0.032
137	0.036
181	0.042
213	0.049
253	0.054

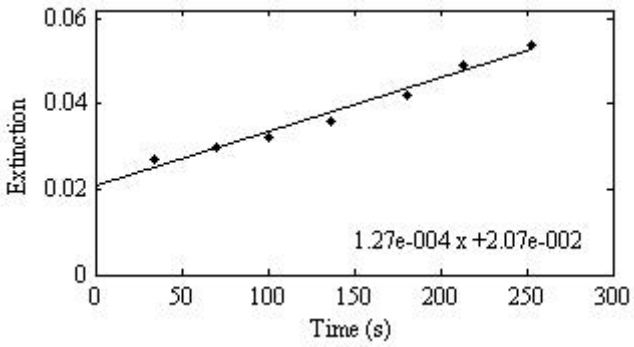


Table B.59: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; repeated measurement sample 6 (B.52)

Time (s)	Extinction
24	0.022
72	0.029
100	0.032
158	0.040
190	0.044
220	0.048
254	0.051

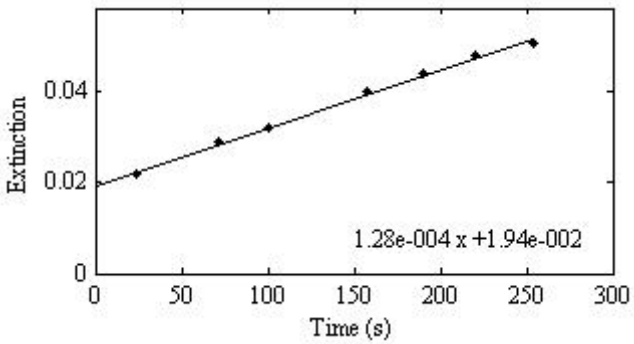


Table B.60: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; repeated measurement sample 1 (B.54)

Time (s)	Extinction
29	0.032
71	0.034
110	0.031
150	0.032
190	0.033

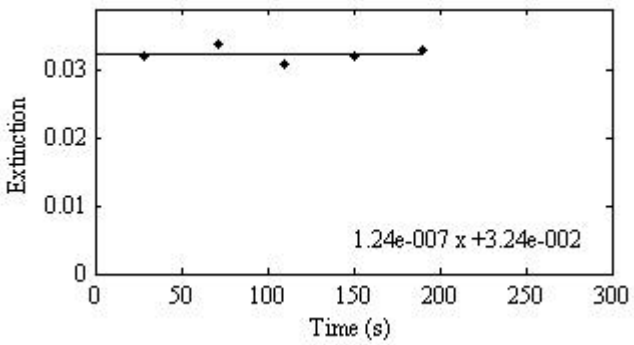


Table B.61: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; repeated measurement sample 2 (B.55)

Time (s)	Extinction
36	0.030
76	0.031
114	0.032
160	0.032
202	0.034
258	0.037
310	0.038
350	0.042
400	0.044

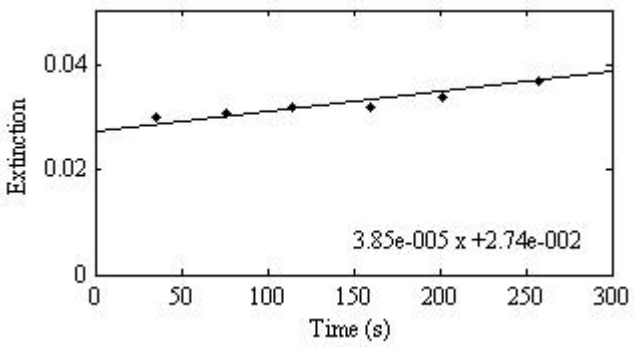


Table B.62: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; repeated measurement sample 3 (B.56)

Time (s)	Extinction
40	0.032
80	0.032
121	0.026
150	0.027

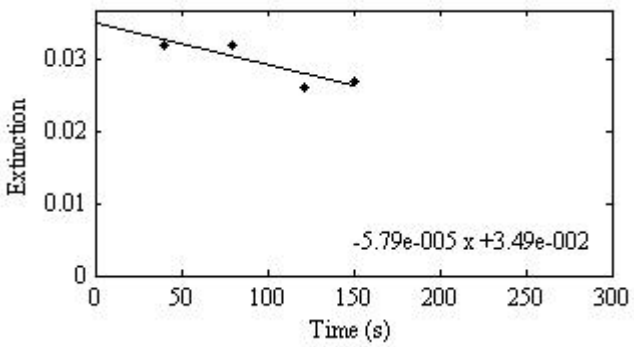


Table B.63: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; repeated measurement sample 4 (B.57)

Time (s)	Extinction
30	0.030
67	0.033
110	0.031
159	0.032
212	0.035
280	0.035
360	0.038
426	0.042

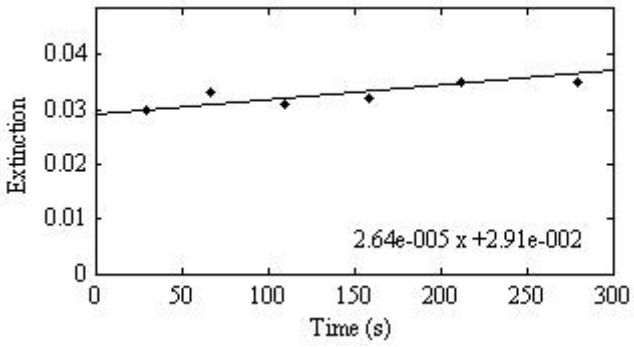


Table B.64: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; repeated measurement sample 5 (B.58)

Time (s)	Extinction
30	0.030
95	0.034
143	0.038
200	0.040
247	0.042
295	0.044

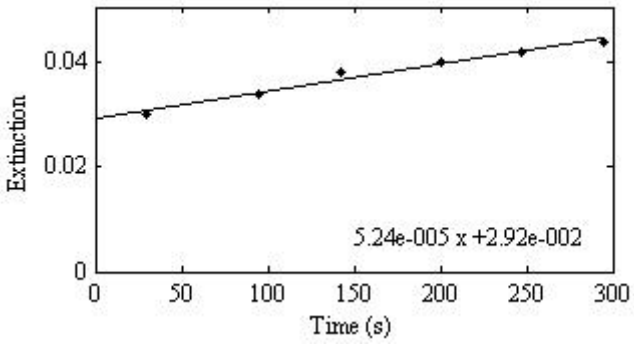
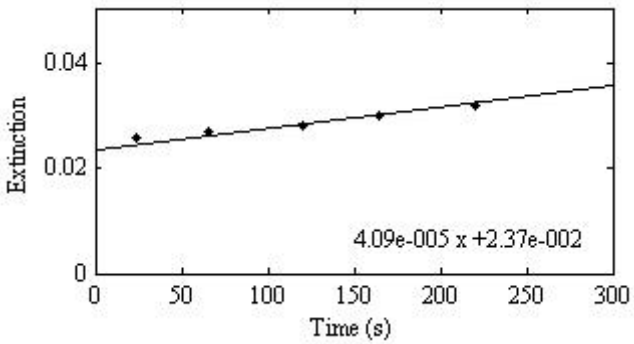


Table B.65: αHRP fragment on glass with s-SMCC, incubated with AP-HRP; repeated measurement sample 6 (B.59)

Time (s)	Extinction
24	0.026
66	0.027
120	0.028
164	0.030
220	0.032
330	0.034
380	0.040
423	0.042
466	0.044



Abbreviations

316-L	surgical stainless steel
ADCC	Antibody dependent cell mediated cytotoxicity
αHRP	Anti HRP antibody
AP	alkaline phosphatase
BAPA	Bacillus anthracis protective antigen
Biocytin	ϵ-N-(+)-Biotinyl-L-lysine
BSA	Bovine Serum Albumin
CD	Cluster of differentiation
CD3	Part of the T cell receptor complex
CD20	Phosphoprotein expressed on the surface of mature B-cells
CD33	Trans membrane receptor expressed on cells of monocytic myeloid lineage
CD147	Basignin (EMMPRIN), extracellular matrix metalloproteinase inducer
CDR	Complementary Determining Region
CMV	Cytomegalovirus
CSGP 17-1A	Cell-surface glycoprotein 17-1A
dαg	Donkey anti goat antibody
DMSO	Dimethyl sulfoxide

EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EGFR	Epidermal growth factor receptor
ELISA	Enzyme Linked Immuno Sorbent Assay
Ep-CAM	Epithelial cell adhesion molecule
EPR-effect	enhanced permeability and retention effect
EtOH	Ethanol
FDA	Food and Drug Administration
Fv	Variable fragment of an antibody
GA	Glutaraldehyde
gαm-HRP	Goat anti mouse antibody HRP conjugate
GD2	Disialoganglioside expressed on neuroectodermal tumours
gp-120	Glycoprotein (120 kD) on the surface of the HIV envelope .
HA	Hemagglutinin
HER2	Human epidermal growth factor receptor 2 (CD 340)
HFC	Hollow fibre cartridge
HRP	Horseradish peroxidase
IFA	Incomplete Freund's Adjuvant
IFNγ	Interferon γ
Ig	Immunoglobulin
IGF-IR	Insulin-like growth factor I receptor
IgG	Immunoglobulin G
IL-2R β	Interleukin-2 receptor β-subunit
IL-6	Interleukin-6
IL-6R	Interleukin-6 Receptor

kD	Kilo Dalton
Lewis-Y	Fucosyltransferase 3 (CD174)
M	Molar; $^{mol}/_{L}$
mαHA	Mouse anti HA antibody
mAb	monoclonal antibody
M_r	Molecular weight
MUC-1	Mucin 1, membrane bound glycosylated phosphoprotein (CD227)
NHS	N-Hydroxysuccinimide
OP	Oligopeptide
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
PTHrP	Parathyroid hormone-related protein
PSMA	Prostate specific membrane antigen
RAAG12	N-linked carbohydrate antigen expressed on solid organ cancers
rαm-HRP	Rabbit anti mouse antibody HRP conjugate
scFv	single chain Fv fragment
SELEX	systemic evolution of ligands by exponential enrichment
SMCC	Succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate
s-NHS	N-hydroxysulfosuccinimide
SPION	Super Paramagnetic Iron Oxide Nano Particle
TBS	TRIS buffer saline
TCF	Tissue culture flask

TGF β1	Transforming growth factor β-1
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF α	Tumour necrosis factor α
Tris	Tris(hydroxymethyl)aminomethane
VEGF	Vascular endothelial growth factor

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