Master-Thesis

Development of lateral flow immunoassays for the detection of Dipyrone and Ovomucoid

Submitted by

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Affidavit

I hereby declare that I have authored this master thesis independently, and that I have not used any assistance other than that which is permitted. The work contained herein is my own except where explicitly stated otherwise. All ideas taken in wording or in basic content from unpublished sources or from published literature are duly identified and cited, and the precise references included.

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Vienna, 29.12.2021   Lisa Mayerhuber

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Abstract – English

A Lateral Flow Assay using colloidal gold-labelled polyclonal antibodies was developed for the rapid detection of allergenic food derived proteins and smaller molecules like water contaminants. Ovomucoid, one of the listed food derived proteins is known to cause allergic reactions and was therefore chosen for this work. On the other hand, Dipyrone a common used pain-killer found as a water contaminant was investigated. Polyclonal rabbit and sheep antibodies were chosen for the development of Lateral Flow devices. Rabbit anti-egg yolk antibodies were purified using protein G. Sheep IgG was commercially obtained. The antibodies were applied in ELISA studies to test der performance for later use. Colloidal gold nanoparticles were prepared according to Turkevich and Frens (1951). The purified antibody was conjugated to the colloidal gold and was applied for the signal generation in LFD. For the assay development, a sandwich assay and a competitive assay were chosen. The optimization of LFDs included the overcoming of false-negative signals, which are often a consequence of insufficient antibody conjugation and unspecific binding. Therefore, different blocking agents and nitrocellulose membranes were investigated. Ficoll® 400 and fish gelatine was shown to exhibit good blocking efficiency and therefore reliable results. The assay procedure could be accomplished after 5-10 minutes and the results of the quantitative assay were evaluated visually. A cut-off level for the developed assay of egg allergens could not be determined, therefore a quantitative determination of egg traced cannot be assured. For the development of the competitive Dipyrone assay a cut-off level of 1 ppm with the addition of fish gelatin as blocking substance was achieved. The versatility of this detection format makes these strips an ideal choice for a rapid, and cost-effective on-site technique. Finally, a comprehensive overview of the results is given and an outlook on further optimization aspects.

Abstract – Deutsch

Im Rahmen dieser Arbeit sollten Schnelltests für die Detektion von Ei-Allergenen und Metamizol entwickelt und optimiert werden. Dazu wurden Antikörper an kolloidales Gold gekoppelt welches für die Signalgenerierung in den Streifen-Tests verwendet wurde. Ei-Das Allergene Ovomukoid ist bekannt allergische Reaktionen hervorzurufen und wurde aus diesem Grund für die Überprüfung ausgewählt. Dipyron ist ein Schmerzmittel, welches häufig als Wasserkontaminant nachgewiesen werden konnte. Für beide Analyten wurden polyklonale Antikörper verwendet. Polyklonale Kaninchen-Antikörper wurden mittels Protein G aufgereinigt. Für die Anwendung von Metamizol, wurden Schaf-Antikörper genutzt. Die Performance der Kaninchen-Antikörper wurde in ELISA Tests überprüft. Die kolloidalen Goldnanopartikeln wurde nach dem Protokoll von Turkevich und Frens (1951) hergestellt. Die Antikörper wurden an das kolloidale Gold konjugiert welche später für die Signalerzeugung genutzt wurden. Für die Assay Entwicklung wurde ein Sandwich und ein kompetitiver Assay Format gewählt. Die Optimierung beinhaltet die Vermeidung von falsch positiven Signalen, welche oft durch unvollständige Kopplungsprozesse hervorgerufen werden als auch durch unspezifische Bindungen. Daher wurden verschiedene Blocking Substanzen und Nitrozellulose Membranen untersucht. Ficoll® 400 und Fish Gelatine zeigte gute Blockierungseffizienz und damit zuverlässige Ergebnisse. Der Assay konnte nach 5-10 Minuten ausgelesen und die Ergebnisse quantitativ bewertet werden. Ein Cut-off Wert für den Test auf Eiallergene konnte nicht bestimmt werden, daher kann eine quantitative Bestimmung von Eiallergenen nicht gewährleitet werden. Für die Entwicklung des kompetitiven Dipyron-Assays wurde ein Cut-Off-Wert von 1 ppm bei Zusatz von Fischgelatine als Blocking Substanz erreicht. Die Vielseitigkeit des Assays macht sie zu einer idealen Wahl für eine schnelle, zugverlässige und kostengünstige Technik für den Nachweis verschiedener Analyten.

1 Theoretical Part

The aim of the work was the application of antibodies coupled to colloidal gold used in rapid test systems for the detection of ovomucoid (high molecular mass molecules) and Dipyrone (low molecular mass molecules). Therefore, lateral flow devices using colloidal gold-labelled antibodies were developed for the detection of these analytes.

Point of care (POC) testing has become more popular of diagnosis in clinical analysis, food safety and environment *(Di Nardo et al., 2019)*. It provides fast results in short times. Lateral flow assays are a strategy for rapid on-site detection by qualitative and semi-quantitative analysis and therefore gaining further attention. A LFA is a paper-based platform for the detection of complex analytes (*Koczula and Gallotta, 2016*). Lateral flow assays provide the advantage of relative short analysis time and easy sample preparation. The sample mixture is placed on the device and test-result is displayed within 5-10 minutes. It is also a technology of low-cost, simple, rapid and a portable device, which finds its application in many different areas of science *(Sajid et al., 2015)*.

Before any potential application a LFA requires thorough optimization of many components to make the lateral flow assay work properly. An important aspect is the choice and purification of antibodies. Stable conjugation of antibodies to different labels is a major concern during the development of antibody-based methods, therefore it requires highly purified antibodies. Another point is the synthesis of gold nanoparticles. Gold nanoparticles are metallic nanoparticles that have attracted more and more attention due to their unique optical properties and multiple surface functionalities. They are non-toxic and are therefore attractive to be applied in many different areas *(Jazayeri et al., 2016)*. There are different techniques to prepare gold nanoparticles, the most popular one it the chemical reduction of gold chloride by sodium citrate *(Alzoubi et al., 2015)*. The most common particles used in lateral flow device are 40 nm diameter gold nanoparticles.

In this work different methods were tested to control size and shape of the nanoparticles. These two parameters are major indicators of the quality of those solutions. The gold surface strongly binds antibodies and robust nanoparticle-antibody conjugates are fabricated. Standard lateral flow assays generate an optical signal that results from coloured particles which bind to test and control line on the test strip. This enables an easy optical readout.

1.1 Food Allergens – Egg

Food allergens are naturally occurring proteins in food but not all food proteins are allergens *(Bannon, 2004)*. Food allergies are non-toxic immune reactions to food. They represent an important health issue in developed countries *(Poms et al., 2004)*.

But what causes a food protein to trigger an allergic response. It requires it presence in higher amounts, its resistance for digestion and its durability during food processing *(Bredehorst and David, 2001)*. Food allergies are caused by abnormal immunological responses to specific food or food components. Furthermore, it is important to distinguish between food allergies and food intolerances, such as lactose-, fructose- or histamine intolerance *(Clare Mills et al., 2007)*. While it is easy to control food intolerances by limiting the amount of the offending food, with allergies it often requires to completely restrict it *(Taylor, 2006.)*. Such an allergic reaction can occur within minutes and can range from mild skin irritation to problems with the gastrointestinal tract to an anaphylactic shock. As already mentioned, in the most cases the elicitors of reactions to food are proteins *(Poms et al., 2004)*.

This work focuses especially on egg allergens. Allergens from egg are one of the most frequent causes of food allergic reactions reported to literature. After cow's milk hen's egg is the second most common allergy in childhood, it affects 1-2% children *(Bartnikas et al., 2015)*. Egg allergens have been found in both, white and yolk. The highest amount of allergens are found in egg white, up to 24 different antigenic protein fractions *(Martorell Aragonés et al., 2001)*.
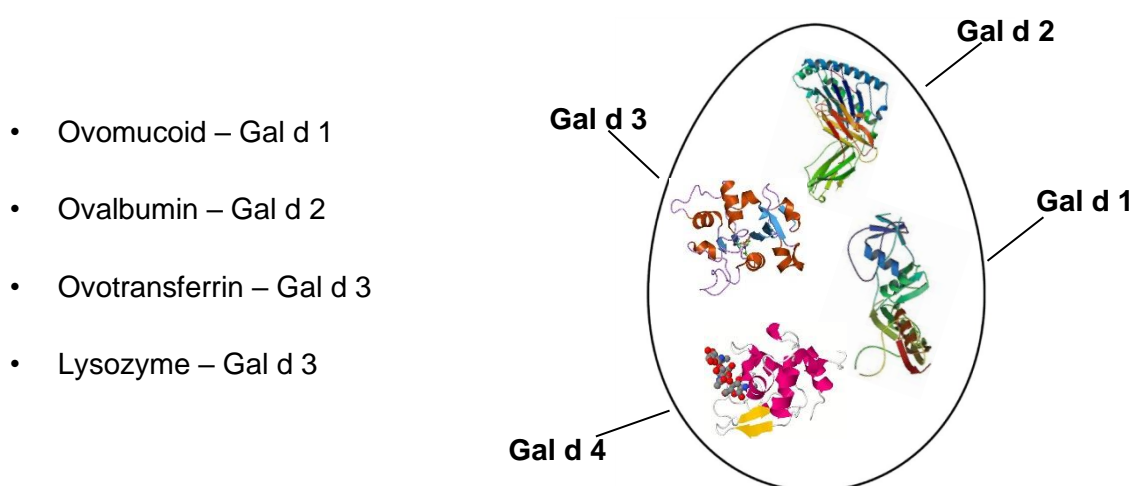
- Ovomucoid – Gal d 1
- Ovalbumin – Gal d 2
- Ovotransferrin – Gal d 3
- Lysozyme – Gal d 3

Figure 1 Main egg allergens components Gal d 1, Gal d 2, Gal d 3, Gal d 4

The dominant allergenic protein in hen's egg is ovomucoid (Gal d 1) *(McNamara, 2013)*. It is very stable against heat and proteolysis. Compared with other egg white components ovomucoid has very strong allergenicity, this can be related to the strong disulfide bonds that stabilize this highly glycosylated protein *(Caubet and Wang, 2011)*. Ovomucoid accounts for 10% of egg white proteins. Other main allergens are ovalbumin (Gal d 2), ovotransferrin (Gal d 3) and lysozyme (Gal d 4). Ovalbumin represents more than 50% of egg white proteins, while ovotransferrin represents only 12% of the total protein in egg white. Lysozyme is a very small protein and is often used as food additive *(Martorell Aragonés et al., 2001)*.

1.2 Water contaminant – Dipyrone

A water contaminant or pollutant can be defined as a physical, chemical or biological factor causing effects on the aquatic life, the environment and the human health (Goel, 2006). Metamizole, our target analyte, belongs to the class of pyrazolones, which are generally used as analgesic drugs. Metamizole also known as Dipyrone (DIP) was among the most widely used drugs acting as a painkiller or fever reliever. Further it was shown to have anti-inflammatory effects. Metamizole is a so-called prodrug. That means it gets hydrolysed in the gastrointestinal tract into the primary metabolite 4-methylaminoantipyrine (MAA) in the gastrointestinal tract and rapidly absorbed in form of MAA. This compound is further metabolized by oxidation to 4-formlyaminoantipyrine (FAAP), which is an end-metabolite and further by demethylation to 4-aminoantipyrine (AAP) in the liver. AA is further transformed by acetylation into 4-acetylaminoantipyrine (AAAP). *(Li et al., 2019; Mayerhuber et al., 2021)*
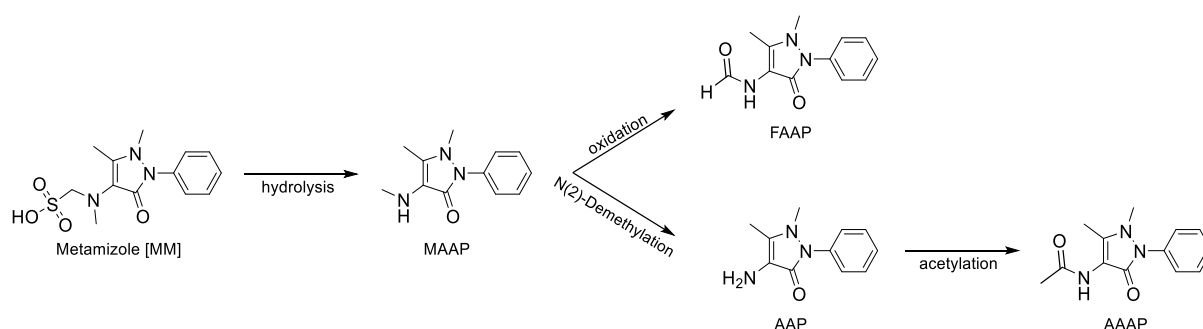

Figure 2 Main metabolic pathway of Dipyrone in humans

Even if Dipyrone is still available and sold under various names, it is banned in several countries due to its potential adverse effects. Dipyrone and its metabolites belong to the pharmaceutical active compounds (PhACs). PhACs are a growing concern based on their occurrence and fate in water and environment. These compounds are a complex topic which is directly related to the environment and human health. A wide range of pharmaceuticals are released in the environment, either directly or indirectly. The most common way that those substances find their way into the environment are medical facilities and excretion via urine and faeces (Figure 3). Even if there are possibilities for the removal of those pollutants, there are still some very small persistent compounds which cannot be removed completely and stay in the environment over a very long period. Trace-levels of PhACs have already been detected in drinking water samples. It is not clear if this small amount can harm the human health but due to their persistence they lead to accumulation and chronical exposure. Based on this, it is highly desirable to develop a possibility for fast and easy pollution test such as e.g. LFD devices.
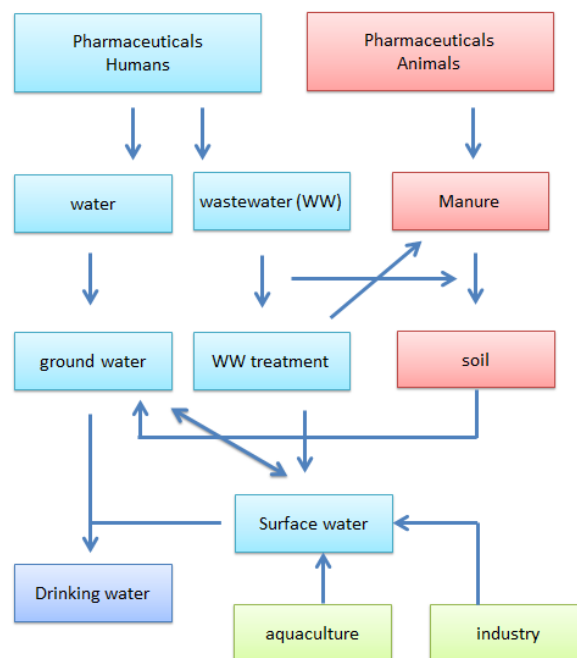

Figure 3 Distribution of pollutants

There are different approaches for the quantification of contaminants, whereas most of them are based on chromatographic methods like GCMS, LCMS or LC-HR-MS(MS). In contrast to these methods also electrochemical sensors as an alternative are used, those methods are using nickel-salen, phthaloycyanine or reduced graphene oxide as sensing element and they are all based on amperometric detection. There are also efforts on the development of potentiometric ion-selective electrodes, since they are simple to use, cost efficient, portable and don't require trained personnel, as described by *(Mayerhuber et al., 2021).*

1.3 Lateral flow device and other fast immunoanalytical methods

A lateral flow device (LFD) is a paper-based platform for the detection and quantification of analytes. It is designed to confirm the absence or presence of a compound. It exists since the 1980s when the pregnancy test for the detection of human chorionic gonadotropin (HCG) in urine was developed *(Helen Hsieh et al., 2017)*. Lateral flow devices are immunochromatographic test strips which work with the same principle as an ELISA. The great

advantage of the strips compared to an ELISA is the easy handling, therefore it fulfils the requirement of a simple test, since there is just no need for further detection equipment. It finds its application in different areas like detecting drugs, hormones, pathogens, feed/food and in environmental settings. The immunochromatographic test strips use the principle of antigen-antibody binding on thin-layer chromatographic background. Therefore signal generation happens due to a labelled analyte specific antibody which requires the conjugation of antibodies to coloured nanoparticles *(Posthuma-Trumpie et al., 2009)*. Such assays should be easy to perform without the need of trained personnel *(Rudolf et al., 2009)*. They offer many advantages including economical, convenience, simplicity and rapid results. Another advantage is, that they are cheap to produce, show a rapid response and are portable. Lateral flow assays as mentioned rely on visual detection of coloured lines, furthermore they enable easy handling, portability can be used at any time by non-technical personnel *(Helen Hsieh et al., 2017)*.

One trade-off of this application is, that the test results are generally qualitative. This means that this type of assay can only be used for tests in which a yes and no answer is enough *(Koczula and Gallotta, 2016)*. Another drawback is, since it is antibody-based other proteins with similar structure can cause a positive result.
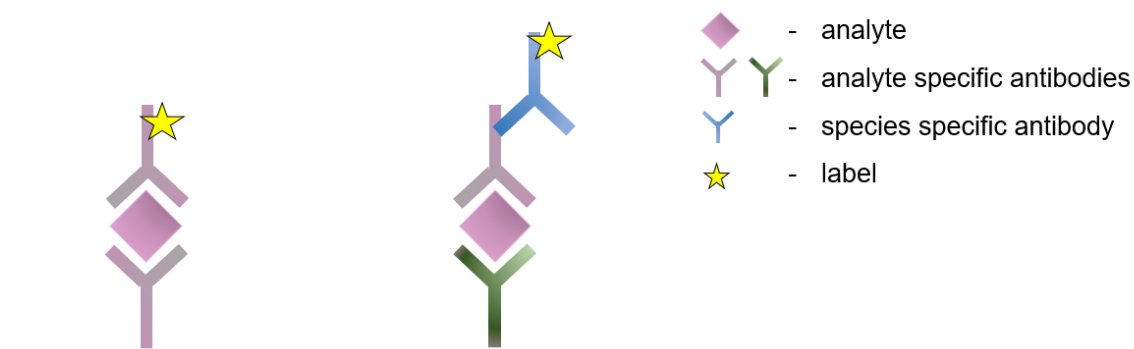
Figure 4 Sandwich assay assemble with direct (left) and indirect (right) detection

Another technology which had its breakthrough in the 1980's is the enzyme-linked immunosorbent assay (ELISA). Due to its specificity, sensitivity and simplicity it finds its application in many areas. It enables high sample through put and is currently the most widely applied technique for routine measurement *(Schubert-Ullrich et al., 2009)*. Lateral flow devices work on the same principle as ELISA, both are antibody-based tests.

1.4 Principle and components of the lateral flow assay

The assay is composed of many elements, therefore problems can be caused by imperfect material characteristics, by material incompatibility or flaws in the connection of the overlapping elements. A very important aspect is choosing the best suitable antigen or antibody or using the right membrane to produce a consistent and proper working product.

The membrane used as strip material is one of the most important components of the test system. For those assays nitrocellulose is the most common used material. But also other polymeric materials are available e.g. nylon, polyethersulfone, polyethylene or fused silica but these are used less often *(Edwards and Baeumner, 2006; Noguera et al., 2011; Posthuma-Trumpie et al., 2009).*
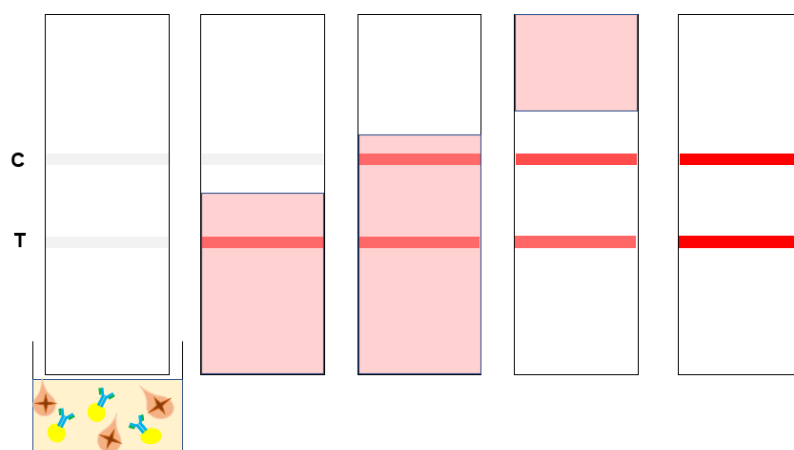

Figure 5 Showing the lateral flow assay principle and colour development of test and control line after 1, 2, 3 and 5 minutes

One reason for the common use is that nitrocellulose absorbs protein at a high level and also large molecules are able to travel the membrane in a reasonable time (*Abdo et al., 2010).* Nitrocellulose membranes are available in different grades, means with different capillary flow rate or pore size. Important parameters are the capillary forces or speed of the membrane which affects sensitivity, specificity and response time, as well as pore size and material *(Sajid et al., 2015).* Also, the ease of binding and immobilizing proteins is a crucial factor. A range of nitrocellulose pore sizes are available, but since the pores are not equally distributed, the capillary flow time is a more accurate parameter. The capillary flow time is the time which the liquid requires to travel the whole membrane and this is expressed as seconds per centimetre. The speed with which the whole complex is transported through the membrane allowing optimal reaction time. That means that the run time is an important parameter because the

sample is only able to react with the test line during the period when it is passing over it *(Mansfield, 2005; Posthuma-Trumpie et al., 2009)*.

A liquid sample containing the analyte or an extract of the analyte is moving with external capillary forces through various zones of the strip and interacts with the analyte attached to the membrane. On a backing card, the thin and fragile nitrocellulose membrane is attached for better handling. On this membrane a test- and control line are sprayed onto. At the bottom of the strip the sample pad is mounted with 1 – 2 mm overlapping on the membrane. It is made of cellulose and/or glass fibre.
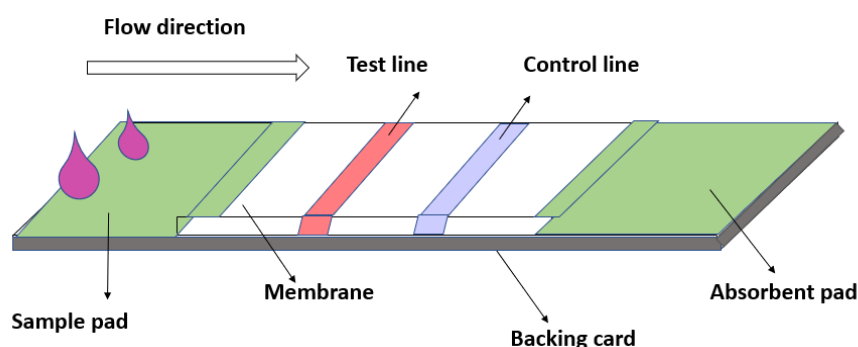

Figure 6 Typical configuration of a lateral flow assay

The sample pad or application pad has multiple roles, one of the most important is to evenly distribute the sample and transport it in a smooth and continuous way toward the membrane. It also ensures that the analyte solubilized in a liquid will be able to bind on the membrane. Furthermore, the pores of the sample pad act as filter and remove redundant material. It is common that the sample pad is impregnated e.g. with proteins or various detergents to influence the flow rate or increase the reaction time.

The absorbent pad works as a sink at the end of the strip. It is attached to the top of the strip with 1-2 mm overlapping on the membrane. The absorbent pad wicks the fluid through the membrane and finally collects the processed liquid. The capacity of the pad results in increased test sensitivity. The porous membrane has specific biological components immobilized in lines. These lines react with the analyte present in the sample. The control line, which is presented on the top indicates a proper flow and if the strip is working correctly. The test line indicated if whether the analyte is present in the sample or not. The read-out can be assessed by eye after 5-10 minutes *(Koczula and Gallotta, 2016; Sajid et al., 2015)*.

Within this work sandwich and competitive format LFD were developed, since two very different molecules were investigated.
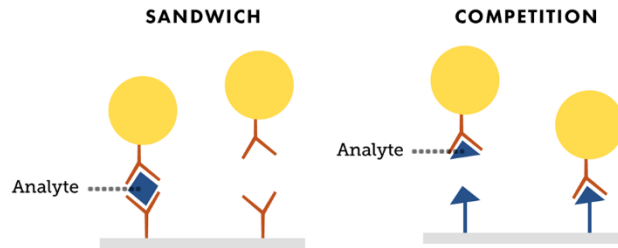
Figure 7 Difference of sandwich and competitive assay

1.5 Sandwich Assay

The sandwich or direct assay is used for detecting larger analytes, as well as analytes with multiple binding sites like HCG used in pregnancy tests *(Frankle, 1976).*

An antibody against the target analyte is immobilized on the lest line. An anti-species antibody against the labelled conjugate antibody is immobilized on the control line. A sample containing the analyte mixed with antibody-gold conjugate is applied to the sample pad and migrates to the upper parts of the strip. The target analyte is captured by the gold antibody conjugate and results the formation of a gold antibody conjugate – analyte complex *(Sajid et al., 2015).*

Through capillary forces this complex reaches the nitrocellulose membrane. At the test line the antibody gold-conjugate/analyte complex is captured by the immobilized antibody which is primary to the analyte. At this point the analyte is sandwiched between the primary immobilized antibody and the antibody-gold conjugate *(Koczula and Gallotta, 2016).* The excess antibody-gold conjugate binds to the anti-species antibody, which is immobilized as a control line, regardless how much analyte is present. This demonstrates that the assay is working correctly. In this assay the signal intensity at the test line is direct proportional to the amount of analyte present in the sample.
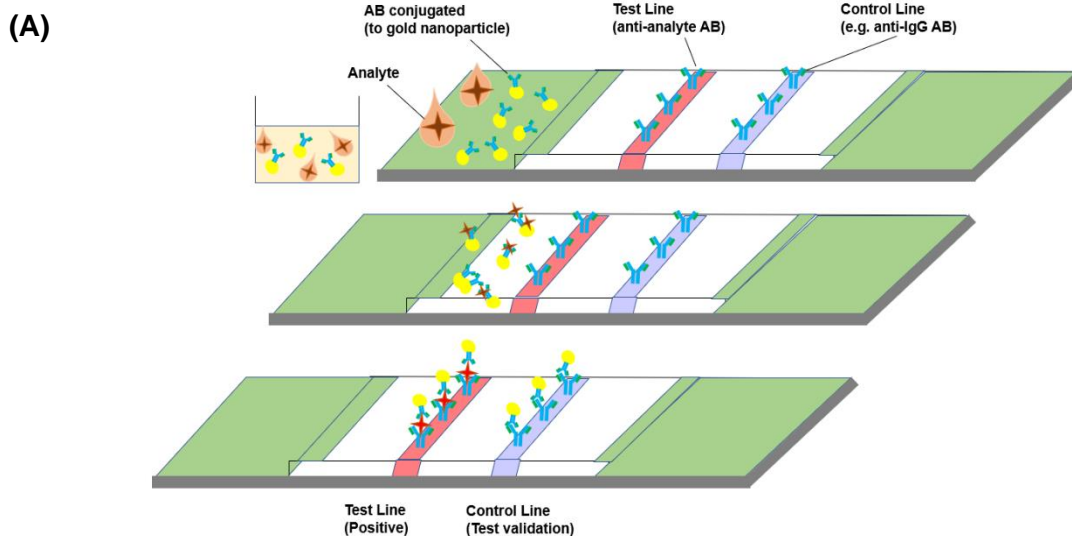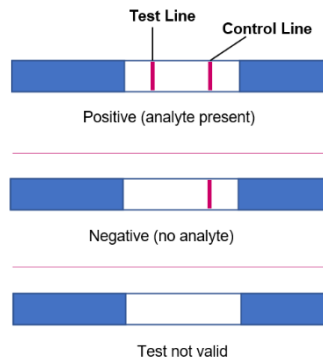
(B)

Figure 8 Operation mode of a lateral flow device

(A)Schematic representation of the mechanism. First one, the sample is placed on the sample pad and migrates with the flow direction. The strip in the middle shows how the conjugate antibody binds the target analyte and on the third strip the mixture migrates to the test line and the bound target analyte is captured. (B) Shows the possible results and the meaning of such a test.

1.6 Competitive Assay

The competitive assay is applied if the analyte is too small for multiple antibody binding events, such as drugs, in this case *(Bose and Sarma, 1975)*. In this format the test line contains a protein-analyte complex. The detection antibody-gold conjugate is present in the reaction tube together with the analyte. If the analyte is present in the sample, the analyte will bind to the antibody-gold conjugate and will therefore prevent the binding of the conjugate at the test line. If no analyte is present, the antibody-gold conjugate will bind to the analyte on the test line, yielding a positive signal, while appearance of colour both at test and control line indicates a negative result. In this case the signal intensity is indirect proportional to the amount of analyte present in the sample.
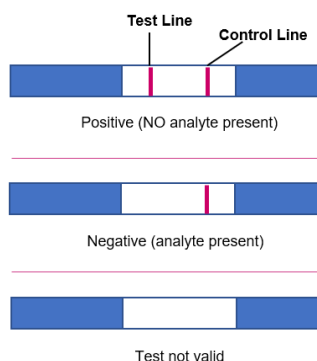

Figure 9 Shows the working principle of a competitive assay - no analyte is present no colour development on the test line,

1.7 Antibodies

An antibody, also known as an immunoglobulin, is a large Y-shaped protective protein produced by the immune system. Antibodies are grouped into five classes according to their constant region. Each class is marked with a letter: IgG, IgM, IgA, IgD and IgE. The antibody which is the physiological basis of food allergies is human IgE (*Valenta et al., 2015*). It is activated by ingestion and later on responsible for the occurrence of symptoms. For the detection of allergens within immunoanalytical assays, poly- and monoclonal antibodies, which are IgGs from various animal species, are applied. Polyclonal antibodies are produced by different immune cells, segregated into the blood and can be found in the serum after removing red blood cells. They are targeting the same antigen but different epitopes. Monoclonal antibodies are using identical immune cells which are clones of a specific parent cell *(Singh et al., 2014).* For this work only polyclonal antibodies were used. They are relatively inexpensive, have a higher overall antibody affinity due to the recognition of multiple epitopes and they are also easy to couple with antibody labels and are rather unlikely to affect binding capability. There are still some drawbacks such as batch-to-batch variability since produced in different animals and there is also a higher chance of cross-reactivity due to the recognition of multiple epitopes *(Benjamin Caballero Paul Finglas Fidel Toldrá, 2016).*

The basic structure of all antibodies is the same, as shown in Figure 10.The four polypeptide chains are connected by non-covalent forces. The four chains form a symmetrical molecular structure. Each Y contains two identical copies of a "heavy chain" with around 440 amino acids and two identical copies of a "light chain", with around 220 amino acids. There is a hinge in the centre between heavy chains to allow the protein to be flexible *(Nezlin, 1998; Schroeder and Cavacini, 2010).*
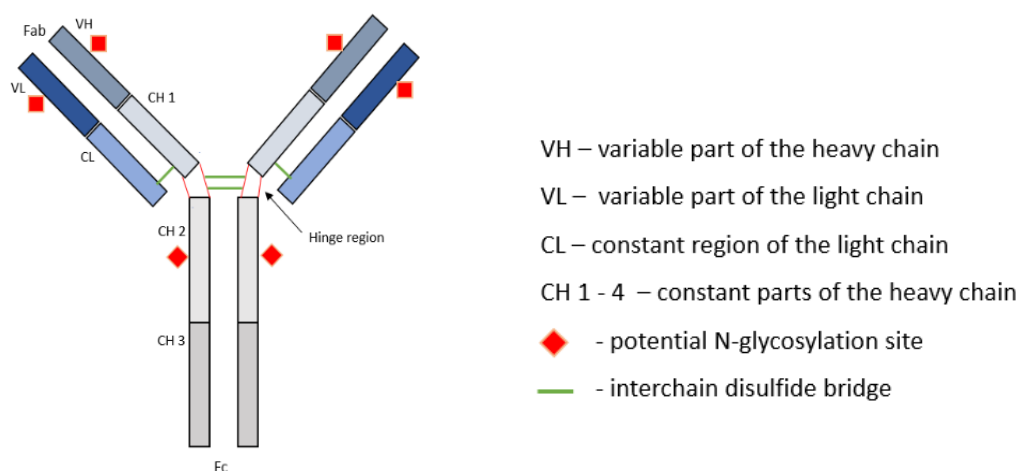

Figure 10 Structure of Immunoglobulin G

1.8 Antibody purification

For the application of antibodies in immunoanalytical detection systems highly purified antibodies are required. Affinity chromatography offers highest selectivity for clean-up of polyclonal and monoclonal antibodies, other common methods are size exclusion chromatography, ammonium sulphate precipitation and ion exchange chromatography.

Resins contain immobilized ligands which bind to the Fc parts of the required antibodies. This type of chromatography is based on high affinity of protein A and protein G for the Fc-region of IgG. These two proteins which are derived from microorganisms are commonly applied for this purpose. Staphylococcal protein A (SPA) was one of the first discovered immunoglobulin binding molecules. SPA is a cell wall associated protein domain, exposed on the surface of the gram-positive bacterium *(Hober et al., 2007)*. It consists of 5 highly homogenous IgG binding domains which all bind the Fc part of IgG from different species such as human and rabbit. Additionally, each domain has high affinity to the Fab part of certain antibodies. Protein G is a streptococcal cell wall protein with two albumin and two immunoglobulin binding domains. The IgG binding domain interacts with the Fc and the Fab parts, but the binding of Fab is much weaker. The affinity of protein A and G generally varies among species and subclasses *(Nezlin, 1998)*.
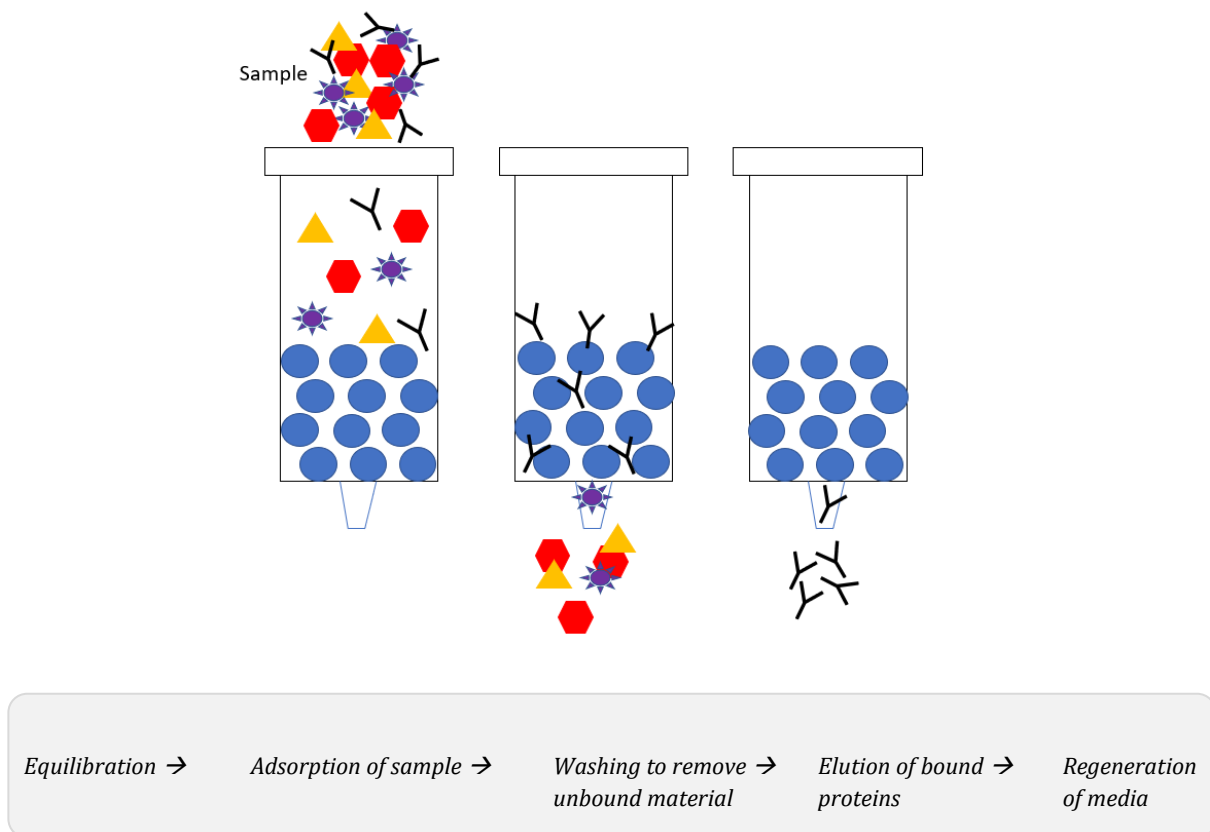

| *Equilibration →* | *Adsorption of sample →* | *Washing to remove → unbound material* | *Elution of bound → proteins* | *Regeneration of media* |

Figure 11 Steps of antibody purification with affinity chromatography

11

The IgG binding proteins A and G are coupled to agarose. The serum which contains IgG is applied on the column and unbound substances are removed under mild conditions at a neutral pH. For the elution of bound IgG more harsh conditions as applied and the pH is lowered.

1.9 Labelling the antibodies

Common labels for antibodies in lateral flow assays are made of coloured nanoparticles with varying sizes (*Posthuma-Trumpie et al., 2009*) based on different materials such as e.g.:

- colloidal gold
- latex particles
- silver nanoparticles
- enzymes
- liposomes and others.

However, any used material should be detectable at low concentrations and maintain its properties upon conjugation with biorecognition molecules *(Sajid et al., 2015)*. An easy conjugation as well as long term stability are favourable features for a good label. Labels which give a direct signal are preferable for LFDs. There are different coloured nanoparticles that can be applied as labels, this work focuses on frequently used approach utilizing GNP, reasons for that decision can be found in the next chapter.

1.10 Colloidal Gold – Gold nanoparticles

The gold nanoparticles have been often studied due to their unique optical and physical properties, which are strongly dependent on their size, shape, colour, surface structure, charge and agglomeration state (*Chegel et al., 2012)*. Nanoparticles are defined as particles between 1 and 1000 nm that have a range of unique properties *(Jazayeri et al., 2016)*. Colloidal gold particles are the most widely used label in commercial lateral flow assays, also because they give perfect spherical particles, have high affinity towards biomolecules and can easily be functionalized. A general understanding of the chemistry is needed to be able to synthesize and functionalize GNP. The nanoparticles can be produced easily according to the Turkevich and Frens method, which is the most commonly used protocol for GNP synthesis but there are also other commercial available sources based on the chemical reduction of gold chloride trihydrate in aqueous solution *(Kimling et al., 2006).*

Briefly, this method can be characterized as the reduction of gold chloride with sodium citrate. Due to their surface plasmon resonance and the presence of citrate ions on the surface of the particle, they carry a negative surface charge *(Freitas de Freitas et al., 2018).* For the application in lateral flow assays a diameter of around 40 nm in recommended. Therefore, the nanoparticles have an absorption maximum at around 530 nm *(Byzova et al., 2020).*

1.11 Conjugation of GNPs

If the particles are supposed to be used for further immunoanalytical applications, it is necessary to choose a target component such as a monoclonal or polyclonal antibodies and attach the particles surface to them. Physical or chemical interactions can be used to attach proteins and other molecules to the surface of the gold nanoparticle. These antibody – gold particle interactions are based on different phenomena. There is hydrophobic attraction between the antibody and the gold surface. Additionally, the ionic attraction between the negatively charged gold and the positively charged antibody takes place, as well as chemical interaction of antibody and nanoparticle. This can be achieved through chemisorption via thiol derivates, bifunctional linkers and adapter molecules. For conjugation of the antibody to gold particle both, covalent and non-covalent immobilization techniques can be used. For the non-covalent methods, the antibodies in this case are non-specifically absorbed onto the surface. The nanoparticles are still negatively charged and remain therefore stable in the colloidal gold solution. This technique is a spontaneous absorption of antibodies of the citrate stabilized particles. *(M. Ijeh, 2011, Ljungblad, Jonas, 2009)*

Another method of bioconjugation is the covalent immobilization mode. This involves the modification of the surface of nanoparticles with reactive groups such as carboxyl and amine groups. They can be coupled covalently to amino side chains on the surface of the antibody using methods such as 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide/ N-hydroxysuccinimide (EDC/NHS). Therefore the gold nanoparticles are coated with thiolated polyethylene glycol (PEG-SH) and conjugated to biomolecules through EDC/NHS chemistry, which yield a covalent bond without the addition of a spacer. *(Wu et al., 2014; Zhou et al., 2004)* It is always desired to enhance the biocompatibility of nanomaterials. Using spacers enables to work with a defined system, meaning in case of PEG non-toxic, hydrophilic, and commonly used in various areas. This system is also known to prevent unspecific binding and showing a extend live time of the particles. Even if spacers should prevent aggregation of nanoparticles the problem should still be taken into account when working with nanoparticles *(Leopold et al., 2017).*
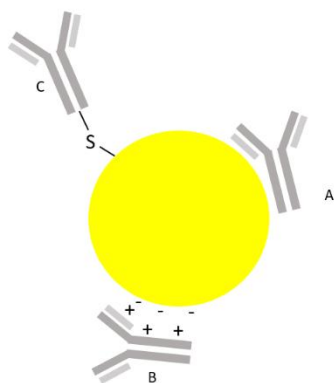
Figure 12 Different interactions between antibody and gold nanoparticle surface. A) hydrophobic interaction B) ionic interaction C) covalent bond is formed.

This approach bears a great potential for sensitivity improvement since there is defined loading of GNP possible. Due to better stability and stronger binding to the nanoparticles, stronger interactions will occur. Another major advantage of thiol-based self-assembled monolayers as an interface between a metal surface such as gold and biological molecules is that denaturation can be avoided. Drawbacks of thiol-SAMs are limited thermal long-term stability under ambient conditions. However, both systems, either covalent or non-covalent has its advantages and disadvantages, there usability usually depends on the type of application *(Jazayeri et al., 2016).*

2 Materials and Methods

Reagents and Materials. Metamizole Sodium (98%), Gold (III) Chloride Trihydrate (49% AU Basis), Horseradish peroxidase (HRP)-conjugated, Coomassie Brilliant Blue G-250 was purchased from Sigma-Aldrich (Vienna, Austria) as well as fish gelatin (gelatin from cold water fish skin) and bovine serum albumin (96%). Salts used for buffers: Na_2HPO_4 (99.5%), H_3PO_4 (85%), NaCl (99.8%), Citric Acid (99.5%), were bought from ROTH (Karlsruhe, Germany), also Tween20, Ficoll® 400, and Milk Powder. Sodium azide and (3 3' 5 5'-tetramethylbenzidine) (TMB) were purchased from Sigma Aldrich (Vienna, Austria). The antibodies of rabbit were prepared in-house. Donkey Anti-Sheep IgG, unconjugated (95%) was purchased at Thermo Fisher. Metamizole HAS-conjugate was obtained from Squarix biotechnology (Germany). Water was purified by reverse osmosis before use. Samples for extracts were prepared in-house.

2.1 Purification of polyclonal rabbit sera

Buffers and reagents
- Binding buffer (A): 0.02 M Na_2HPO_4, pH 7 with 85% H_3PO_4
- Elution buffer (B): 0.1 M Citrate-Buffer, pH 2.7 with 85% H_3PO_4
- 20% Ethanol

Equipment
- Protein A Column: 1 mL HiTrap
- Peristaltic Pump Pharmacia LKB
- FPLC system Agilent Technologies 1260 Infinity x
- UV/VIS Spectrometer

2 mL rabbit serum was diluted with binding buffer to a final volume of 12 mL. The peristaltic pump was first rinsed with water and 10 mL of binding buffer at a flow rate of 6 mL/h^{-1}to remove all air bubbles from the tubes. The column was attached to the pump and equilibrated with 5 column volumes of binding buffer. The rabbit serum dilution was then applied onto the column and before finishing it was rinsed three times with 5 mL of binding buffer to remove serum molecules that have not bound to the column. The column was disconnected and either stored at 4°C or directly used for purification with the FPLC system.

The valves and tubes of the FPLC system were rinsed with open purge valve and rinsed again with closed purge valve with milliQ, buffer A and B to remove all air bubbles from the tubes.

The system was stopped, the column was connected and rinsed with binding buffer. The eluent was changed to elution buffer and the bound protein was eluted into collection tubes at a flow rate of 0.6 mL/min. After collecting all protein, the column was rinsed 20 minutes at a flow rate of 1 mL/min with elution buffer, followed by milliQ and 20% ethanol, disconnected and stored at 4°C. The purge valve was opened and rinsed with milliQ and ethanol again and kept like this. The fractions containing the protein were measured at 280 nm. The concentration was determined using 1.4 as extinction coefficient. Absorbance values higher than 2.5 were diluted and measured again. For the calculation the dilution factor had to be considered. Fraction 1 and 2 showed quiet low concentrations, therefore they were combined and up-concentrated with cut-off filters (cut off at 100 kDa) and measured again as explained in Chapter 2.2. – Enrichment of antibodies.

2.2 Enrichment of the antibodies

Buffers and reagents
- 0.2 M PBS-Buffer: Na_2HPO_4, NaH_2PO_4, NaCl, pH 7
- Rabbit Anti-Egg yolk (in-house produced)

Equipment
- Thermofisher Amicon Ultra-15 Centrifugal Filter Device 100K
- Centrifuge: Beckman Coulter Allegra X-22R

The ultrafiltration membranes in the tubes can contain trace amounts of glycerine, therefore the tube had to be rinsed before use. It was rinsed with a suitable buffer, in this case 0.2 M PBS-buffer was used. This was done for 5 minutes at 5.000 rpm. After the step was completed the rabbit anti-egg yolk was added to the tube. For this application a fixed-angle rotor was used and the sample was spinned at 5000 rpm for 14 minutes. The fraction remaining in the upper part of the filter contained the up concentrated antibody. The absorbance was measured, the samples were portioned in 500 µL and stored at -20°C until further use.

2.3 Preparation of colloidal gold

Buffer and reagents
- 0.010% Au-Solution
- 1% Na-Citrate solution
- 0.2 M K_2CO_3 for pH adjustment

Equipment

- Reflux condenser
- Heating mantle
- Magellan Reader, Sunrise Basic, Fa. Tecan

According to the procedure colloidal gold particles with an average size of 40 nm were produced through chemical reduction with 1% sodium citrate at 100°C. All glassware and magnetic stir bars utilized in the synthesis of AuNPs were washed with milliQ water to avoid undesirable nucleation during the synthesis and aggregation of the gold solution. For the preparation of 200 mL 0.010% chloride trihydrate solution the gold (III) chloride trihydrate was weight in and solved in milliQ water. It was heated to reflux using a heating mantel which assures a very homogenous temperature distribution within the solution. During the whole procedure the round bottom flask remained connected to a reflux condenser to maintain a constant volume of the reaction mixture. The liquid was constantly stirred by coated magnetic bars. As soon as the solution started to boil, the reduction process was induced by adding 2 mL of a 1% citrate solution in a ratio of 1:100. That lead to a colour change from light yellow, to black, to deep purple and finally to brilliant red, demonstrating the formation of AuNPs. The solution was boiled for another 15 minutes and then cooled down. The pH was adjusted to 8.0-8.5 using 0.2 M K_2CO_3 and stored at 4°C until further use.

2.3.1 Size variation through varying concentration and pH

The influence of varying concentration and pH conditions was investigated. Parameters like temperature or pH but also different concentrations of the reactants can have a strong influence on the morphology of the gold particles. For size variation different concentrations of the chloride trihydrate were investigated, without varying any other parameters. Therefore, three batches of each concentration step were produced. It was started with the standard procedure with 0.010% of gold. Further 0.008%, 0.009%, 0.011% and 0.012% of colloidal gold solution was produced following the same procedure. Gold spectra/ UV-Vis spectra were recorded using Magellan Reader and the maximum absorbance of the colloidal gold suspension was determined photometrically from 450 to 650 nm. Depending on the size of the particles they have different absorption maxima. For the 40 nm colloidal gold produced here, the typical lambda max was between 526-531 nm.

For further investigation regarding the size of the gold nanoparticles the pH of the sodium citrate solution was varied by adding citric acid. A 1% solution of citric acid and tri-sodium dihydrate solution was prepared and mixed in a proper relation to obtain acidic buffer solutions with pH 3-6. The pH of the gold solution without adding any citric acid was about 7.85. For

each pH, three batches were prepared the same way as stated before and gold spectra were recorded to determine the maximum absorbance.

2.3.2 Structural characteristics

The structural characteristics and sizes of the particles were measured using scanning electron microscope (SEM). Therefore 500 µL of each batch were filled in an Eppendorf tube and dried in the warming cupboard/ heat cabinet overnight at 37°C. The heat cabinet was connected to a vacuum pump. After two days the liquid was evaporated and the samples were ready for SEM measurements. All measurements were performed on a FE REM from Zeiss, Modell "SIGMA HD VP" with a EDX system from EDAX type "TEAM Pegasus". The EDX detector is a EDAX Octane Plus SSD.

2.4 Antibody-gold titration

Buffer and reagents

- 0.05 M PBS buffer
- 10% NaCl solution (0.05M PBS)
- Purified antibody [3.45 mg/mL]

Equipment

- Magellan reader, Sunrise Basic, Fa. Tecan
- 96-well non-binding plates

The titration of colloidal gold solution was performed using clear non-binding 96 well plates with a flat bottom. The purified antibody was diluted with 0.05 M PBS buffer to obtain a concentration of 0.2 mg/mL.

Table 1 Titration table for antibody-gold conjugation

AB [µg]	AB [µL]	+	H$_2$O [µL]	+	AU [µL]	Total vol. [µL]	µg AB / mL AU
0	0		50		450	500	0
1	5		45		-''-	-''-	2
2	10		40		-''-	-''-	4
3	15		35		-''-	-''-	6
4	20		30		-''-	-''-	8
5	25		25		-''-	-''-	10
6	30		20		-''-	-''-	12
7	35		15		-''-	-''-	14

8	40	10	-"-	-"-	16
9	45	5	-"-	-"-	18
10	50	0	-"-	-"-	20

A dilution series in tubes was prepared, containing antibody in a concentration range from 0 to 0.2 mg/mL. 450 µL of the colloidal gold solution were added, mixed and incubated. After 10 minutes 100 µL of a 10% NaCl solution were added, mixed and incubated another 5 minutes. 200 µL of each concentration was pipetted in the non-binding plate. The absorption was measured at the maximum absorption of the gold suspension, which was at about 530 nm and as a reference/blank at 600 nm on a plate reader. For the evaluation the antibody concentrations were plotted against the absorption difference ($abs_{max} - abs_{600}$) to obtain the optimum amount of antibody needed for the gold conjugation. The optimum is reached where the absorption is on a constant level.

2.5 Preparation of colloidal gold-labelled polyclonal antibodies

Buffers and reagents
- 0.05 M PBS buffer
- Purified Antibody [3.45 mg/mL]
- 1% BSA in 0.05 M PBS
- 6% BSA in 0.05 M PBS

Equipment
- Overhead-Shaker: stuart rotator SB3
- Centrifuge: Beckman Coulter Allegra X-22R

The calculated optimal concentration of antibody and 20 mL of the colloidal gold solution were mixed for 1 hour at room temperature using an overhead shaker. 2 mL of 1% BSA were added and mixed for another 20 minutes. The BSA was added to block the free binding sites on the surface of the gold nanoparticles. The mixture was centrifuged at 15°C, 9500 rpm for 20 minutes to remove unbound proteins. The supernatant was removed with a pipette, discarded and the pellet was washed with the same amount of milliQ water and centrifuged again. After removing the supernatant, the precipitate of about 600 µL was redissolved and transferred in a 1 mL tube. If there was still some gold remaining in the tube, it was flushed again with about 100 µL of the supernatant and also transferred in the 1 mL tube. 100 µL of a 6%-BSA solution were added, mixed and stored at 4°C until further use.

2.6 Extraction of egg allergens

Seven different egg samples were used for the following protein extraction. The aim was to identify the best standard that could be used for the LFDs and to see against which egg material the antibody works best. All egg samples were already available from other/old projects and stored at -20°C.

- Egg yolk cooked
- Egg yolk raw
- Whole egg cooked
- Whole egg raw
- Whole egg powder spray dried
- Egg white powder spray dried
- Egg yolk powder (not defatted)

Real food samples, to which the developed LFD should be applied as real application study, were bought from the supermarket. Samples which contain egg and samples which had no egg in it. They were shredded and stored at 4°C for further use.

- Tassenküchlein Schoko Glutenfrei
- Tassenküchlein Schoko
- Clever Schokokuchen
- Clever Strataltella Kuchen
- Semmelknösel
- Eierwaffeln
- Eierbiskotten

Contains egg

- Eiswaffeln
- Kartoffelgnocchi

Contains NO egg
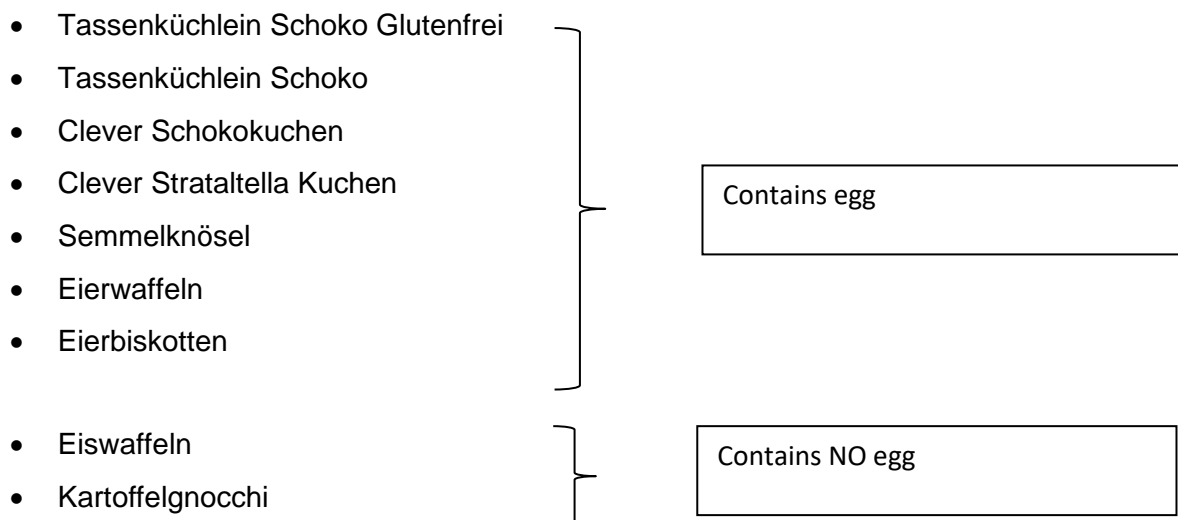
2.7 Protein/Egg extraction

Buffer and reagents
- 0.1 M TBS buffer + 1% Tween 20, pH 8.2 with 1 M HCl

Equipment
- Waterbath GFL technology laboratory equipment
- Centrifuge: Beckman Coulter Allegra X-22R

Extraction was done with 0.5 g of each egg sample in 10 mL of extraction buffer. After mixing the extraction was performed for 15 minutes at 60°C using a water bath. Each 3 minutes the extracts were shaken by hand for a short moment to enhance extraction. Afterwards the extracts were centrifuged for 15 minutes at 4°C and 9.500 rpm. The supernatant was aliquoted in 2 mL Eppis and stored at -20°C until further use. The real food samples for application study were treated the same way.

2.8 Protein determination according to Bradford

Buffers and reagents
- 0.2 M PBS buffer
- BSA in PBS buffer
- Coomassie Brilliant Blue G-250,

Equipment
- ELISA non-binding plates: Thermo Scientific
- Magellan Reader, Sunrise Basic, Fa. Tecan

The Bradford assay was used to determine the concentration of total protein in the extracts. Eight BSA dilutions were prepared as standards. The BSA stock solution had a concentration of 1000 µg/mL. The stock solution was diluted seven times 1:2 with 0.2 M PBS buffer. The last standard was PBS buffer and used as a blank.

Table 2 Bradford pipetting scheme

	BSA Concentration [µg/mL]	BSA Solution [µL]	+	0.2 M PBS[µL]
1	500	500 µL Stock solution		500
2	250	500 µL solution 1		500
3	125	500 µL solution 2		500
4	62.5	500 µL solution 3		500
5	31.25	500 µL solution 4		500
6	15.63	500 µL solution 5		500
7	7.81	500 µL solution 6		500
8	0	-		500

The egg samples with unknown protein concentration were also diluted with three different dilution steps. For getting an overview about the protein content in the samples 1:50, 1:500, 1:1000 dilutions were prepared. All solutions were prepared with 0.2 M PBS buffer. 20 µL of standard and protein solution were pipetted in triplicate into separate microplate wells. 200 µL of Coomassie Brilliant Blue were added using a multichannel pipette and mixed carefully. ELISA non-binding 96 well plates were used. Everything was incubated for 10 minutes at room temperature and the absorbance was measured at 595 nm with a plate reader.

A standard curve (cubic spline) of the measured BSA standards was created to calculate the protein content of each extraction dilution. For those samples in which the chosen dilutions were not in the linear range of the standard curve, new dilutions were prepared and measured.

Table 3 Egg extract dilutions

Egg yolk cooked	Egg yolk raw	Whole egg cooked	Whole egg raw	Whole egg powder	Spray egg protein	Egg chicken yolk powder
1:5	1:10	1:5	1:10	1:50	1:50	1:10
1:10	1:50	1:10	1:50	1:100	1:100	1:50
1:50	1:100	1:50	1:100	1:200	1:200	1:100

The samples were measured the same way and the protein concentration was calculated. The given values of the mean concentration in [µg/mL] of each dilution were multiplied with its dilution factor to get the protein concentration of the measured extract. The values of the three dilution steps were averaged to get the total protein concentration.

2.9 Enzyme-linked immunosorbent assay

Buffers and reagents

- Coating buffer: 12 mM Na_2CO_3, 38 mM $NaHCO_3$ and 0.01% NaN_3
- Blocking solution: Coating buffer + 1% Ficoll
- Washing buffer: 0.01 M PBS, 0.1% Tween20
- Assay buffer: 0.2 M PBS, BSA, Tween20, H_2O
- TMB stock solution: 375 mg Tetramethylbenzidine, 5 mL Dimethylsulfoxide, 25 mL MeOH
- Substrate solution: Substrate buffer, 30% H_2O_2, TMB stock solution
- HRP 1:40.000
- Stop solution: 1M H_2SO_4

Equipment

- 96-well ELISA high-binding plates: Thermo Scientific
- Washer: Tecan 96PW^{TM}
- ELISA reader: Sunrise from Tecan Austria GmbH
- ELISA software: Magellan5 Tecan Austria GmbH

For the application the competitive ELISA format was chosen. It is an end-point reaction, this means the reaction is stopped after a certain time and the color intensity is measured with a photometer. The standard curve shows a typical sigmoid function. For calculations the 4-parameter equation for sigmoid curves is used. The calculation range lies always in the linear range of the curve. This is the general equation for a dose response curve and it shows the response as a function of the logarithm of concentration.

Working with a competitive assay means, low concentration, high intensity. The x-axis shows the analyte concentration in µg/mL while the y-axis shoes the absorbance. in [nm]. The analyte was whole egg powder extract. Each dot on the line shows a concentration with the corresponding absorbance. The analyte concentration ranged from 150 µg/mL to 0.2 µg/mL and a blank.

2.9.1 Competitive Assay for egg

To find out which egg extract was the most suitable for the application as standard on the LFD as control line the competitive immunoassay was chosen. The goal was to find the best egg extract with the most specific binding.

2.9.2 Coating of the microtiter plate

The coating antigens (all egg extractions) where diluted in coating buffer to a concentration of 5 µg/mL. 12 mL thereof were needed for the coating of one plate. For coating 100 µL were added to each cavity of the high binding microtiter plate. The plates were coated overnight at 4 °C covered with parafilm. After coating the plates where emptied and washed with washing buffer three times.

2.9.3 Blocking of the microtiter plate

For blocking 150 µL of 1% Ficoll® 400 in coating buffer were added into each well of the microtiter plate and blocked for 2 hours at 37°C. The plates were emptied and dried overnight at 37°C in the vacuum oven. The plates can be used immediately or stored at 4°C up to four weeks if needed later.

2.9.4 Assay procedure

An antigen stock solution with 150 µg/mL in assay buffer was prepared. This stock solution was diluted in 1:3 dilution steps to a concentration of 150, 50, 16.6, 5.5, 1.8, 0.6, 0.2 µg/mL. 75 µL of each standard and of the blank were added to the plate. After incubation the plate was washed.

Antibody (Anti-Rabbit Egg yolk) dilutions were prepared in assay buffer (1:200, 1:500, 1:1000, 1:2000) and 25 µL were added in triplicates into the well. The plates were incubated for 1 hour at room temperature. After 3 times of washing 100 µL of anti-rabbit HRP 1:40000 were pipetted into each well and incubated for another hour at room temperature. The plate was washed again and 100 µL of the TMB substrate solution were added.

The TMB solution is converted by the horseradish peroxidase. The unreacted substrate should be colourless or a very slightly yellow. As soon as it is converted by the peroxidase, a blue reaction product is obtained. The TMB solution was incubated for 15 minutes protected from light before the reaction was stopped with 30 µL 1 M H_2SO_4. At this point a yellow solution is produced. The absorbance was measured at 450 nm using an ELISA plate reader. For creating the standard curve the four-parameter equation was used.
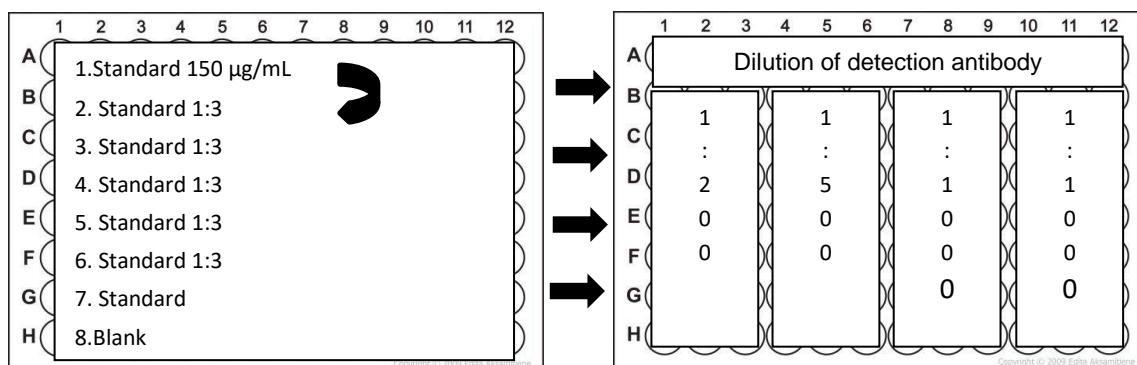
Figure 13 Pipetting scheme for ELISA plates

For further investigations different antibody dilutions were tested: 1:2000, 1:5000, 1:8000, 1:10000 and 1:1000, 1:2000, 1:5000, 1:8000. The evaluation was done as stated above.

2.10 Lateral flow device

Buffers and Reagents

- Running buffer: 0.05 M PBS, 5% Tween 20
- TBS buffer: 23.4 g/L NaCl, 1% (v/v) Tween20 → pH 8.2
- Anti-Rabbit Antibody [8 mg/mL]
- Anti-Rabbit Egg yolk antibody [3.45 mg/mL]
- AB/AU conjugate [8 µg Ab/mL AU]
- Egg extracts
- Metamizol Sodium/Dipyron
- Metamizol-HAS-conjugate [2.5 mg/mL]
- Donkey Anti-sheep Antibody [1mg/mL]

Equipment

- Backing Cards: DCN Diagnostics
- Nitrocellulose membranes
  - HF 75/80/120 Whatman
  - HFC 180 Merck
- Wick pad (CF5, 470 grade cotton liner), Whatman-GE Healthcare
- Cutter: Biodot CM4000
- Sprayer: Biodot ZX1000
- Vacuum oven: Thermo Scientific, Heraeus

2.10.1 Pre-tests for the LFD – Egg

For the lateral flow device production, some pre tests had to be carried out in order to get better knowledge about different formats, membranes and concentration range of antibody and analyte. Different concentrations for control and test line were investigated. At the beginning only spots were dapped onto the membrane using a pipette. For the color development of test and control line antibodies coupled to gold nanoparticles were used, different amounts of this conjugate were tested.

2.10.2 Comparision of different membranes

Membranes with different pore sizes were used for the experiments, such as HF 75, 80, HFC 120 and 180.

2.10.3 Sprayer

Control and test line were sprayed onto the nitrocellulose membrane using non-contact BioJet Quanti instead of dabbing spots. On a backing card the membrane was attached. The sample pad was attached to the bottom of the membrane with 1-2 mm overlapping on the membrane. The absorbent pad was attached to the top of the membrane also with 1-2 mm overlapping on the membrane. Strips were cut as stated above using a Cutter from Bio-Dot. The strips were stored in a plastic container under dry conditions at room temperature.

2.10.4 Blocking reagents

First tested blocking reagents was Ficoll® 400. The reagent was mixed in the running buffer with 8%, 4% 2% and 0.5%. Further the application of BSA, fish gelatin and milk powder were observed.

2.11 Dipyrone

For the assay devolpment of the small molecules the same membranes as stated above were used. Also the procedure was the same and didn't chance. For this part a competitive assay set-upwas used.

2.11.1 Blocking reagents

The blocking experiments were done with membrane FF 120. Two concentration set-ups of the test line were investigated. As blocking substances Ficoll® 400, fish gelatine, BSA and milk powder were tested.

3 Results and Discussion

3.1 Affinity chromatography / Antibody purification

Affinity purification is used as a standards purification procedure. For the use in immunoanalytical test systems it is very important to have highly purified and selective antibodies available. The polyclonal antibodies from rabbit used for the following tests were cleaned up according to (2.1) with purifying by protein A. After the FPLC clean-up the concentration of the purified IgG was measured photometrically at 280 nm. Absorbance values higher than 2.5 were diluted and measured again. The resulting optical density was multiplied by the dilution factor and divided by the extinction coefficient, which is 1.4, to obtain the protein concentration according to Lambert-beer law.

The collected fractions of rabbit anti egg-yolk IgG contained 3.45 mg/mL protein and 5.97 mg/mL. Based on the required concentration these are good results for further coupling to colloidal gold, therefore defined concentrations are needed for the titration experiments.

Table 4 Absorbance values and concentration for the antibody anti-rabbit egg yolk.

Antibody fraction	Absorbance value	Dilution factor	Extinction coefficient	Concentration
1	0.342	10	1.4	2.44 mg/mL
2	0.380	10	1.4	2.71 mg/mL
1 & 2*	0.484	10	1.4	3.45 mg/mL
3	0.767	10	1.4	5.47 mg/mL
4	1.237	10	1.4	8.84 mg/mL
3 & 4	0.837	10	1.4	5.97 mg/mL

*1 & 2 mixed together and up-concentrated

The following figure shows the elution chromatogram from the antibody purification using protein A. The red peak shows the absorbance signal of the eluting protein, when passing the detector, indicating the collected fraction. Amino acids like tyrosine, tryptophan and phenylalanine with aromatic rings are the main reason for the absorbance at 280 nm. But also, cysteine which forms disulfide bonds absorbs at 280 nm.
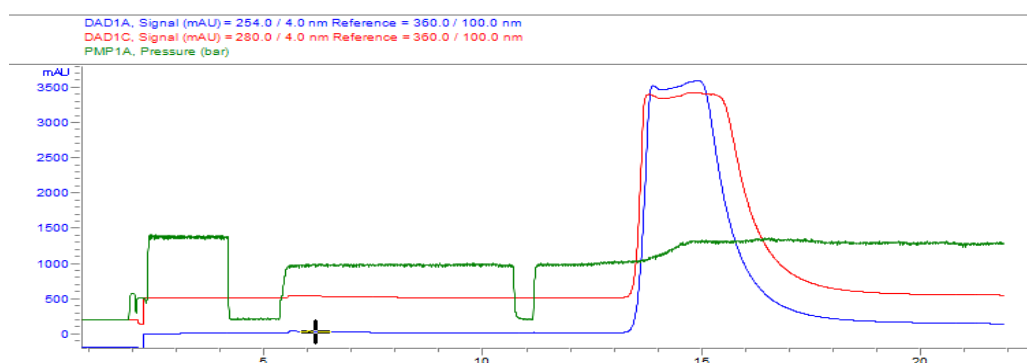
Figure 14 Chromatogram of the protein G purification of the rabbit anti-egg antibody, x-axis in minutes

3.2 Determination of the protein concentration in the egg extracts using the Bradford Assay

The determination of the protein content was done by the Bradford assay. The measurements were done for 7 extracts, only 3 of them were used for further investigations. The obtained results are shown in Table 5, Table 6, Table 7.

Table 5 Protein concentration egg yolk cooked

	Dilution factor	Optical Density (n=3)	Conc. [µg/mL]	Conc. X DF [µg/mL]	Mean concentration in [mg/mL]
Egg yolk cooked	1:5	0.683	145.4	727	
	1:10	0.535	72.7	727	0.7
	1:50	0.382	18.4	925	

Table 6 Protein concentration egg yolk raw

	Dilution factor	Optical Density	Conc. [µg/mL]	Conc. X DF [µg/mL]	Mean concentration in [mg/mL]
Egg yolk raw	1:10	1.191			
	1:50	0.871	248.4	12420	12.8
	1:500	0.659	133.1	13315	

Table 7 Protein concentration whole egg powder

	Dilution factor	Optical Density	Conc. [µg/mL]	Conc. X DF [µg/mL]	Mean concentration in [mg/mL]
Whole egg powder	1:50	1.008	348.7	17433	
	1:100	0.814	215.4	21544	20.2
	1:200	0.626	115.6	23134	

3.3 Affinity testing of antibody towards the analyte using ELISA

The competitive immunoassay was chosen to identify the extract (antigen) that is most suitable as standard. During the assay procedure all parameters remained the same and were not changed. As already mentioned, different extracts where prepared and tested, in the following section some of the tested extracts will be shown. The microtiter plates were coated with antigen-extract and the assays were performed as described in the material and methods section. The antibody was added in four different dilution step and detection was done using a commercially available horseradish-peroxidase labelled anti-rabbit IgG.

3.3.1 Whole egg powder as standard

Figure 15 shows the results of the competitive ELISA where whole egg powder extract was coated as standard onto the plate. The 4 graphs in *Figure 15* demonstrate 4 different groups, they differed in the concentration (dilution step) of the antibody. Group 4 represents the lowest antibody concentration with a dilution of 1:2000, resulting in overall lower OD values. The dilution of 1:1000 showed a slightly higher OD which corresponds with the fact, that there was more antibody present and therefore more antibody could bind to the coated antigen.

The slopes decreased at different points. For group 1, a very high antibody amount only 1:200 diluted was used. The result was, that the well was overloaded with antibody and therefore higher antigen -concentration was needed to see antigen-antibody interaction. The slope of this curves went down at higher antigen-concentration. Using less antibody leads to a more sensitive assay. An important indicator for sensitivity of an ELISA is the IC 50 value (C-value in figure), as it indicated the concentration at which half inhibition was reached.
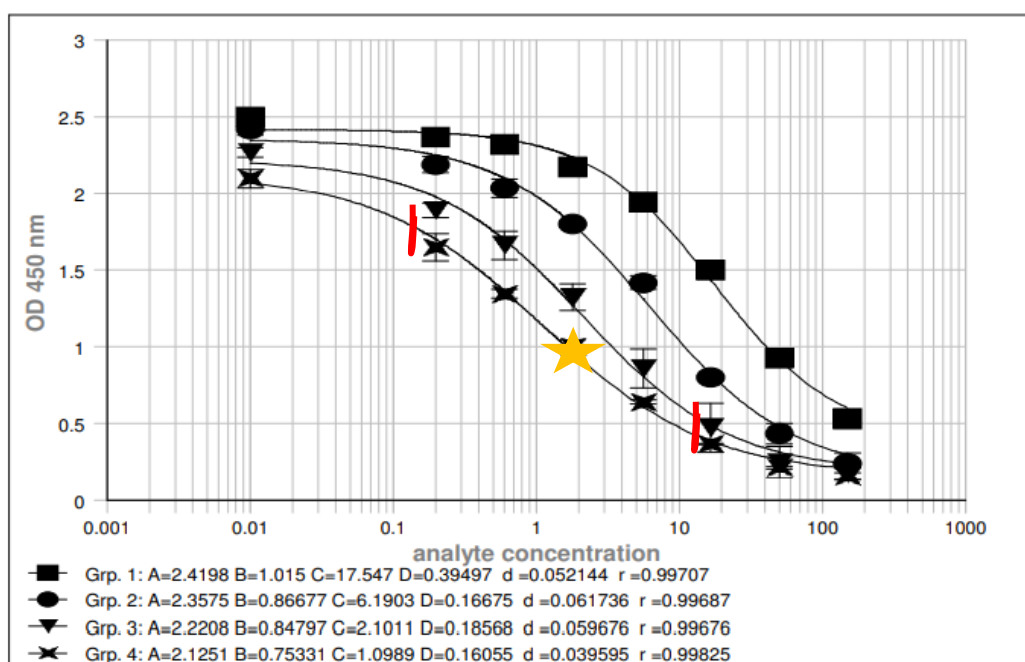
Figure 15 whole egg powder competitive ELISA. Rabbit anti-egg yolk dilution steps 1:200 (Grp. 1), 1:500 (Grp. 2), 1:1000 (Grp.3), 1:2000 (Grp. 4). Analyte concentration is given in µg/mL

The higher the dilution of the antibody the lower the C value. The C values for group four is marked with a star. A low C-value means a more sensitive assay and therefore a lower concentration can be detected.

For the assay a polyclonal rabbit anti egg-yolk antibody was used. Both the 1:2000 and the 1:1000 dilution of the antibody showed a very low C-value. These combinations were very promising for sensitive detection of egg proteins. In terms of being more economical, the dilution of 1:2000 was chosen, as this combination was still very sensitive, but less amount of antibody was needed. Reached maximum absorbances for the different groups lay between 2.5 and 3.0. An absorbance above 3 would mean, that the results are not anymore on the linear range of the detector and so quantitative read out would not be on the linear area of the detector. The obtained IC50-values [µg/mL] were: Grp. 1: 17, Grp. 2: 6, Grp. 3: 2 and Grp. 4: 1.

3.3.2 Raw egg yolk as standard
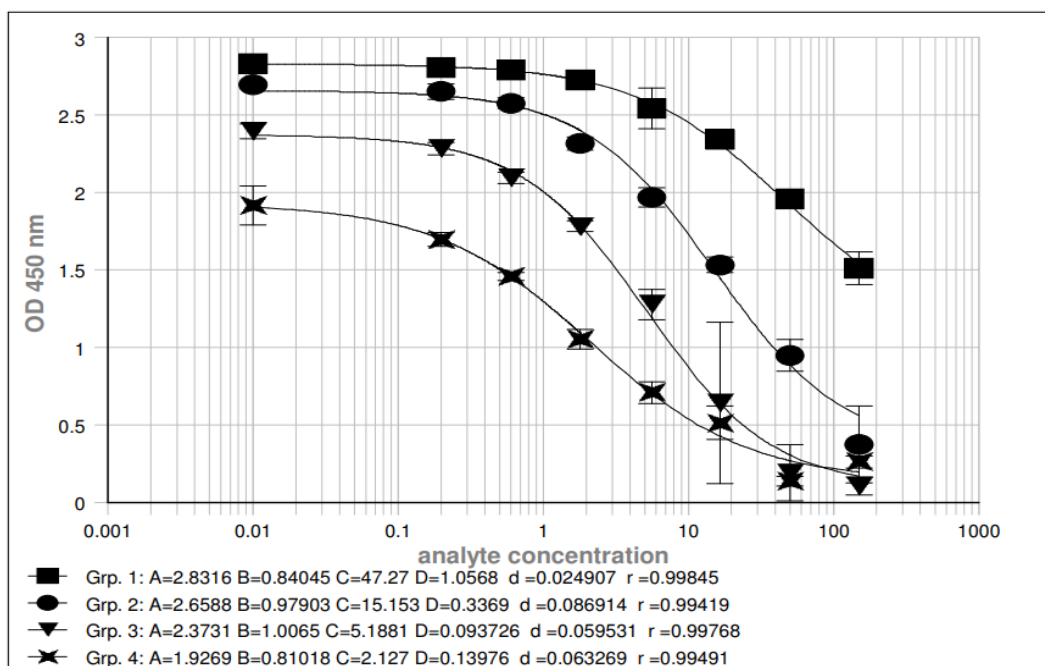
Figure 16 Egg yolk raw competitive ELISA. Rabbit anti-egg yolk dilution steps 1:200 (Grp. 1), 1:500 (Grp. 2), 1:1000 (Grp.3), 1:2000 (Grp. 4).

For the assay where raw egg yolk was used as an analyte the antibody dilutions stayed the same. Egg yolk was coated on the plate and also used as a standard. Since a different analyte was used, the targets are coming from another source in a different ratio, this also results in different curves. In general, higher OD values were achieved.

The different antibody dilutions showed greater differences between the single curves and also higher C-values were obtained. Is seems that the antibody recognized the egg yolk very well and therefore less antibody was needed for the detection. Possibly even higher dilutions then the 1:2000 dilution could have been possible. Group 4 matched the curve parameters of whole egg powder and showed a low C-value. The linear range over 2 orders of magnitude is very favourable. It shows a flatter slope, but this makes the linear area broader and the single concentrations could be distinguished better Obtained C-values are as followed: Grp. 1: 47, Grp. 2: 15, Grp. 3: 5 and Grp. 4: 2.

3.3.3 Egg white as a standard

As shown in Figure 17 the antibody even showed response to the egg white extract. Higher dilution steps of the antibody were applied, the maximum OD for the 1:8000 dilution was at about 1.0 and showed a low C-value. For the competitive assay it is favourable to obtain OD values above 2.0. The slopes were not as steep as for egg yolk or whole egg powder.
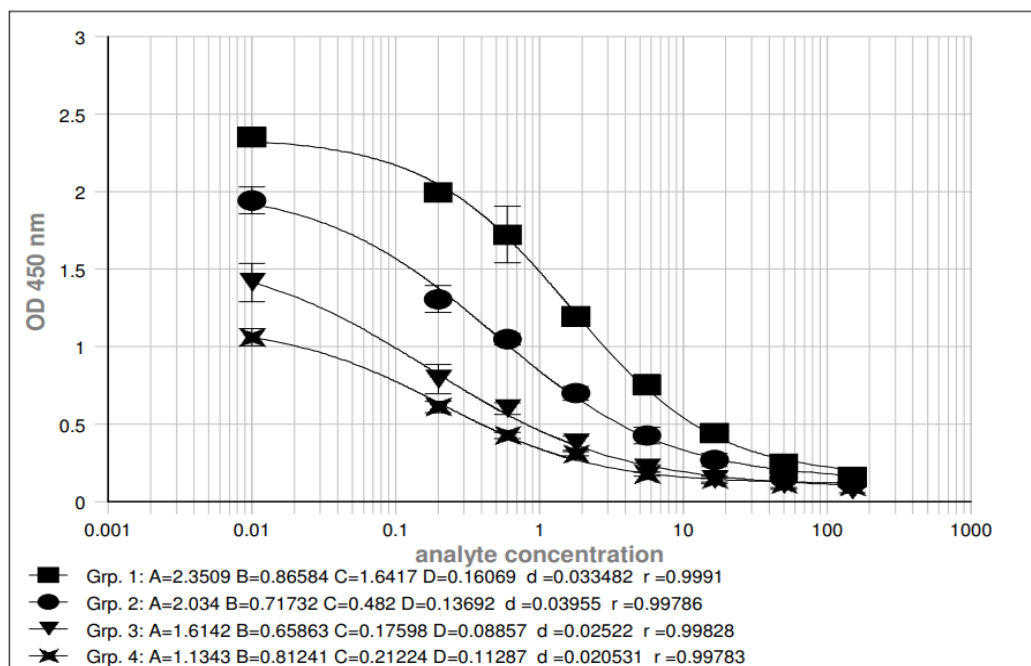
Figure 17 Egg white competitive ELISA; Rabbit anti-egg yolk dilution steps 1:1000 (Grp. 1), 1:2000 (Grp. 2), 1:5000 (Grp.3), 1:8000 (Grp. 4).

The applied dilution steps were for Grp. 1: 1000 with a C-value of 1, for Grp. 2: 1:2000 with a C-value of 0.5, for Grp. 3 a dilution step of 1:5000 with a C-value of 0.2 and for Grp. 4 a dilution step of 1:8000 was applied with a corresponding C-value of 0.1. This showed that the antibody detects also egg white proteins with a high affinity and not only egg yolk proteins. Unfortunately, the set-up was not tested with lower dilutions like for whole egg powder and egg yolk.

3.4 Synthesis and characterization of colloidal gold

The synthesis of gold nanoparticles by reduction of metal salts is a quite simple process, which only requires the mixing of reagents at defined conditions. These conditions can affect the resulting morphology of the metal particles. This work is focused on different gold concentrations and pH values.

Gold nanoparticles with different diameters ranging from 20 nm – 70 nm were prepared as described in 2.3. The recorded spectra showed absorption curves with a distinct maximum, nearly constant 525 nm depending on the size of the particle. The peak absorbance wavelength increased with the particle diameter. The peak optical density (OD) or absorbance of the sample correlated linear to the concentration of nanoparticle in solution. Figure 18 represents the UV-Vis absorption spectrum and the SEM image of the produced gold nanoparticles. The synthesized particles which were used for the experiments had a mean particle diameter of 40 nm. UV-VIS spectra of 40 nm AUNPs exhibited an extinction peak at 530 nm.
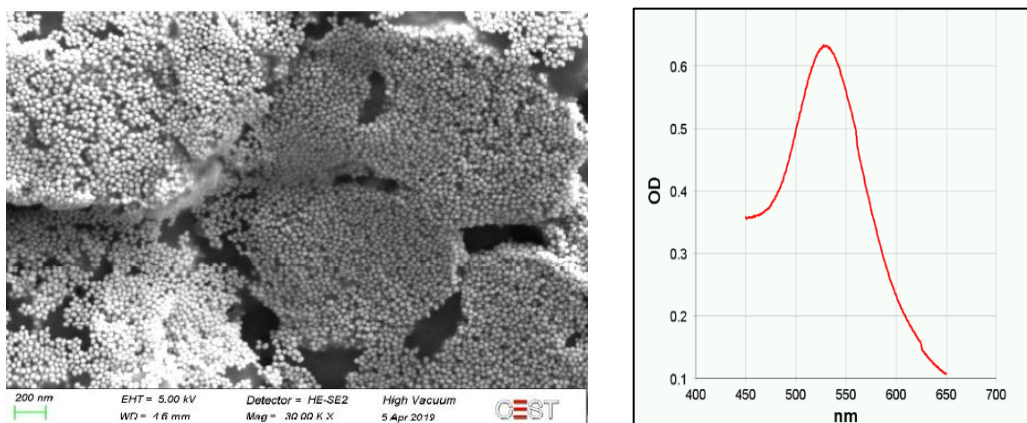
Figure 18 SEM and UV spectra of colloidal AUNPs with an average diameter of 40 nm

Besides the shape and size dispersion of the particles, also the colour was an important quality control in the nanoparticle synthesis. The typical red wine colour of the prepared solution may be considered as a pointer of quality.

Figure 19 Colour of colloidal gold solutions with different lightening

Figure 19 show the prepared batches with different amounts of gold ranging from 0.008%, 0.009%, 0.010%, 0.011% to 0.012%. The solutions were clear and no precipitation occurred.

It was visible that the amount of gold used for preparing the solution showed differences in the colour and intensity of the solution. The more gold used, the darker the solution was. Nevertheless, all GNP solution showed the typical red wine colour and therefore the successful formation of gold nanoparticles.

For the preparation of the antibody-gold conjugate the batch with 0.010% gold was chosen, according to the standard protocol.

The absorption spectra of the above-mentioned samples with varying amounts of gold are plotted in Figure 20.
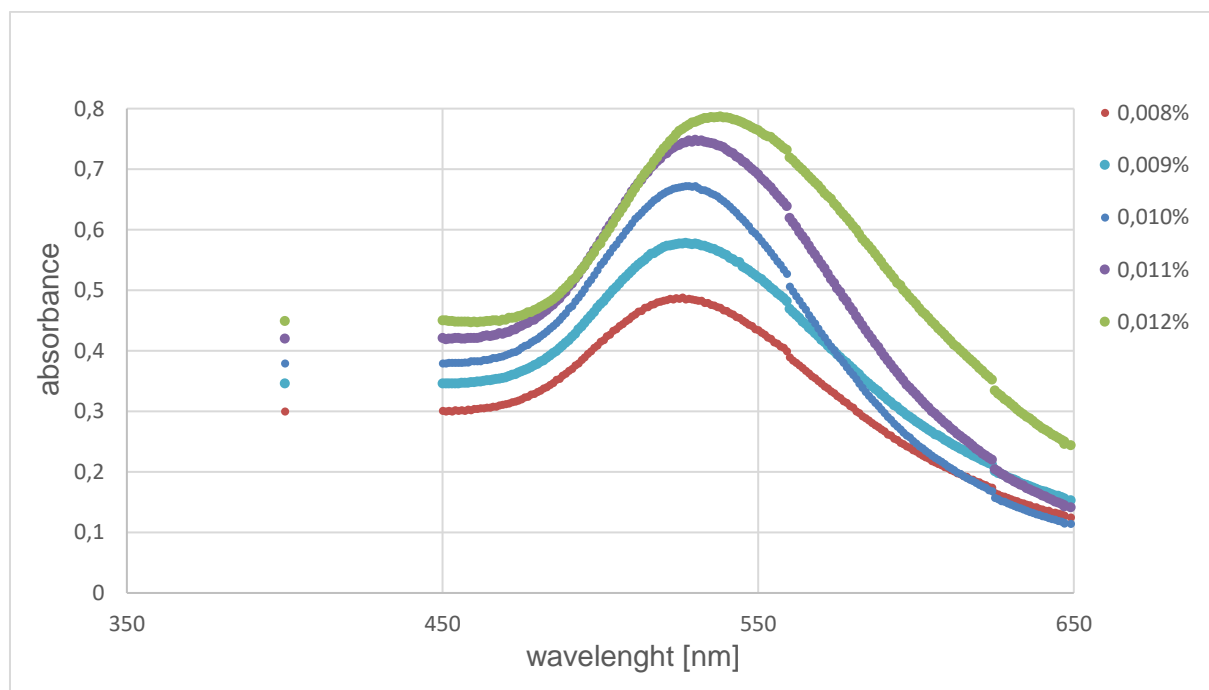
Figure 20 Absorption spectra of colloidal GNPs with different amounts of gold

The maximum absorption of gold nanoparticles occurred at the wavelength of 526 nm for 0.008%, 530 nm, for 0.09%, 527nm for 0.010%, 530 nm for 0.011% and for 0.012% the highest absorption was reached at a maximum of 532 nm. This absorption maximum differences indicated different sizes of the colloidal nanoparticles, which are discussed in more detail on the next page.
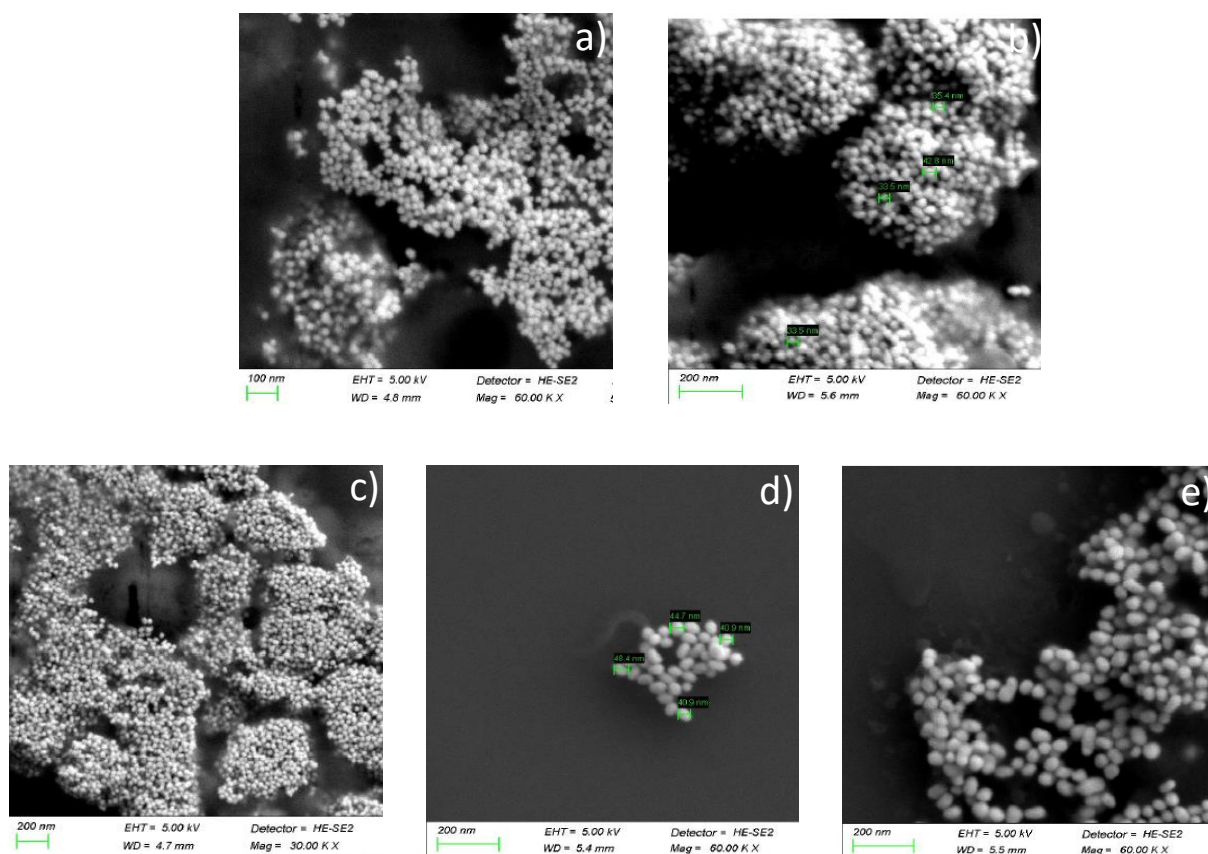
Figure 21 SEM images of colloidal GNP of (a) 0.008%, (b) 0.009%, (c) 0.01%, (d) 0.011% and (e) 0.012%,

SEM images of the five samples described above are listed in Figure 21. The gold nanoparticles had an average diameter 30 (a), 39 (b), 38 (c), 44 (d) and 54 (e) nm. The nanoparticles' shape is nearly spherical. When comparing Figure 19, Figure 20 and Figure 21 the absorption maxima were close to 530 nm which gave also rise to the bright red colour and correlated well with the spherical shape of the ~ 40 nm gold particles.

The aggregation of gold nanoparticles is an easy to visualize process. An unstable solution will show an aggregation of nanoparticles in form of large clusters which will end up with precipitation. This phenomenon was observed in several batches, during long-term stability tests. Another reason for the aggregation could have been the drying treatment of the gold particles before their evaluation in the microscope. The drying process could have led to aggregation of the gold particles.

0.008%		0.009%		0.010%		0.011%		0.012%	
Diameter [nm]	λmax [nm]	Diameter [nm]	λmax [nm]	Diameter [nm]	λmax [nm]	Diameter [nm]	λmax [nm]	Diameter [nm]	λmax [nm]
30	522	39	527	38	527	44	529	54	532
35	526	37	526	35	530	44	530	50	532
38	526	37	527	33	526	45	531	54	532

As seen in Table 8 the size lay in the mentioned range between 520 and 530 nm. The wavelength of maximum absorbance at 530 nm using the standard method [0.010%] correlated with the formation of gold nanoparticles having a diameter of 30-40 nm.

3.5 Stability tests of gold nanoparticles

The following Figure 21 shows the absorption spectra of gold nanoparticles with varying concentrations of gold, recorded over a period of 9 month.

The samples were prepared in March, stored for almost 9 months and tested 3 times within this period (March, June and December) via UV/VIS spectrometer. The suspensions were stored at 4°C in the dark. The blue line shows the batch prepared in March, measured shortly after preparation. The red line shows the measurement in in June and the green line presents the measurement in December, which is 9 months after preparation.

As seen in the spectra a, b, c and d of Figure 22, the stored AUNPs presented rather the same spectral appearance as the corresponding freshly prepared solutions. However, if it was not visible in the spectra, but the solution b) showed insoluble aggregates, a change in colour and turbidity. When having a look at spectra e) it was visible that the absorbance after 9 month storage is way lower than for the other solutions. The optical density after preparation was 0.578. After 6 months there was a slight drop to 0.507. For the last measurement after 9 month an optical density of only 0.124 was obtained. This huge drop visible in the spectra corresponded well to the shown picture f). When comparing Figure 22 to Figure 19 where the freshly prepared solution is shown, it could be seen that the colour was less intense and a visible precipitate was observed.
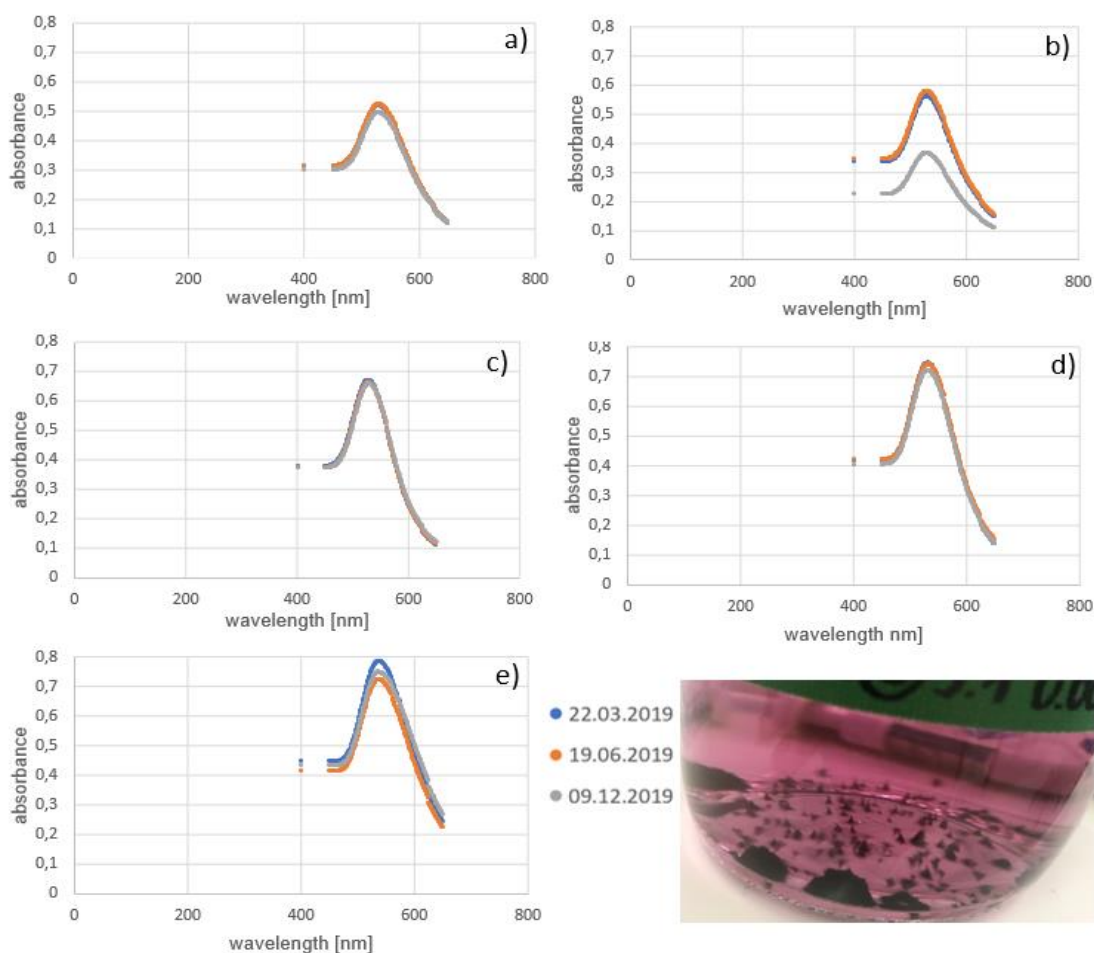
Figure 22 UV-Vis spectra of gold nanoparticles (AuNPs) with varying gold concentrations. a) 0.008%, b) 0.009% c) 0.010%, d) 0.011%, e) 0.012%, , f) corresponding picture to e)

Due to the higher negative charge (0.010 – 0.012% of gold) of the particles the stability was increased. If the particles are charged positive to some extent, the negative zeta potential is weakened, this causes faster aggregation and precipitation. The zeta potential provides important information on surface charge. Nevertheless, zeta potential measurements were not carried out within this work.

3.6 Varying pH for the preparation of gold nanoparticles

Gold solutions with different pH were prepared. It is known that size can be changed and determined by controlling the pH value of the solution within the preparation process. In this study, four samples with different pH values were prepared and observed. Samples are shown in Figure 23. The results are shown in Table 9.

UV/VIS spectra were recorded. SEM images were taken once for each batch shortly after preparation.

Table 9 Summary of the size distribution of the gold nanoparticles from the first batch

pH 3		pH 4		pH 5		pH 6	
Diameter [nm]	λmax [nm]	Diameter [nm]	λmax [nm]	Diameter [nm]	λmax [nm]	Diameter [nm]	λmax [nm]
73	560	45	539	30	528	30	528
67	557	40	533	25	528	30	530
60	549	30	531	30	530	25	530

For the preparation of gold solutions with different pH values citric acid and trisodium citrate dihydrate was chosen. Because the ions which absorb to the surface of gold particles are OH$^-$ but also citrate ions. Citrate ions play a major role since they act as reductant and convert gold ions (Au^{3+}) into gold atoms (Au^0) but they also reduced the impact of counter ions and enhance the stability of colloidal gold solutions, by preventing particle growth and aggregation via electrostatic repulsion *(Contreras-Trigo et al., 2018)*. The absorption of citrate ions, gives the gold particles an overall negative charge and this is responsible for the formation of a stable colloids. The color of the GNP solutions obtained showed its typical color presented in Figure 23.

Figure 23 Colour of colloidal GNPs solutions (from left to right: pH = 3, pH = 4, pH = 5, pH = 6)

When looking at Figure 23 the difference in colour is clear visible. The colloidal GNP solution changed to wine red (right, pH = 6), a lighter turbid red for pH = 5 and a dark red for pH = 4. The tests showed that gold solutions with pH = 3 changed to a purple – blueish colour. The difference in colour was caused by the size difference of the nanoparticles. When looking at Table 9 at pH 3 it's shown that the size was way bigger as for the pH 4, 5, and 6, but when looking at Figure 24 it demonstrated that the absorbance is much lower, only at 0.35, while for pH 4 it is at ~0.7. It's possible that the particles approached and aggregated into larger and larger clumps. This formation of agglomerates is connected to an optical change in the spectrum, the higher absorbance peak and the blueish colour of the solution. After some time, the gold nanoparticles aggregate to a point at which they started to precipitate. The solution

became almost white and clear, and a black precipitate could be seen on the bottom of the bottle.

The growth and stability of colloidal suspensions are a result of the sum of the attractive and repulsive forces between individual particles. One important indicator is the zeta potential, which can be changed by controlling the pH value of the solution. Solutions with a low pH value show positive surface charge, an increase in H^+ ion concentration and this increases the repulsion of positive ions through the bulk. While solutions with a higher pH value, show negative surface charge, an increase in the OH^- ion concentration a repulsion of negative ions is created. In both cases the zeta potential increases and consequently the stability of the GNPs solutions increases *(Alzoubi et al., 2015).*
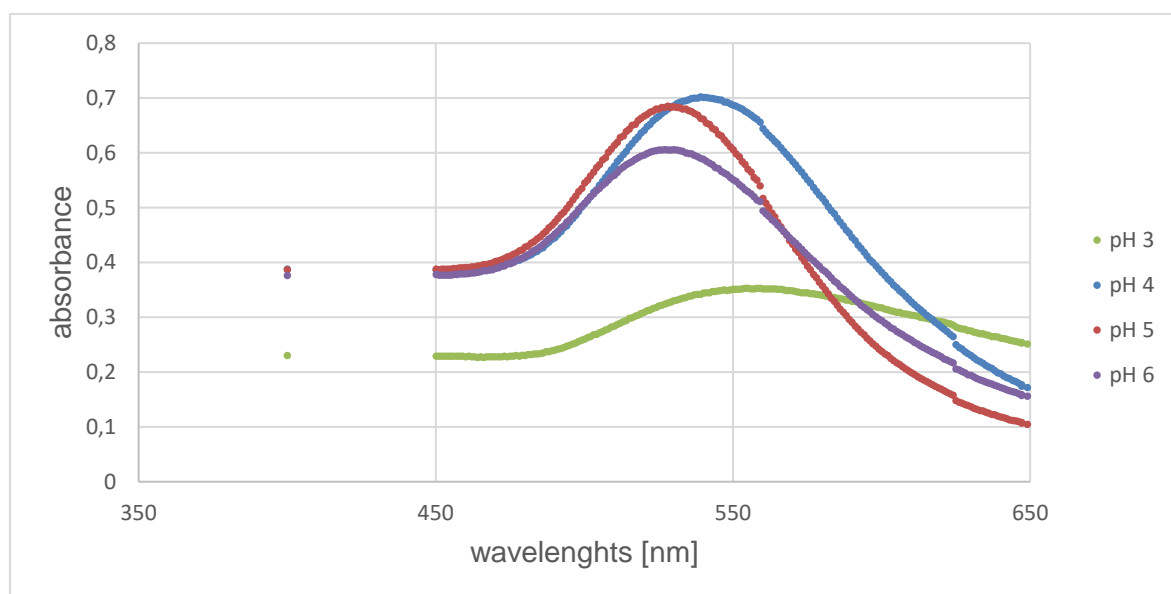
Figure 24 Absorption spectra of gold nanoparticles obtained by citrate reduction at pH 3 (green line), 4 (blue line), 5 (red line) and 6 (purple line)

Figure 24 presents the absorption spectra of the AuNPs solutions synthesized at variable pH values (3, 4, 5 and 6). It was measured within a range of 450 – 650 nm. The maximum occurred at a wavelength of 560 nm for pH = 3, 539 nm for pH 4, 528 nm for pH = 5 and 528 nm for pH = 6. The pH value had a relevant effect on the curves and the absorption maximum. The average size at pH 3 was about 66 nm with aggregated clusters, while at pH 4 the average size was only 38 nm almost half the size of pH 3. For pH 5 and 6 similar results with an average of 28 nm were obtained.

Agglomeration of GNP is a major problem because of the different properties of particles. To get a clear vision about the effect of the pH on the size and the state of agglomeration, several

samples at different pH values were prepared. SEM images are presented in Figure 25. SEM micrographs of gold nanoparticles (pH 4,5,6,) were predominantly spherical with a smooth looking morphology.
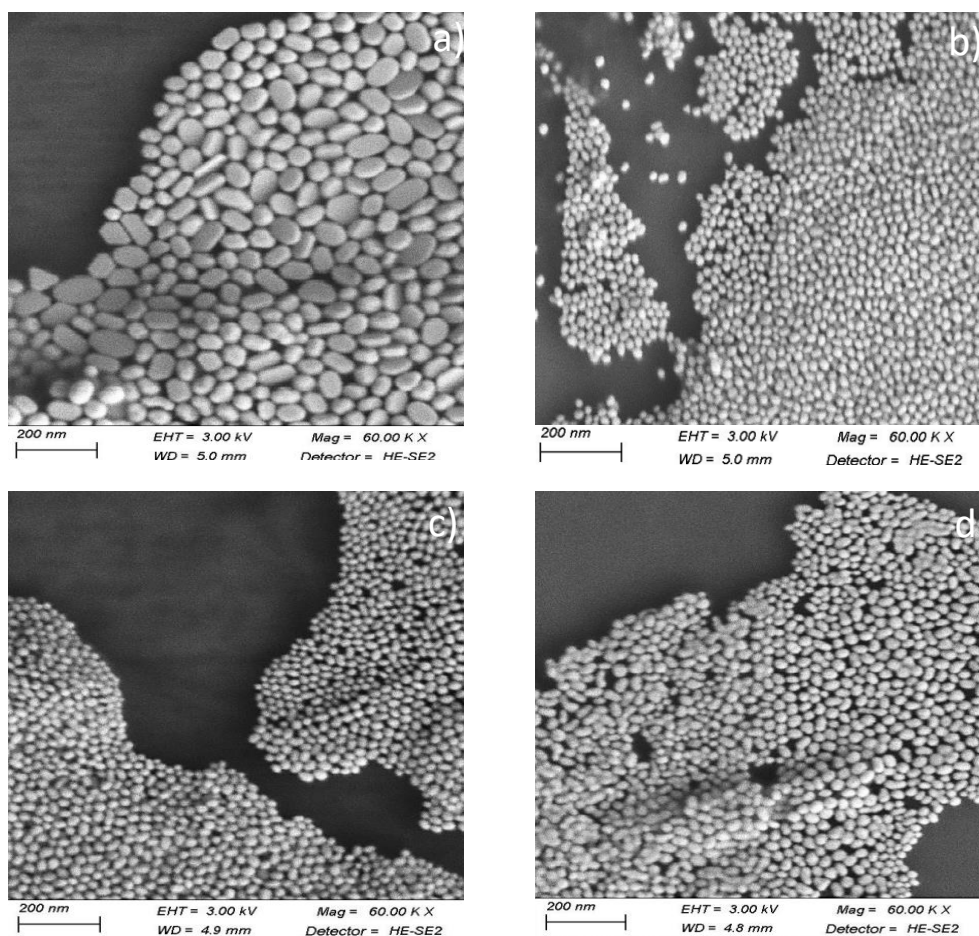
Figure 25 SEM images of colloidal GNPs of pH values (a) 3, (b) 4, (c) 5 and (d) 6.

The size of the synthesized particles ranged between of 25-40 nm. It was noticeable that for pH less than 4 the particles changed in their morphology and size, with an average of ~66 nm. The size of the nanoparticles clearly increased with decreasing pH. When the synthesis was done at pH 3, the nanoparticles tended to have oblate shapes. Another prevailing shape were polyhedrons and long nanoparticles. As the pH of the citrate solution increased to 6 both the mean and the particle diameter decreased and reached a lower limit of 25 nm. In this range the spherical shape was more abundant. *(Derjaguin and Landau 1941; Verwey and Overbeek 1948)*

Storage stability study of gold nanoparticles with varying pH

Figure 26 presents the absorptions spectra of the AuNPs solutions synthesized at variable pH values. The same storage stability tests of plain AuNPs were carried out for the gold solutions with varying pH in a period of 8 month (April – June – December). The gold samples of pH 4, 5 & 6 presented the same visual appearance as the fresh prepared solutions. The UV/VIS absorption spectra of AUNPs after storage were very similar to those of freshly prepared solutions. As expect for pH 3, there was a drop in the optical density for the measurements in June and December, as gold particles aggregated and precipitated.
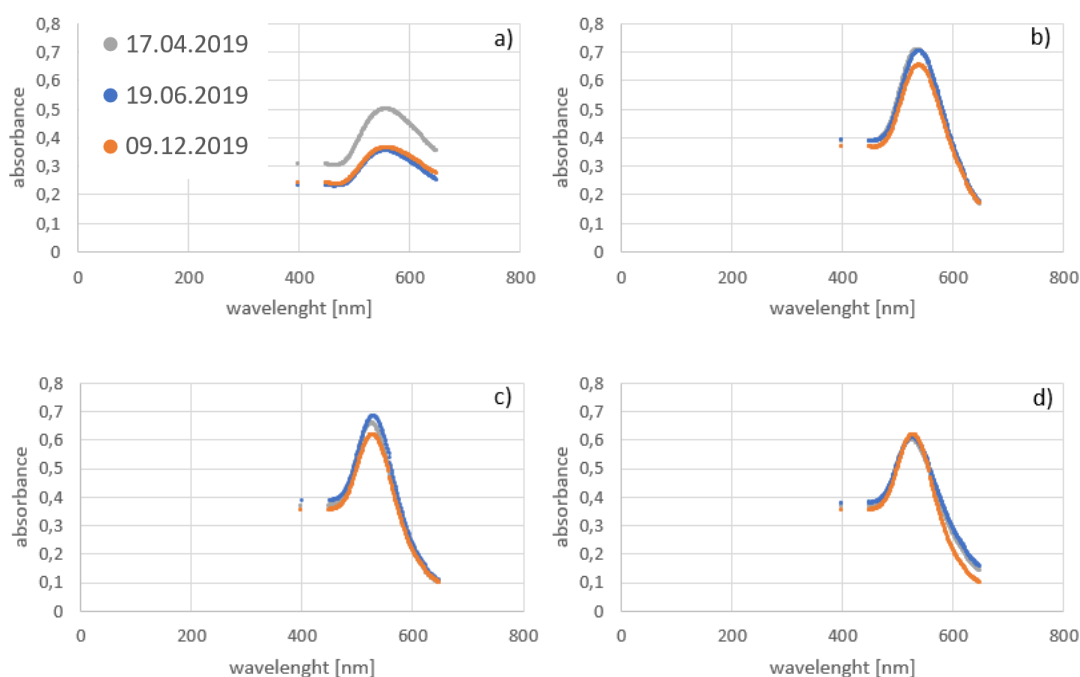
Figure 26 UV-Vis spectra of gold nanoparticles (AuNPs) with different pH values; a) pH 3, b) pH 4, c) pH 5, d) pH 6 – presenting three lines for stability control

The recorded spectra show three lines. The grey curve represents the absorbance values recorded shortly after preparation in April. The blue line shows the measurement in June, 2 months after preparation. The orange line presents the measurement in December, 8 months after preparation. All batches were stored at 4°C in the dark.

When comparing the recorded spectra, they all, except for pH 3, showed the same behavior and remained stable. The gold solution with prepared with pH 3, showed a drop in optical density, also dark precipitates were observed at the bottom of the flask, which were likely large AuNP aggregates settling out. For some samples, a colour gradient was observed in the solution. Big particles/aggregates on the bottom showed a darker colour and the smaller AuNPs a lighter in the upper part of the suspension.

3.7 Colloidal gold solution titration experiments

According to the procedure, described in 2.3 colloidal gold was prepared with a particle size of around 40 nm. The prepared gold solution was used for the coupling of the previous affinity purified antibodies.

3.7.1 Titration of rabbit IgG with colloidal gold

The titration experiments were performed according to the procedure described in 1.9. Concentrations in a range from 0 to 20 µg antibody/mL gold solution were prepared. After incubation NaCl was added to identify the antibody coverage rate of the colloidal gold particles.
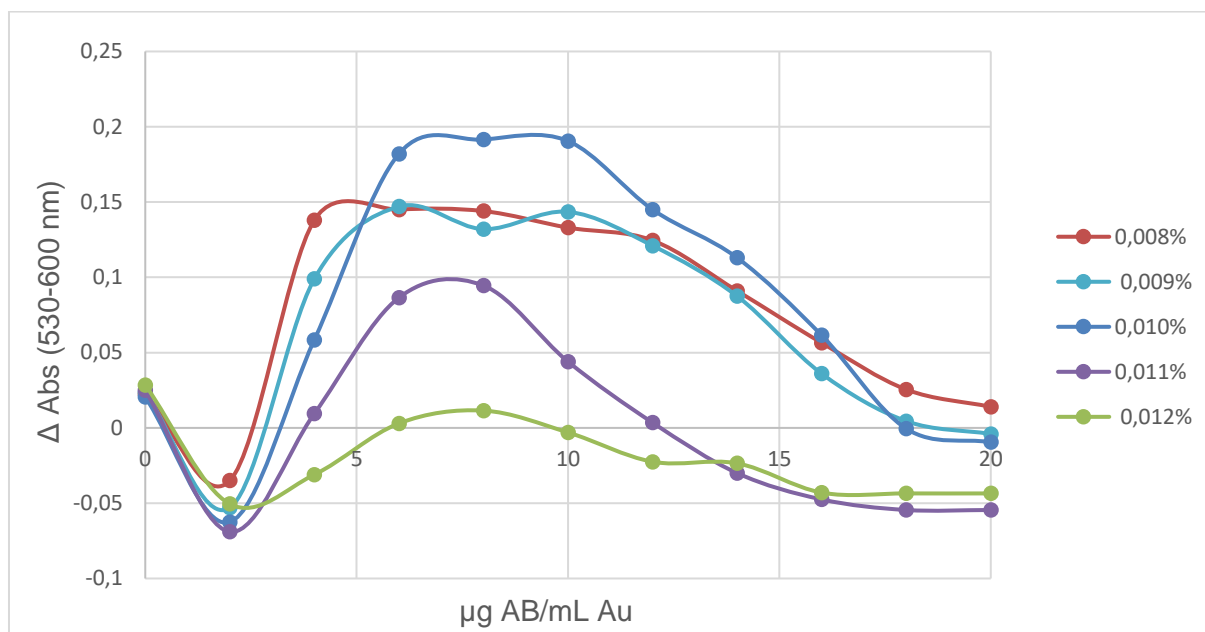
Figure 27 Titration curve of antibody from rabbit with gold in 4 different concentrations; Absorbance difference between 530 – 600 nm

At this point the surface of the gold particle was supposed to be covered with antibody, if not, free negatively charged binding sites remain. The addition of the positively charged sodium ions led to agglomeration of the gold particles and due to this reaction, a change in the absorption maximum occurred. The photometrical measurement allowed an estimation of the optimal coupling ratio of gold and antibody. For the first part within this work, the titration and conjugation experiments were carried out using Protein G affinity purified rabbit IgG.
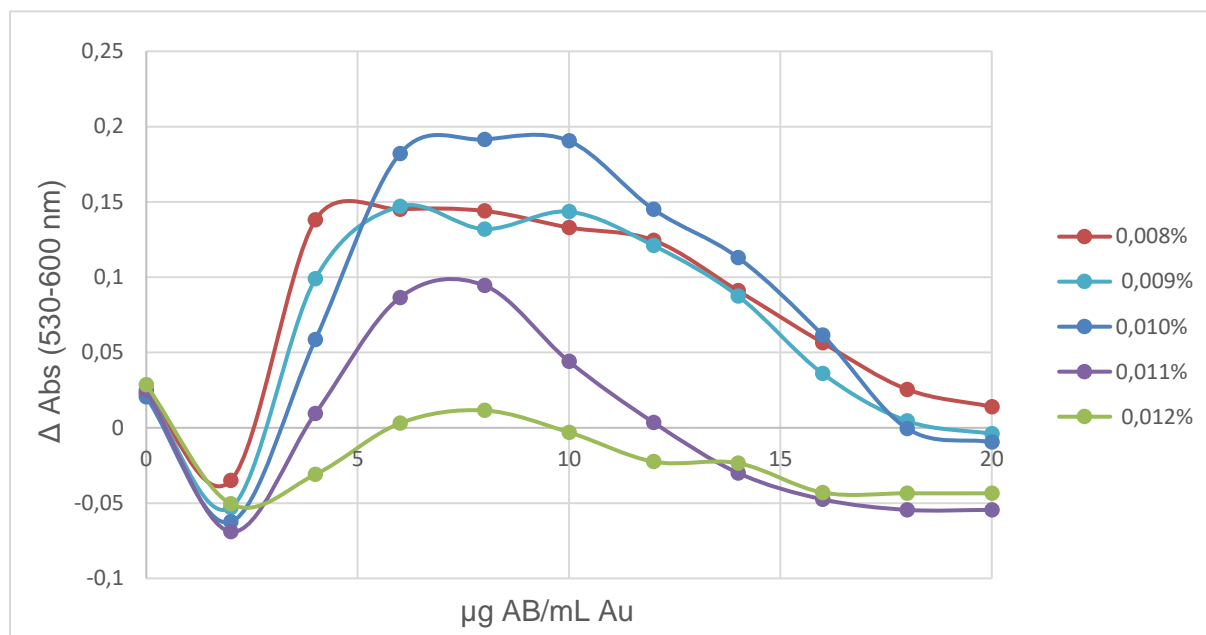
Figure 27 the solution with 0.010% gold, worked best for the experiment, as the highest absorbance difference was reached. The batch with 0.008% showed also quiet stable plateau but the absorption maximum was not as high as for 0.010%.

At about 5 µg rabbit IgG/mL gold the curve reached the beginning of the saturation curve and the asymptotic region of the x-axis. No asymptotic region could be reached for the other solutions. Therefore, they were not used for further experiments. Even for the standard solution with 0.01% of gold only a very short stable plateau was reached.

As already mentioned additionally to the gold batches with different amounts of gold, pH variation experiments were prepared according to 2.3.1 and titration experiments were conducted.
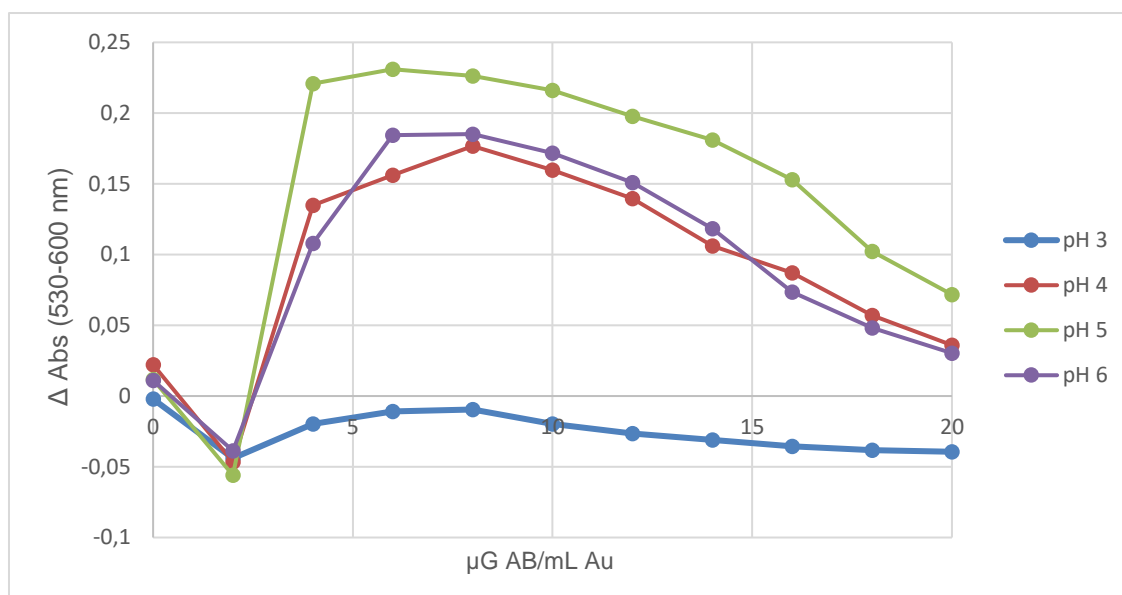
Figure 28 Titration curve of antibody (ab) from rabbit IgG with gold at 4 different pH values. Absorbance difference between 530 and 600 nm.

The pH range of colloidal gold was chosen between 3 to 6. Figure 28 represents titration experiments for rabbit IgG at 4 different pH values. Three batches of each pH value were prepared and measured. It was shown that the colloidal gold with pH 3 gave an unstable curve and no saturation curve could be achieved. A possible reason for the obtained curved for pH 3 and 4 is that the gold solution is not stable under acidic conditions.

An absorbance increase could be observed at pH 4 and 6. The colloidal gold with pH 4 and 6 showed a better behavior and a quiet stable plateau and saturation curve was reached. The optimum curve was reached by using a pH of 5. This led to the assumption that binding direction of the antibody starts to change with pH values below 4 or above 6. Titration curves of all batches were recorded.

3.7.2 Titration of sheep IgG with colloidal gold

In comparison for later work a commercially available polyclonal donkey anti sheep antibody was used. In this part of the work, not ovomucoid was tested, but water contaminant Dipyrone. The antibodies were already affinity purified and ready to use. The following figure shows the titration curve of polyclonal sheep IgG and the colloidal gold solution.
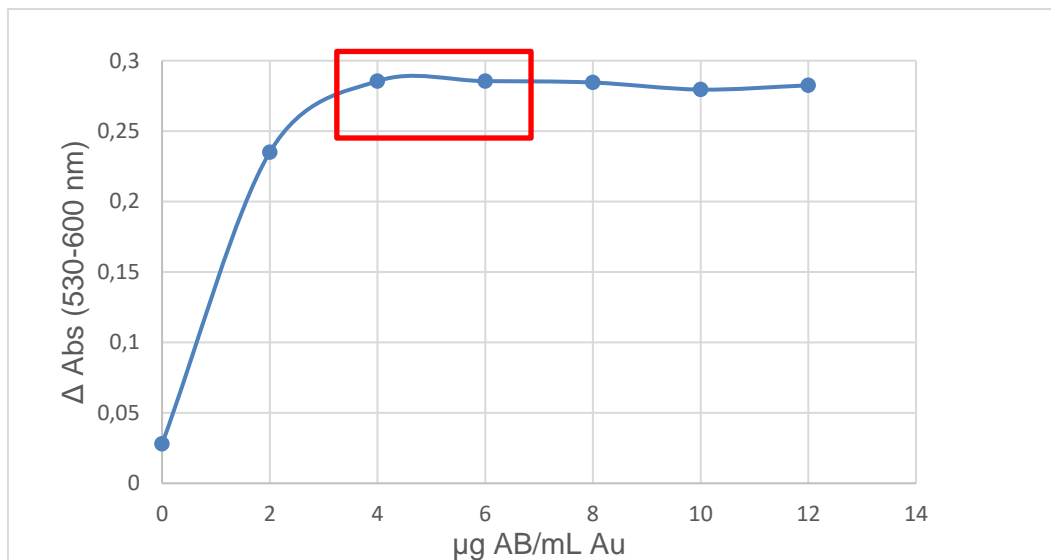
Figure 29 Titration curve of polyclonal antibody (ab) from sheep-IgG, using colloidal gold batch 1.3 (0.010%) at pH 8.5, absorbance difference between 530 nm and 600 nm.

In this case different concentrations ranging from 0 to 12 µg antibody/mL gold solution were prepared. The evaluation of the photometrical measurement allowed an estimation of the optimal coupling ratio. All other titration experiments were carried out testing antibody concentrations ranging from 0 to 20 µg/mL gold. In this case the maximum amount was 12 µg/mL gold solution. Furthermore, the other experiments showed that the saturation was reached earlier. Within this titration experiment a very stable plateau could be reached, while the beginning of the saturation curve was at about 5 µg sheep IgG/mL gold. As a result of these experiments a concentration of 5 µg/mL was therefore selected for the conjugate production with the 40 nm gold nanoparticles. The titration experiments represent the best and smallest antibody concentration for the further coupling experiments. The rabbit antibody reached the beginning of the asymptotic region of the x-axis at 8 µg rabbit IgG/mL gold solution. The commercially purchased sheep IgG reached its saturation at around 5 µg antibody/mL gold solution. The titration experiments were done for the further lateral flow test production. Further coupling procedures were based on the obtained results in the titrati9on experiments.

3.8 Lateral Flow test production and test procedure – Egg

Polyclonal antibodies were produced in rabbits and purified by affinity chromatography. Whole egg powder was extracted with 0.1 M TBS pH 8.2 containing NaCl and Tween20. The LFD was studied as visual qualitative assay which shows a simple presence or absence of the target analytes. Therefore, the colour intensity is an important aspect. It must be strong enough to differentiate between the negative control and the samples. The principle of the LFD functioning is shown in Figure 6. Briefly, the sample with the probe was added to the sample pad. From this point it was transferred by capillary forces across the detection membrane where it reacted with the recognition elements anchored as test and control lines. The colloidal gold labelled antibodies which were solubilized in the well, reacts with the analyte if it was present.

In detail, the test line was formed by Anti-Rabbit Ab, while the control line was formed by Anti-Rabbit egg-yolk Ab. The colour of the test line was directly correlated to the amount of allergen in the sample. Means, qualitatively, the more intense the colour of the test line, the higher the level of allergen in the sample and vice versa.

The signal was first visible after about 30 seconds. The colour and signal formation for test and control line was complete after 5 -10 minutes and were evaluated visually. Optimization tests, including the choice of the best working membrane, the optimal immobilization concentration for test and control line and optimal incubation time were studied.

As mentioned in the material and methods part, various membranes with different pore sizes were investigated. Since each membrane has unique characteristics the selection of an appropriate membrane is a crucial step in the development process of a lateral flow test. Within this work membranes such as Millipore FF75, FF80, FF120 and FF 180 were tested. The number basically indicated how long the liquid takes to process 4 cm of membrane, f.e. 70 or 180 seconds. Membranes with smaller pores have a higher assay run time and therefore the liquid has more time to interact with the antibody striped at the test line. Pre-tests were carried out applying spots of anti-rabbit as "control-spot" and whole-egg powder as "test-spot", on the membrane. These dot-assays were done to find out which concentration range can be applied for further tests as they were a quicker approach than already spraying the material with the sprayer. The amount of antibody-gold conjugate was varied.

First test was done with membrane FF75, which had a relative large pore size, resulting in a fast capillary flow rate. This kind of nitrocellulose membrane was already assembled on a backing card, which means only the wick/absorbend pad and sample pad had to be attached. Stripes of 4.5 mm width were cut using the Biodot cutter. Purified anti-rabbit was dotted in the

"Control line" position [1 mg/mL], while a whole egg extract was dotted in the "test line" position [1 mg/mL].

At the beginning spots were directly applied to the membrane without using a sprayer, since its much faster.Therefore a pipette was used, dots in 0.5 µL steps were directly applied on the membrane and dried at 37°C in the vacuum oven. The amounts ranged from 0.5 µL – 5 µL. After drying, the strips were dipped into the reation mixture and incubated for about 3 minutes. The mixture consisted of 10 µL [8 µg AB/mL AU] antibody-gold conjugate, 50 µL running buffer and 50 µL extraction buffer. The gold concentration was varied to check if the signal is still strong enough at a lower concentration. Therefor only 2 µL [8 µg Ab/mL AU [16 ng] were pipetted into the mixture. In Figure 30 (b) it can be seen, that the anount of antibody-gold-conjugates was too low for the dotted amount of whole egg powder extract because the control line is much weaker than at the previous strips.
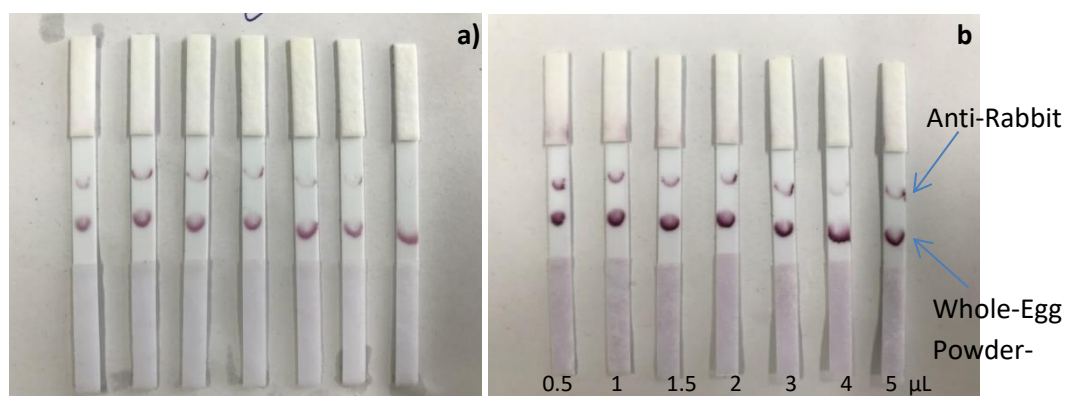
Figure 30 (a) recorded after 3 minutes, (b) recorded after 5 minutes; the amounts is in [µg] – whole egg powder protein

The next test was done using a direct antibody sandwich assay, using the same strategy of dabbing spots directy on the membrane, but this time, anti-rabbit was dotted at the control line position and anti-rabbit egg yolk at the test line position. For the test line purified Rabbit Anti-Egg yolk spots in a range of 0.5 µL – 3 µL were dabed on the membrane. The reaction mixture consisted of antibody-gold conjugate [16 ng/10 µL], running buffer and a standard of whole egg powder protein extract diluted in extraction puffer, with portein concentrations of 20, 10, 2 and 0.2 µg/mL. The whole egg powder extract with a protein concentration 0.2 µg/mL was tested again with a higher AB-AU conjugate concentration to see a difference in the intensity of the colouring.
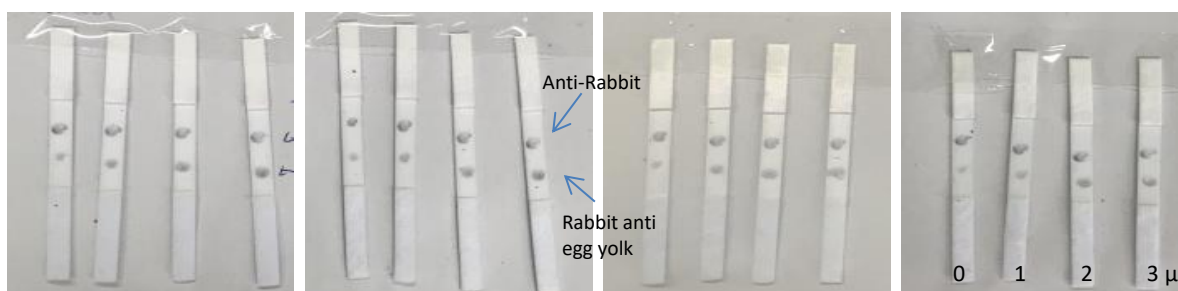
Figure 31 (a) 20µg of whole egg powder protein in 1 mL (b) 10 µg, (c) 2 µg and (c) 0.2 µg/mL

For further tests, membrane FF 80, FF 120 andFHF 180 was added. FF 80 has similar properties but slightly smaller pores than FF 75. Membrane FF 120 and FF 180 has a smaller pore size resulting in a slower capillary flow rate but therefore a higher resulting sensitivity. The use of this membrane may increase the time for the nanoparticles to bind to the test line. The procedures were done the same way as stated above only using a different membrane. In addition, membrane FF 120 was tested. Strips of all membranes were blocked with different BSA concentrations: 0%, 2%, 5%, 10% and 20%. This was only done with the sandwich format. Control line and test line contained an amount of 0.5 µg. It was tested for blank stripes, with no analyte in the reaction mixture and second 20 µg/mL whole egg powder protein were in the reaction mixture. In Figure 32, the BSA was added to the reaction tube.
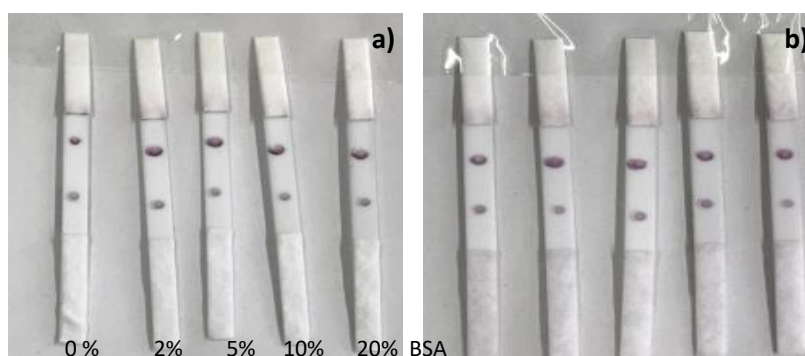
Figure 32 Membrane FF 180 with BSA concentration from 0- 20% mixed in the reaction tube; (a) no analyte in the tube (b) 20 µg/mL whole egg powder protein
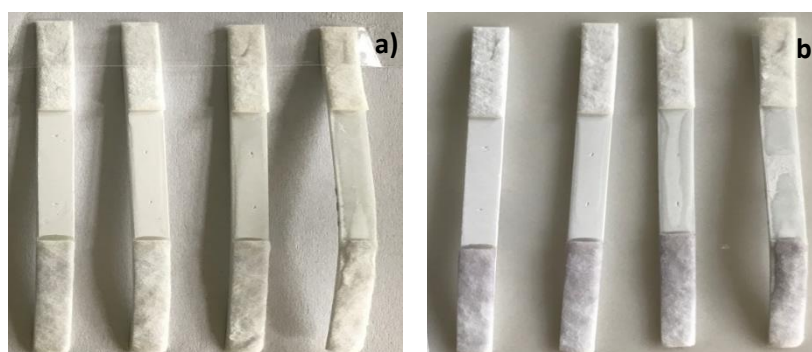
Figure 33 Membrane FF 180 incubated with BSA in a concentration from 0 – 20%; before analysis, (a) no analyte in the tube (b) 20 µg/mL whole egg powder protein

In Figure 33, strips were incubated in BSA mixtures for 30 minutes and dried overnight, before analysis. The tests showed no results at all, meaning no colour development for control and test line. Due to a BSA overload no specific binding of the antibodies could take place anymore.

After finishing the pre-tests, the set up was changed. For creating control line and test line a sprayer was used instead of dabbing spots on the membrane. Also different membranes were tested to see the running behaviour and to be able to make further optimization steps.

Based on the previous tests, two different concentrations for the test line were chosen. Anti-Rabbit was dispensed on the top of the membrane as the control line in a concentration of 1.785 mg/mL. For the test line [0.5µg] 1.785 mg/mL and [1 µg] 3.57 mg/mL analyte specific antibody were applied with a flowrate of 0.7 µL/cm on the membrane. A distance of 5 mm was chosen between test and control line.

Figure 34 Test and control line right after spraying

For the lateral flow device procedure 5 µL [8 µg Ab/mL Au] antibody-gold-conjugate were diluted in 55 µL running buffer and 50 µL extraction buffer containing the analyte in different concentrations were dripped into the sample holder. The first whole egg extract concentration set up was ranging from 0, 0.1, 4, 8, 12, 16, 20 µg/mL. Those amounts were applied to each membrane. The sample pad side was dipped in the reaction mixture and allowed the liquid to migrate to the top of the LFD. After 3 – 7 minutes the test results were evaluated visually.

After the first experiment, membrane FF 80 and FF 120 were chosen, for further experiments. For the test and control line 0.5 µg [1.785 mg/mL] was applied on the membrane. The applied concentration range of whole egg powder protein in extract rangded from [0, 0.2, 0.4, 4, 8, 16, 20, 30, 40, 100 µg/mL].
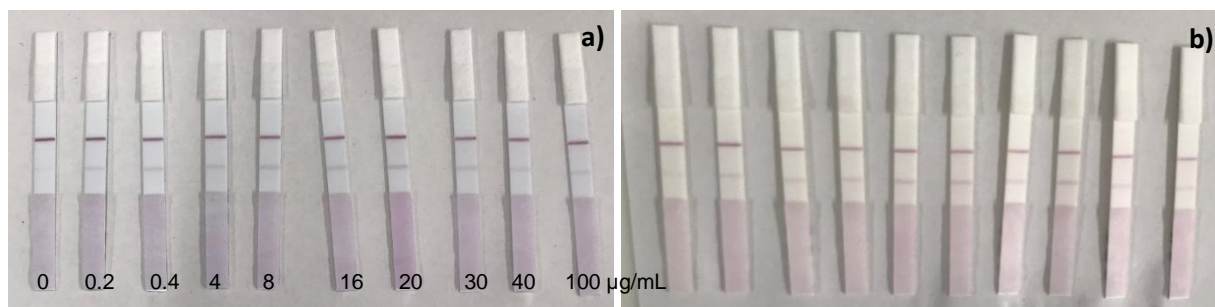
Figure 35 (a) shows membrane FF 80 and (b) FF 120; ranging from 0 to 100 µg/mL egg protein

A closer look at Figure 35 shows that membrane FF 80 gave a more intense colouring of the control line, also the blank signal showed almost no test line. To obtain a more intense signal for both lines and a clear blank signal, concentrations for control – and test line and gold-antibody conjugation were varied.

A higher amount of 2 µg [7.14 mg/mL] for the test line was chosen. The control line stayed the same with 0.5 µg. Different amounts of gold-antibody conjugate in the well were tested. It was done with 2 µL, 5µL and 8 µL [8 µg Ab /mL AU] of antibody-gold conjugate, shown in Figure 36. The protein concentration of whole egg extract in the well ranged from 0, 0.1, 0.2, 1, 2, 4 µg/mL. Since the results were not as good as expected the set-up was changed again. All lines showed the same intensity. There was no difference between test and control line or high and low concentration of the analyte.
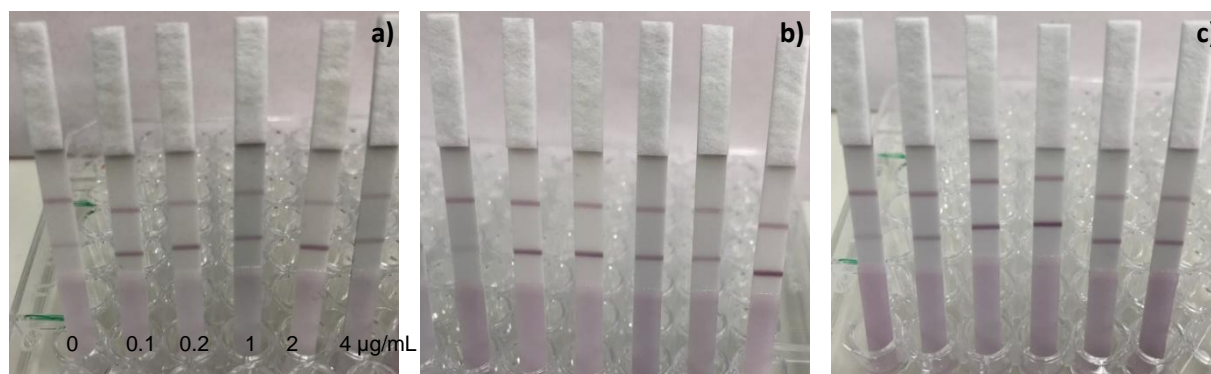
Figure 36 Membrane FF 80; (a) 2 µL AU, (b) 5 µL AU, (c) 8 µL AU [8 µg Ab/mL AU] in the reaction tube

Finally, membrane FF 80 was chosen for our tests, since it suited best in combination with our analyte. For striping test and control line the reagent concentration in [mg/mL], dispense speed [cm/sec] and dispense rate [µL/cm] have to be considered. Test and control line were applied with a flowrate of 0.7 µL/cm. To yield 1 µg of anti-rabbit on the control line and 1 µg of anti-rabbit egg yolk on the test line with the mentioned flowrate a solution of 3.57 mg/mL was prepared and sprayed on the membrane which had a width of 0.4 cm.

For optimization reasons the concentration of control and test line were changed to 1 µg [3.57 mg/mL] for both lines. The whole egg concentrations stayed the same and the amounts of the Ab-Au-conjugate remained the same. As demonstrated in Figure 37 the colour intensity of the test line correlated with the increasing amounts of analyte concentration Different amount of gold-conjugate were tested to see if it makes a difference in signalling.
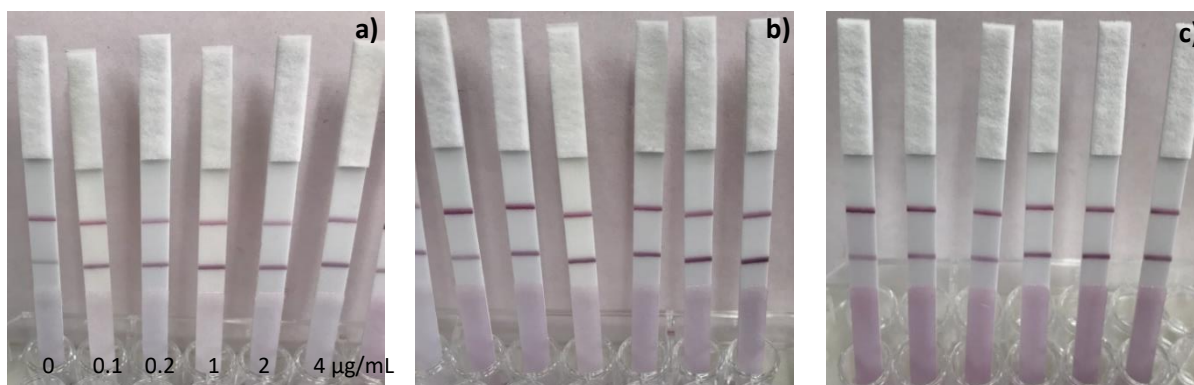
Figure 37 the concentration the well ranged from 0 to 4 µg/mL whole egg powder protein, using different amounts of antibody-gold-conjugate; (a)2 µL, (b) 5 µL and (c) 8 µL [8 µg Ab /mL AU]

There was still the problem with false positive results. That means the test strips showed a test line in the absence of the target, which might indicate non-specific absorption. To overcome this phenome different blocking agents where applied.

Since already many different concentrations for test and control line were investigated, different membranes, gold amounts and various blocking reagents were tested to obtain correct signals. One of the main problems was to completely get rid of the test line when no analyte was in the sample. A very common strategy to reduce non-specific bonding or adsorption is to use blocking reagents. The best results were obtained using Ficoll® 400 as a blocking agent, as shown in Figure 38.
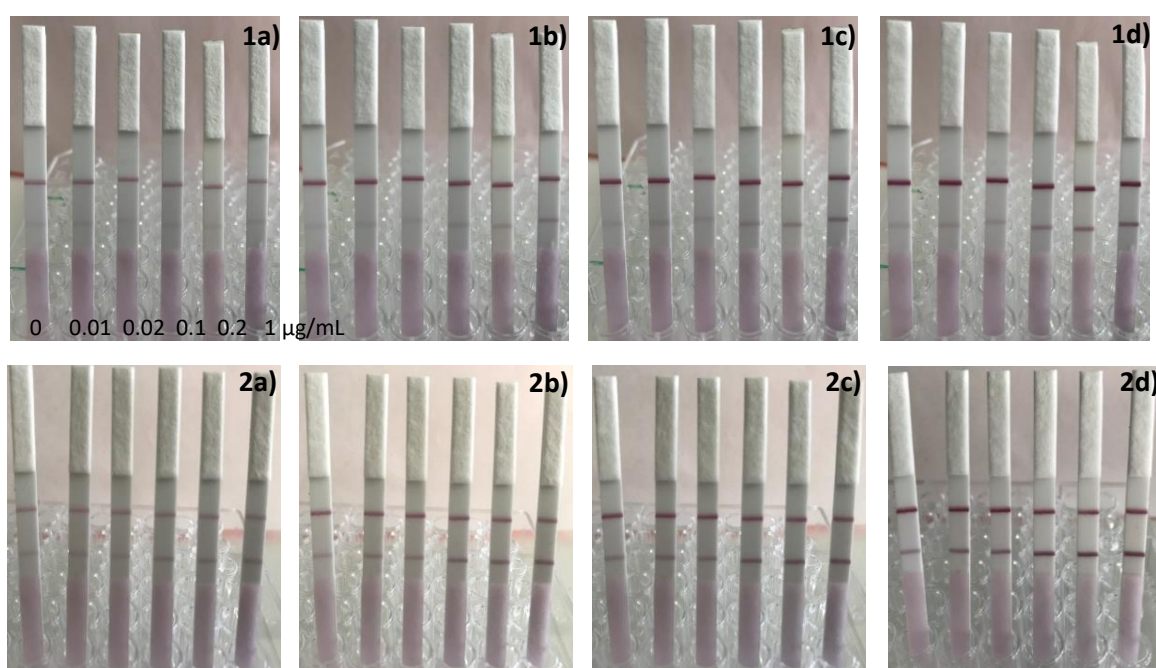
Figure 38 Detection of egg-yolk in standard samples, 1) was done with the addition of 0.1% Ficoll® 400 and 2) no Ficoll® 400 was added as blocking agent. The standard solutions of egg yolk at final concentrationof 0, 0.01, 0.02, 0.1, 0.2, 1 µg/mL whole egg powder protein in each well

Figure 38 was recorded after 1, 3, 5 and 10 minutes, the final visual readout was done after 10 minutes. The difference between the picture series 1 and 2 is the use of 0.1% Ficoll® 400, as blocking reagent. It is visible that the test line in general is more intense without Ficoll® 400, but also shows a control line at the blank position. At the upper pictures the wanted colour gradient from left to right is visible, as it is supposed to be. But still no clear distinction could be achieved between the negative (0.05 M PBS) and positive samples. None of the concentration could be detected reliable with the in house developed LFD. This is possible due to some nonspecific binding.

3.8.1 Determination of matrix effects – in Results

For the determination of the matrix effects the same membranes were used. The matrix substances were shortbread and cornflakes, both did not contain egg naturally. To evealuate the detection performance of the LFDs the samples were spiked with different concentrations of whole egg powder protein: 0, 0.2, 2, 4, 8, 16, 20 µg/mL.
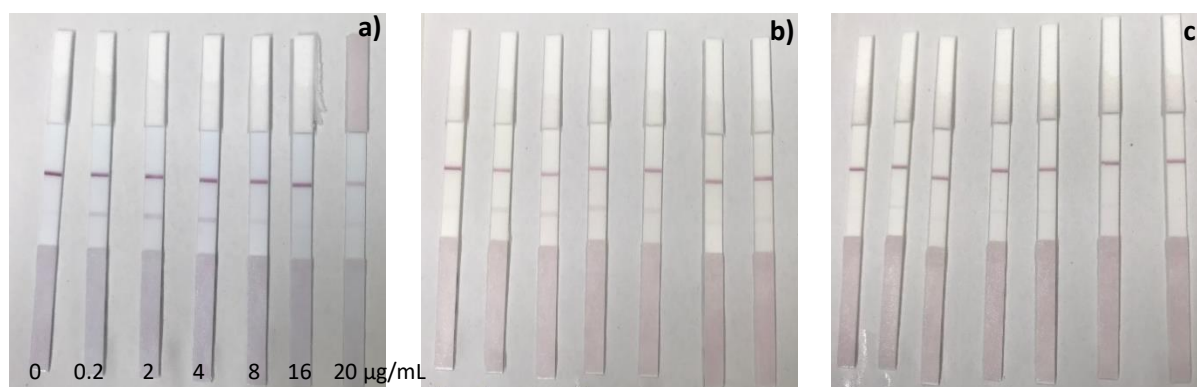
Figure 39 Determination of matrix effects in shortbread, using different membrane pore sizes; a) FF 80, b) FF 120, c) FF 180
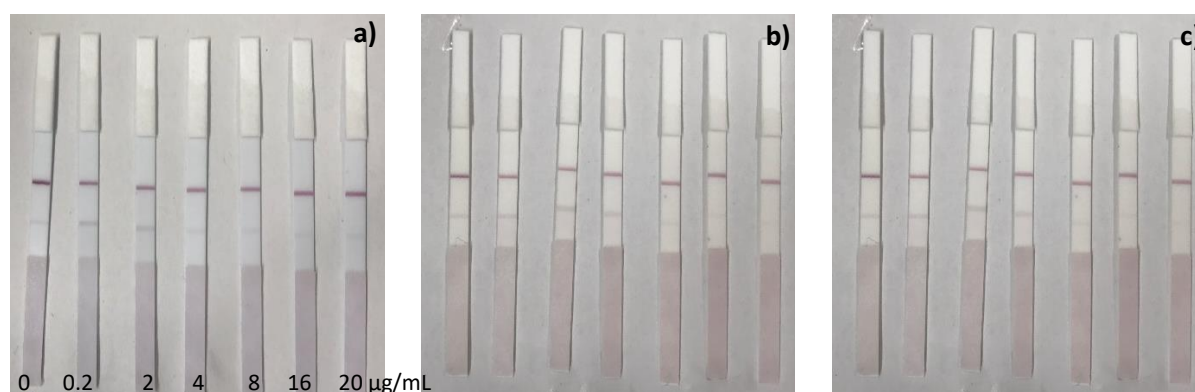
Figure 40 Determination of matrix effects in Cornflakes; using different membrane pore sizes; a) FF 80, b) FF 120, c) FF 180

The matrix had no effect on the intensity of the control line. It was visible that membrane FF 80 showed the strongest colour development. But the resulting colour intensities of the test line generated by the matrix samples did not show the wanted results, because it was not strong enough and it was not possible to determine a reliable YES or NO result.

3.8.2 Application in real matrix samples

To see, if the developed LFD can distinguish between real food samples that contain egg and real food sampled that do not contain egg, exctracts of real foods were tested.

Table 10 Used products for matrix application

		Product	**Allergen**	**Signal**
a)		Tassen Schokoküchlein Glutenfrei	Volleipulver Hühnereiweißpulver	
b)		Tassen Schokoküchlein	Hühnereiweißpulver	
c)		Clever Schokoladenkuchen	Vollei 21%	
d)		Clever Stracciatellakuchen	Vollei 19%	
e)		S-Budget Kartoffelgnocchi	KEIN EI	
f)		SPAR Semmelknödel	VOLLEI	Signal
g)		S-Budget Eiswaffel	KEIN EI	
h)		SPAR Enjoy Eierwaffel	Vollei 32%	
i)		S-Budget Eierbiskotten	Vollei 29%	

For this trial also membrane FF80 was chosen. An amount of 1 µg [3.57 mg/mL] of anti-rabbit egg yolk was applied for the test line an the same amount of anti-rabbit was applied for the control line. In the reaction tube the undiluted extract, the running buffer, 0.1% of blocking reagent, Ficoll® 400 and the antibody-gold conjugate was mixed together. For each of the nine samples a blank, which is running buffer was tested too. After 10 minutes the readout was done.
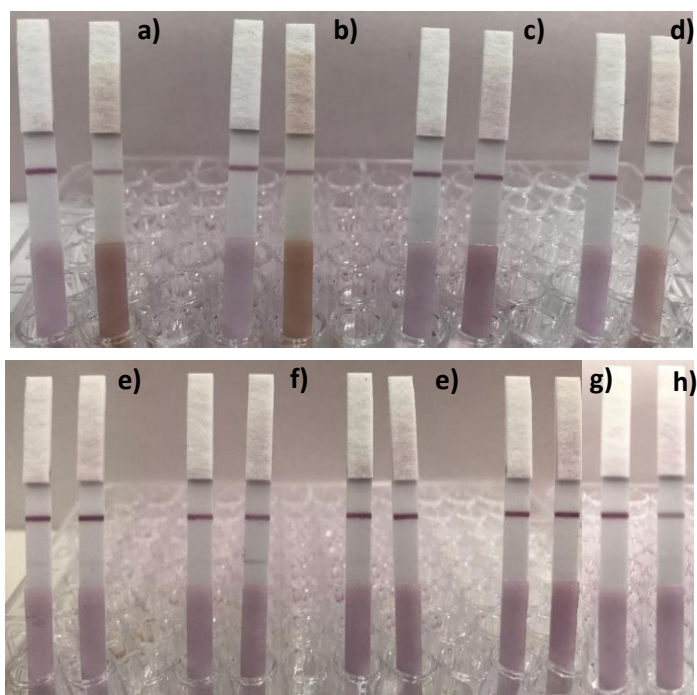
Figure 41 Results of matrix trials, letters according to Table 10

The tests were done in complex matrix samples with other ingredients present. Only for f) a weak positive signal was obtained, which also contained whole egg, the test line for the blank did not show colour development, as expected. All other samples did not show a test line no matter if analyte was present or not. Further optimization steps need to be done for the application in real matrix samples. Measurements were done, detecting the egg standard at a concentration of 0, 0.01, 0.02, 0.1, 0.2, 1 µg/mL whole egg protein in 0.05 M PBS buffer and 8 µL [8 µg Ab/mL AU] of antibody-gold conjugate, using the optimal conditions. The read out was done with the naked eye.

3.9 Dipyrone

Besides the deveopment of an LFA for egg, also for Metamizole an assay was developed. Blank trials were carried out for each membrane. The reaction mixture consited of running buffer, extraction buffer and antibody-gold-conjugate in two different concentrations to check if there is a difference in intensity. For pre-concentration tests, spots in amounts of 1, 2, 4 and 8 µg [1 mg/mL] were dripped in 0.5 µL steps with a pipette onto the membrane and dried at 37°C, this is shown in Figure 42. After drying, the strips were assembled and cut as stated above.
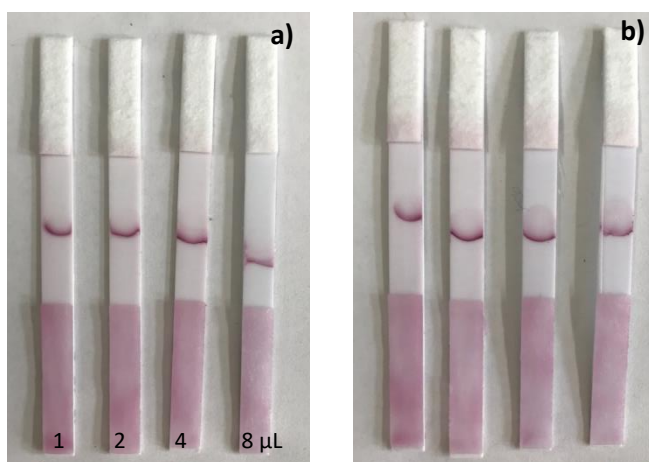
Figure 42 Pre-concentration tests of metamizole HAS-conjugate; (a) FF 120 (b) FF 180

A competitive format was applied which means the signal intensity is indiretcly proportional to the analyte concentration. Therefore 1 µg [1 mg/mL] HAS-conjugate was applied on the membrane and 0, 0.5, 1, 2, 5 10 million ppm metamizole was mixed with 5 µg [5 µg Ab/mL AU] in the reaction tube. All three membranes were checked. In the next set-up 2 µg of the HAS-conjugate were applied on the membrane
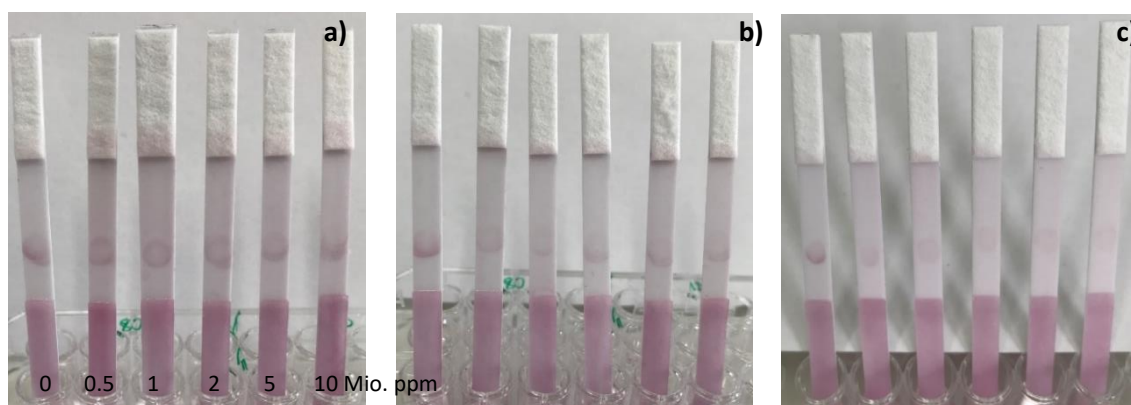
Figure 43 (a) FF80; (b) FF 120; (c) FF 180

Figure 43 shows well which effect the right membrane can have on the assay performance. For membrane HFC 180 is visible that the intensity of the dots decreases from left to right.

Since metamizole is a very small molecule, the best results were obtained with FF 120 and FF 180, therefore further tests were carried out with these two. 2 µg of the conjugate were applied von the membrane. In the next step concentrations in a lower range were tested: 0, 30.000, 60.000, 125.000, 250.000, 500.000 and 1.000.000 ppm of metamizole.

Since the HAS-metamizole conjugate presents the test line of the strip, also the donkey anti-sheep was tried on how it corresponds to the antibody-gold conjugate. The donkey anti-sheep antibody functions as a control line in this case. Undiluted donkey anti-sheep IgG [2.5 mg/mL] was spotted on the top of the membrane as a control line. An amount of 0.5 µL which equals 1.25 µg were applied via pipette on the membrane. After spotting, the membrane dried at 37 °C assembled and cut in stripes. Running buffer, extraction buffer and antibody-gold conjugate were placed in the test tube. The strip was dipped into the solution at the sample/absorbent pad side for 10 minutes.

After pre-concentration tests, test and control line were sprayed again all on the three different membranes, this shown in Figure 44. On the test line HAS-Metamizole conjugate was applied, while anti-sheep was sprayed on the control line. The volume dispensed on the control line was 0.5 µL per cm line which equals 0.5 µg [2.5 mg/mL]. For the test line 1,25 µL were applied in two steps to obtain 0.5 µg [1 mg/mL]. In a second set-up the amount of the test line was changed to 0.25 µg [1 mg/mL]. The reaction tube was filled with running buffer, a dilution series of metamizole 0, 0.5, 1, 5, 10, 50, 100, 500, 1.000 and 10.000 ppm Metamizole and 5µL Ab-Au-conjugate [5 µg Ab/mL Au].
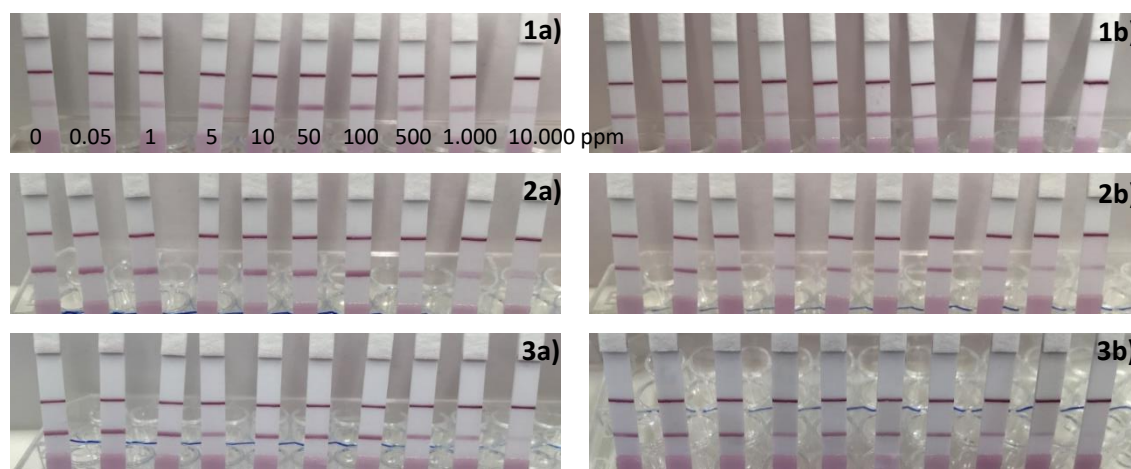
Figure 44 (1) FF 80; (2) FF 120; (3) FF 180; (a) 0.25 µg (b) 0.5 µg HAS-Metamizole conjugate

To maintain a flowrate of 0.7 µL per cm line, diluted Anti-Sheep [1.785 mg/mL] was sprayed on the membrane, to obtain an amount of 0.5 µg for the control line. The 0.25 µg for the test line showed good results therefore only the flowrate was adapted to 0.7 µL per cm line and the HAS-conjugate was diluted [0.89 mg/mL]. In a next step the amount of HAS-Metamizole conjugate was further diluted to 0.1 µg [0.357 mg/mL]. Even though Metamizole is a small molecule FF 80 and 120 showed the best performance, visible in Figure 45.
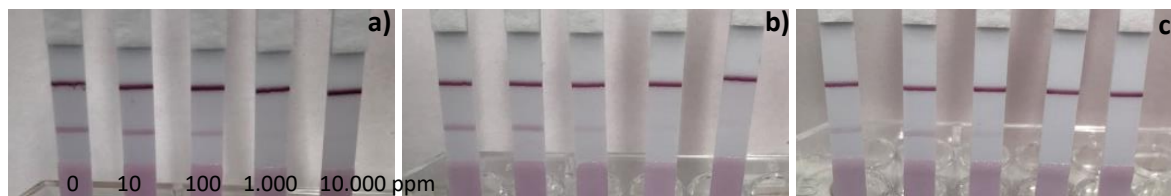
Figure 45 (a) FF 80; (b) FF 120; (c) FF 180; concentration of Metamizole from left to right: 0, 10, 100, 1.000 and 10.000 ppm

Therefore, further tests were carried out with different concentration ranges of Metamizole. Both membrane set-ups 0.25 µg and 0.1 µg for the test line were investigated. In the reaction tube 3 µL and 4 µL [5 µg Ab/mL Au] of gold-coupled antibody was used. The smallest amount of 2 µL of gold-ab conjugate resulted in poor colour development at the test line.

Pictures are recorded after 10 minutes, 4 µL [5 µG AB/mL AU] of antibody-gold conjugate was added to the reaction mixture. The colour intensities with the quantity of gold conjugate used resulted in better colour development for FF 80 and 120, than for HFC 180. For the test demonstrated in Figure 45 a first cut-off level could be defined at 1.000 ppm for membrane 80 and 120.

Further tests with lower amounts of analyte in the well were carried out. The concentration on the test line was varied between 0.1 µg and 0.25 µg. The amount on the control line was kept constant at 0.5 µg Anti-Sheep. From left to right 0, 10, 50, 75, 100, 250, 500, 750, 1000, 2500, 5000 ppm Metamizole.

Figure 46 shows that the amount of 0.25 µg [0.89 mg/mL and 0.7 µg/cm] HAS-Metamizole conjugate gives a more intensely coloured test lines for both membranes. For (1a) is it visible that the intensity of the colour decreased from 250 ppm. There is no test line visible at 2500 ppm. Also for (2a) the colour decreased from 250 ppm. All four possibilities showed good results in terms of line colour intensity and assay sensitivity.
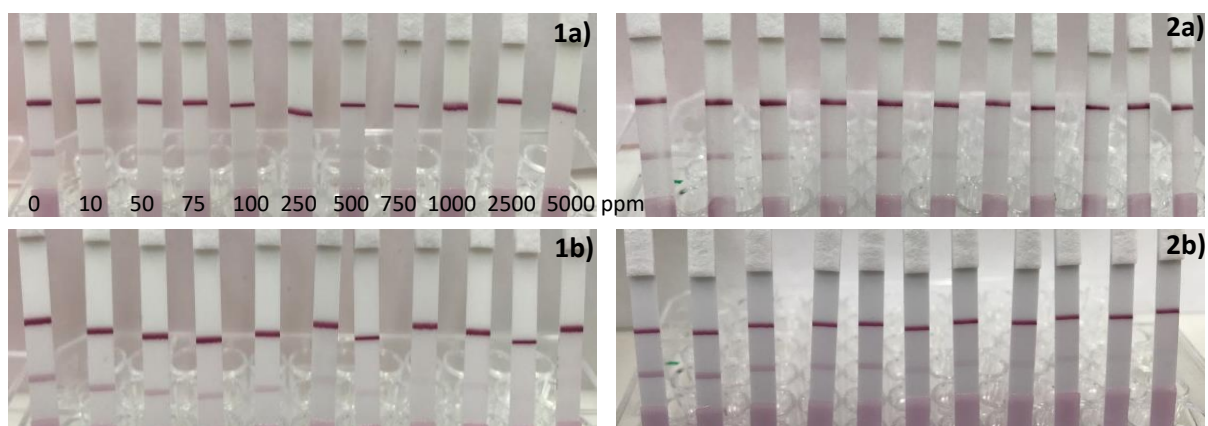
Figure 46 (1b) FF 80 with 0.1 µg HAS-Metamizole conjugate (1b) FF 80 with 0.25 µg HAS-Metamizole conjugate; (2a) FF 120 with 0.1 µg Metamizole conjugate; (2b) FF 120 with 0.25 µg HAS-Metamizole conjugate

As the results looked very promising and the sensitivity could be improved throughout the experiments, various blocking agents were added to the reaction mixture in different concentrations. Previous tests showed, that preblocking of the strips is not advisable, since the nitrocellulose strips have already well-balanced properties and with washing of blocking these properties will be destroyed. Even in several publications preblocking was mentioned. HAS-Metamizole was striped at the test line position with 0.25 µg [0.80 mg/mL] and the control line was kept constant with 0.5 µg [1.785 mg/mL]. This time 8 µL [5 µg AB/mL AU] antibody-gold conjugate was used.
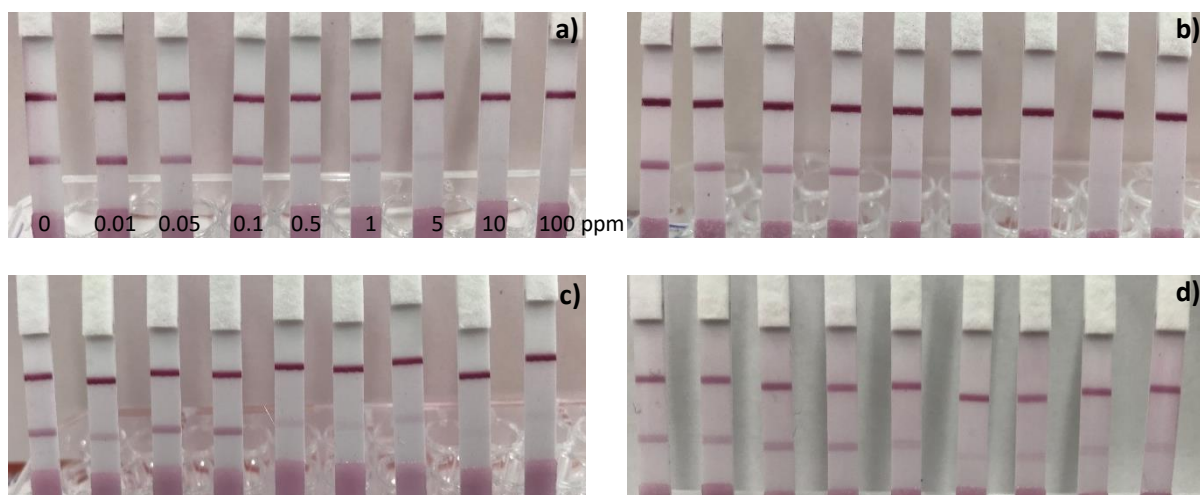
Figure 47 Showing all blocking reagents in a concentration of 0.1% (a) milk powder, (b) BSA, (c) Fish gelatin, (d) Ficoll® 400

Metamizole in concentration range from 0, 0.01, 0.05, 0.1, 0.5 1, 5, 10 and 100 ppm were dipped in the sample holder. For the blocking reagents, milk powder, BSA, Ficoll® 400 and

fish gelatin the concentrations of 0.1%, 0.25%, 0.5% and 1% were investigated. The results are shown in Figure 47.

The effects of milk powder in concentrations of 0.1%, 0.25%, 0.5% and 1% were compared. The same was done with BSA and fish galantine. Also for Ficoll® 400 0.25%, 0.5%, 1% and 2% were added to the set-up.

The blocking reagent was added in the reaction tube. The metamizole concentrations in the tube ranged from 0, 0.05, 0.1, 0.5, 1, 5, 10 to 100 ppm Metamizole. As demonstrated in Figure 47 (c) showing fish gelatin, the intensity of the test line starts decreasing at 1 ppm analyte. Therefore the fish gelatin was tested again with 0.1%, 0.5%, 1% and 5%.
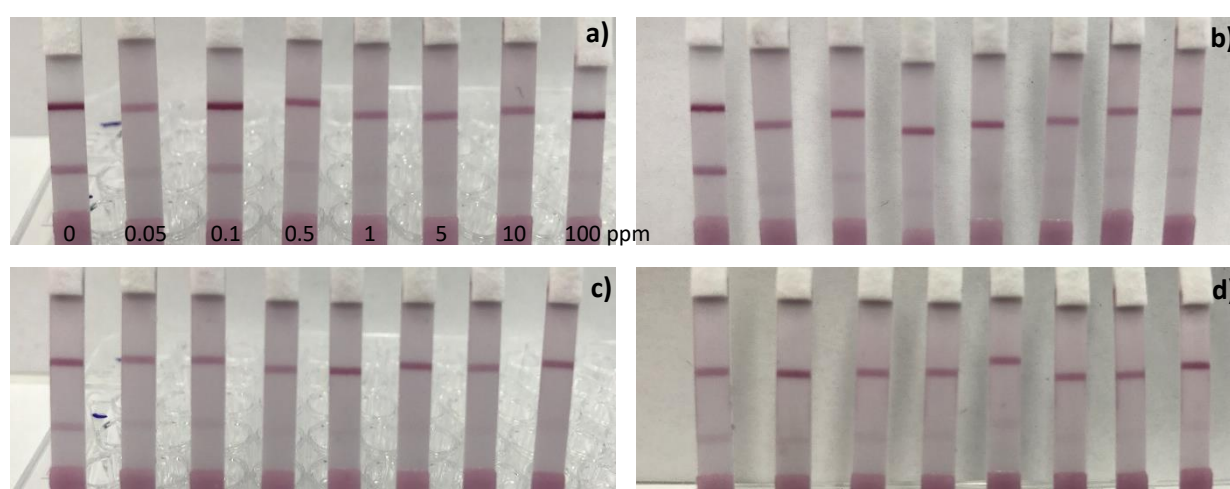
Figure 48 Blocking reagent – fish gelatin: (a) 0.1%, (b) 0.5%, (c) 1% and (d) 5%.

Figure 48 shows the strips with four different concentrations of blocking reagent applied. Comparing a, b, c and d it is visible that with increasing blocking reagent concentration also the intensity of the control line decreases. For (b) the intensity already decreased at 0.05 ppm, same for (c). Picture (d) presents less colour development in general also for the blank, this indicates that the concentration of 5% fish gelatin is too high.

For the assay development of Dipyrone the optimal conditions were as follow: HAS-Metamizole was striped at the test line position either with 0.1 µg [0.357 mg/mL] or 0.25 µg [0.80 mg/mL], the control line with 0.5 µg [1.785 mg/mL] anti-sheep was kept constant. Membrane FF 80 and FF 120 were chosen as the most suitable in combination with the analyte because of the colour development and the sensitivity. The read-out was done after 10 minutes. Fish gelatin was chosen as a blocking substance and was tested in a concentration range of 0.1% - 1%. While 0.1% showed the strongest colour development for control and test line, 0.5% and 1% weaker intensities but the test line was hardly visible anymore at 0.5 ppm.

4 Conclusion and future outlook

In the present study, a colloidal gold-based lateral-flow immunoassay for the rapid detection of egg allergens and Dipyrone was developed and optimized. For the development of Lateral Flow Devices polyclonal antibodies from rabbit serum were used. Within several experiments the gold nanoparticle synthesis was optimized by varying the parameters and concentrations of gold and citric acid salts. The most uniform shaped particles and narrowest size variation was obtained by mixing a solution of 0.010 % gold chloride and 1 % sodium citrate in water. Titration experiments with colloidal gold for the determination of the optimum conjugation ratio was done. The synthesis of the conjugates was done using citrate stabilized AuNPs and rabbit IgG or sheep IgG depending on the intended investigation. The antibodies were nonspecifically adsorbed onto the gold nanoparticles. The non-covalent technique is a spontaneous absorption of antibodies onto the surface of the nanoparticle. The application of the LFD is demonstrated in two examples: egg allergens, such as Ovomucoid and Dipyrone a drug. Based on the different size and structure of the analytes, two assays with different working mechanisms were optimized. For the bigger molecules, the egg proteins a sandwich assay was applied while for the small molecules a competitive format was chosen. The prevention of non-specific binding is an essential part for the development of an immunoanalytical assay. Conventional blocking substances such as fish gelatin, Ficoll® 400 and bovine serum albumin (BSA) were compared and tested.

None of the egg allergen samples could be detected with the in-house developed LFDs reliable. Although protein concentrations up to 100 µg/mL were applied. By adding 0.10% Ficoll® 400 as a blocking substance into the reaction mixture, a difference in the intensity of the signals was shown, nevertheless it was not possible to obtain a clear negative result when no analyte was present. This can have several reasons, on one hand it is possible that the antibodies were not sensitive enough to detect the egg extract. On the other hand, unspecific binding might be a reason, and this is most likely because the sensitivity and selectivity of the antibody towards the analyte was tested in an ELISA set-up and promising IC50-values of 1.0989 for the highest dilution was obtained. The buffer composition also affects the assay performance, as well as the extraction buffer can have an effect on the performance. Also the amount of the conjugate dispensed in the reaction mixture has a huge influence on the performance of the assay. Varying volume of conjugates were tested on the developed strips.

A cut-off level which is defined as the amount of whole egg powder in the standard samples that does not result in a red colour development at the test line, could not be determined for the egg allergens. Concluding it can be stated that the quantitative determination of egg traces

in food products or in the used extract cannot be assured by now. Therefore further test would be necessary.

For the development of the competitive assay more reliable results were obtained. With the addition of 0.1% blocking substance fish gelatin, a cut-off level of 1 ppm Dipyrone was achieved. This means, that no test line was visible anymore. One reason for this might be the type of antibody used for the application. The commercially obtained Anti-sheep IgG showed a different coupling behavior to the colloidal gold, as shown in Figure 29. A very stable plateau could be reached, which was not the case for the anti-rabbit IgG. The system was tested only in a controlled environment with no matrix interferences.

Lateral flow assays provide many advantages being selected as detection system. The benefits include rapidity, point of care testing and simplicity. One of the key elements is developing a sensitive and stable conjugate. For the improvement of the system the labels being used could be further investigated. Colloidal gold which are the primary labels used in such systems, can be attached covalently to the antibody, instead of passive conjugation. Some benefits of covalent conjugation are, that less antibody is needed to maximize the sensitivity. The stability can be increased in sample matrices and non-specific interactions of the system can be reduced. Also better coverage of the AuNPs by IgGs due to orientation on the particles surface is achieved

This study summarizes some important factors which can be considered during lateral flow device development for egg allergens and Dipyrone. The results could provide a better understanding of the influence of colloidal gold nanoparticle conjugation to different antibodies for the application in lateral flow devices.

ABBREVIATIONS

AB	Antibody
AU	Gold
AUNPs	Gold Nanoparticles
BSA	Bovine Serum Albumin
DIP	Dipyrone
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked Immunosorbent Assay
FPLC	Fast Protein Liquid Chromatography
GNP	Gold Nanoparticles
HCG	Human Choriongonadotropin
LFA	Lateral Flow Assay
LFD	Lateral Flow Device
NHS	N-hydroxysuccinimide
SPA	Staphylococcal protein A
SEM	Scanning Electron Microscope
PEG	Polyethylene Glycol
POC	Point Of Care
PPM	Parts Per Million

FIGURES

TABLES

REFERENCES

Abdo, J., Kristersson, T., Seitzer, U., Renneker, S., Merza, M., Ahmed, J., 2010. Development and laboratory evaluation of a lateral flow device (LFD) for the serodiagnosis of Theileria annulata infection. Parasitol. Res. 107, 1241–1248. https://doi.org/10.1007/s00436-010-1994-8

Alzoubi, F.Y., Alzouby, J.Y., Alqadi, M.K., Alshboul, H.A., Aljarrah, K.M., 2015. Synthesis and Characterization of Colloidal Gold Nanoparticles Controlled by the pH and Ionic Strength. Chin. J. Phys. 53, 100801-1-100801–9. https://doi.org/10.6122/CJP.20150601E

Bannon, G.A., 2004. What makes a food protein an allergen? Curr. Allergy Asthma Rep. 4, 43–46. https://doi.org/10.1007/s11882-004-0042-0

Bartnikas, L.M., Sheehan, W.J., Tuttle, K.L., Petty, C.R., Schneider, L.C., Phipatanakul, W., 2015. Ovomucoid Specific Immunoglobulin E as a Predictor of Tolerance to Cooked Egg. Allergy Rhinol. 6, ar.2015.6.0135. https://doi.org/10.2500/ar.2015.6.0135

Benjamin Caballero Paul Finglas Fidel Toldrá, 2016. Encyclopedia of Food and Health.

Bose, K.S., Sarma, R.H., 1975. Delineation of the intimate details of the backbone conformation of pyridine nucleotide coenzymes in aqueous solution. Biochem. Biophys. Res. Commun. 66, 1173–1179. https://doi.org/10.1016/0006-291x(75)90482-9

Bredehorst, R., David, K., 2001. What establishes a protein as an allergen? J. Chromatogr. B. Biomed. Sci. App. 756, 33–40. https://doi.org/10.1016/S0378-4347(01)00069-X

Byzova, N.A., Zherdev, A.V., Khlebtsov, B.N., Burov, A.M., Khlebtsov, N.G., Dzantiev, B.B., 2020. Advantages of Highly Spherical Gold Nanoparticles as Labels for Lateral Flow Immunoassay. Sensors 20, 3608. https://doi.org/10.3390/s20123608

Caubet, J.-C., Wang, J., 2011. Current Understanding of Egg Allergy. Pediatr. Clin. North Am. 58, 427–443. https://doi.org/10.1016/j.pcl.2011.02.014

Chegel, V., Rachkov, O., Lopatynskyi, A., Ishihara, S., Yanchuk, I., Nemoto, Y., Hill, J.P., Ariga, K., 2012. Gold Nanoparticles Aggregation: Drastic Effect of Cooperative Functionalities in a Single Molecular Conjugate. J. Phys. Chem. C 116, 2683–2690. https://doi.org/10.1021/jp209251y

Clare Mills, E.N., Wichers, H.J., Hoffmann-Sommergruber, K., Fernández-Rivas, M., Ballmer-Weber, P.D.B., Nicoletti, C., Temblay, J.N., Ivory, K., de Blok, B.M.J., Dubois, T., O'B. Hourihane, J., Breiteneder, H., Jenkins, J.A., Clare Mills, E.N., van Ree, R., Edwards, C.A., Khanna, S., Amarri, S., Doré, J., Gil, A., Adam, R., Norrin, E., Clare Mills, E.N., Sancho, A.I., Moreno, J., Kostyra, H., Hoffmann-Sommergruber, K., Sancho, A.I., Gilissen, L.J.W.J., Shewry, P.R., Jones, H.D., Paschke, A., Schütte, L., Paschke, A., Gremmel, S., Wichers, H.J., Wal, J.M., Løvik, M., Baumgartner, S., Rudolf Krska, Welzig, E., Poulsen, L.K., Bindslev-Jensen, C., Crevel, R., Frewer, L., Pfaff, S., Miles, S., 2007. Contributor contact details, in: Managing Allergens in Food. Elsevier, pp. xi–xv. https://doi.org/10.1016/B978-1-84569-028-1.50021-6

Contreras-Trigo, B., Díaz-García, V., Guzmán-Gutierrez, E., Sanhueza, I., Coelho, P., Godoy, S., Torres, S., Oyarzún, P., 2018. Slight pH Fluctuations in the Gold Nanoparticle Synthesis Process Influence the Performance of the Citrate Reduction Method. Sensors 18, 2246. https://doi.org/10.3390/s18072246

Davatgaran Taghipour, Y., Kharrazi, S., Amini, S.M., 2018. Antibody Conjugated Gold Nanoparticles for Detection of Small Amounts of Antigen Based on Surface Plasmon Resonance (SPR) Spectra. Nanomedicine Res. J. 3. https://doi.org/10.22034/nmrj.2018.02.007

Di Nardo, F., Cavalera, S., Baggiani, C., Giovannoli, C., Anfossi, L., 2019. Direct vs Mediated Coupling of Antibodies to Gold Nanoparticles: The Case of Salivary Cortisol Detection by Lateral Flow Immunoassay. ACS Appl. Mater. Interfaces 11, 32758–32768. https://doi.org/10.1021/acsami.9b11559

Edwards, K.A., Baeumner, A.J., 2006. Optimization of DNA-tagged dye-encapsulating liposomes for lateral-flow assays based on sandwich hybridization. Anal. Bioanal. Chem. 386, 1335–1343. https://doi.org/10.1007/s00216-006-0705-x

Frankle, R.T., 1976. Nutrition education in the medical school curriculum: a proposal for action: a curriculum design. Am. J. Clin. Nutr. 29, 105–109. https://doi.org/10.1093/ajcn/29.1.105

Freitas de Freitas, L., Varca, G., dos Santos Batista, J., Benévolo Lugão, A., 2018. An Overview of the Synthesis of Gold Nanoparticles Using Radiation Technologies. Nanomaterials 8, 939. https://doi.org/10.3390/nano8110939

Goel, P.K., 2006. Water pollution : causes, effects and control. New Age International, New Delhi.

Helen Hsieh, Jeffrey Dantzler, Bernhard Weigl, 2017. Analytical Tools to Improve Optimization Procedures for Lateral Flow Assays. Diagnostics 7, 29. https://doi.org/10.3390/diagnostics7020029

Hober, S., Nord, K., Linhult, M., 2007. Protein A chromatography for antibody purification. J. Chromatogr. B 848, 40–47. https://doi.org/10.1016/j.jchromb.2006.09.030

Jazayeri, M.H., Amani, H., Pourfatollah, A.A., Pazoki-Toroudi, H., Sedighimoghaddam, B., 2016. Various methods of gold nanoparticles (GNPs) conjugation to antibodies. Sens. Bio-Sens. Res. 9, 17–22. https://doi.org/10.1016/j.sbsr.2016.04.002

Kimling, J., Maier, M., Okenve, B., Kotaidis, V., Ballot, H., Plech, A., 2006. Turkevich Method for Gold Nanoparticle Synthesis Revisited. J. Phys. Chem. B 110, 15700–15707. https://doi.org/10.1021/jp061667w

Koczula, K.M., Gallotta, A., 2016. Lateral flow assays. Essays Biochem. 60, 111–120. https://doi.org/10.1042/EBC20150012

Leopold, L.F., Tódor, I.S., Diaconeasa, Z., Rugină, D., Ştefancu, A., Leopold, N., Coman, C., 2017. Assessment of PEG and BSA-PEG gold nanoparticles cellular interaction. Colloids Surf. Physicochem. Eng. Asp. 532, 70–76. https://doi.org/10.1016/j.colsurfa.2017.06.061

Li, Y., Liu, L., Kuang, H., Xu, C., 2019. Development of a lateral flow immunoassay for the simultaneous detection of four dipyrone metabolites in milk. Anal. Methods 11, 3041–3052. https://doi.org/10.1039/C9AY00774A

Ljungblad, Jonas, 2009. Antibody-conjugated Gold Nanoparticles integrated in a fluorescence based Biochip [WWW Document]. URL http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-50619

M. Ijeh, 2011. covalent gold nanoparticle—antibody conjugates for sensitivity improvement in lfia.

Mansfield, M.A., 2005. The Use of Nitrocellulose Membranes in Lateral-Flow Assays, in: Wong, R.C., Tse, H.Y. (Eds.), Drugs of Abuse: Body Fluid Testing. Humana Press, Totowa, NJ, pp. 71–85. https://doi.org/10.1007/978-1-59259-951-6_4

Martorell Aragonés, A., Boné Calvo, J., García Ara, M.C., Nevot Falcó, S., Plaza Martín, A.M., 2001. Alergia a las proteínas de huevo. Allergol. Immunopathol. (Madr.) 29, 84–95. https://doi.org/10.1016/S0301-0546(01)79023-4

Mayerhuber, L., Trattner, S., Luger, S., Weigelhofer, G., Hametner, C., Fruhmann, P., 2021. Development of ion-selective electrodes for antipyrine and its derivatives as potential tool for environmental water monitoring. J. Electroanal. Chem. 886, 115110. https://doi.org/10.1016/j.jelechem.2021.115110

McNamara, D.J., 2013. Eggs, in: Encyclopedia of Human Nutrition. Elsevier, pp. 132–138. https://doi.org/10.1016/B978-0-12-375083-9.00086-6

Nezlin, R., 1998. General Characteristics of Immunoglobulin Molecules, in: The Immunoglobulins. Elsevier, pp. 3–73. https://doi.org/10.1016/B978-012517970-6/50001-1

Noguera, P., Posthuma-Trumpie, G.A., van Tuil, M., van der Wal, F.J., de Boer, A., Moers, A.P.H.A., van Amerongen, A., 2011. Carbon nanoparticles in lateral flow methods to detect genes encoding virulence factors of Shiga toxin-producing Escherichia coli. Anal. Bioanal. Chem. 399, 831–838. https://doi.org/10.1007/s00216-010-4334-z

Poms, R.E., Klein, C.L., Anklam, E., 2004. Methods for allergen analysis in food: a review. Food Addit. Contam. 21, 1–31. https://doi.org/10.1080/02652030310001620423

Posthuma-Trumpie, G.A., Korf, J., van Amerongen, A., 2009. Lateral flow (immuno)assay: its strengths, weaknesses, opportunities and threats. A literature survey. Anal. Bioanal. Chem. 393, 569–582. https://doi.org/10.1007/s00216-008-2287-2

Rudolf, J., Führer, M., Galler, B., Ansari, P., Hasenhindl, C., Baumgartner, S., 2009. Differences in usability of rabbit IgG and chicken IgY after clean-up and impact on gold labelling properties. J. Immunol. Methods 350, 79–88. https://doi.org/10.1016/j.jim.2009.08.006

Sajid, M., Kawde, A.-N., Daud, M., 2015. Designs, formats and applications of lateral flow assay: A literature review. J. Saudi Chem. Soc. 19, 689–705. https://doi.org/10.1016/j.jscs.2014.09.001

Schroeder, H.W., Cavacini, L., 2010. Structure and function of immunoglobulins. J. Allergy Clin. Immunol. 125, S41–S52. https://doi.org/10.1016/j.jaci.2009.09.046

Schubert-Ullrich, P., Rudolf, J., Ansari, P., Galler, B., Führer, M., Molinelli, A., Baumgartner, S., 2009. Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: an overview. Anal. Bioanal. Chem. 395, 69–81. https://doi.org/10.1007/s00216-009-2715-y

Singh, A., Chaudhary, S., Agarwal, A., Verma, A.S., 2014. Antibodies, in: Animal Biotechnology. Elsevier, pp. 265–287. https://doi.org/10.1016/B978-0-12-416002-6.00015-8

Taylor, S., 2006. The nature of food allergy, in: Detecting Allergens in Food. Elsevier, pp. 3–20. https://doi.org/10.1533/9781845690557.1.3

Tripathi, K., Driskell, J.D., 2018. Quantifying Bound and Active Antibodies Conjugated to Gold Nanoparticles: A Comprehensive and Robust Approach To Evaluate Immobilization Chemistry. ACS Omega 3, 8253–8259. https://doi.org/10.1021/acsomega.8b00591

Valenta, R., Hochwallner, H., Linhart, B., Pahr, S., 2015. Food Allergies: The Basics. Gastroenterology 148, 1120-1131.e4. https://doi.org/10.1053/j.gastro.2015.02.006

Wu, R., Nguyen, T., Marquart, G., Miesen, T., Mau, T., Mackiewicz, M., 2014. A Facile Route to Tailoring Peptide-Stabilized Gold Nanoparticles Using Glutathione as a Synthon. Molecules 19, 6754–6775. https://doi.org/10.3390/molecules19056754

Zhou, Y., Andersson, O., Lindberg, P., Liedberg, B., 2004. Reversible Hydrophobic Barriers Introduced by Microcontact Printing: Application to Protein Microarrays. Microchim. Acta 146, 193–205. https://doi.org/10.1007/s00604-003-0174-2