SCIENTIFIC BULLETIN OF THE TECHNICAL UNIVERSITY OF LODZ
No. 1081 Food Chemistry and Biotechnology, Vol. 74 2010

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# IMMOBILIZED ANTIBODIES FOR BIOSENSORIC DETECTION OF PROTEIN FOOD ALLERGENS IN GROCERY PRODUCTS

Review: **Elżbieta Sobiecka, Ph.D.**

*The following work describes theoretical background for antibody immobilization and exemplifies solutions, which can be applied when designing a test for the presence of food allergens in groceries. Symptoms of an allergy occur as a consequence of ingestion of foods whose components show antigenic properties. Detection of allergenic proteins is being done mainly with the use of immunometric assays). First biosensor systems were composed of specific antibodies immobilized on polystyrene plate. Further steps were taken release functional groups of the matrix polymer so that the antibody binding is less random (nitrocellulose) and remains more stable. Then, there were complex matrices capable of binding an antibody with higher density and reduced denaturating effect (polycaprolactam) as well as elaborate chemical structures, known as "Self-assembled layers". Physical distance between both elements is favorable and allows to minimize disturbance from steric effects. Due to the use of a messenger coupled to the antibody results can be obtained.*

## Introduction

Many people suffer from food allergies today and so it is crucial to work out effective, cheap, small-sized, fast and easy to use detectors, capable of telling immediately if a threatening allergic factor is present in food and this way avoid the risk of its consumption. According to that fact biosensoric systems are being invented. They are based on a specific reaction of binding between antibodies and a given protein responsible for immune response in human organism. Despite technological advance in this area of science, some issues remain unsolved such as dynamising the receipt of results, effective incorporation of the analytical signal

generator and conceiving the whole idea of what functional components of the biosensor should consist of. What is presented in the following paper is the theoretical basis for the subject of antibody immobilization and examples are given that can find a successful application when designing a test for the presence of food allergens in grocery products.

## Food allergies

It is the technological process of production where sources of food allergens can be perceived. In food products, unintended presence or contamination with an allergen can be due to the lack of adequate analytical methods in the industrial plant or unawareness of the hazard. Occurrence of food allergies in people compels special discipline in production process. The only way for the consumer to steer clear of the effects associated with this ailment is to avoid consumption of products containing the antigen which irritates immune system. It is therefore desired to completely eliminate antigens causing immune response or to precisely describe ingredient list on the label. It is one of the main approaches to prevent, often life-threatening, symptoms as the allergy itself remains incurable. It is required to utilize modern and costly technical solutions to determine the presence of undesired chemical structures especially that, the problem addresses detection of biologically active compounds in the vestigial concentration range. Not being able to treat allergy effectively, it is to draw up reliable, fast and sensitive techniques for allergens detection that becomes a challenge for modern science [1]. It poses a problem as while a hundred years ago only 1% of population was afflicted with an allergy, it is now estimated to reach 50% in 2020 [2, 3].

Food and chemicals belong to the group of the most allergizing factors [2]. Immune response in food allergy-afflicted people is reported after ingestion of such products as nuts, sesame, chocolate, eggwhite, cow milk, soy, fish, flour, fruit (for example: kiwi, apples, apricot, mango, pears, cherries, peaches) and vegetables (for example: potatoes, carrot, celery), spices, cereals [3-5]. Small group of allergens (i.e. milk, eggs, fish, shellfish, wheat, soya, nuts) is responsible for 90% of food allergies [6]. That is why it is so important to label foods properly, which in EU is legally established by European Commission Directive 2007/68/WE dated 27th November 2007. It defines all ingredients of food whose presence should always be indicated on the label, even if they come in vestigial amounts. It enlists the so-called ‘great twelve’ of allergens: gluten containing cereals, crustaceans, eggs, fish, peanuts, soy, milk, treenuts, celery, white mustard, sesame seeds, sulphur dioxide at levels above 10mg/kg, or 10 mg/liter, expressed as $SO_2$. Later, lupin and molluscs were included [7]. The law doesn’t consider concentration thresholds for the above mentioned products. It is mainly because it depends on an individual how much allergen elicits immunological reaction [8].

## Tests for the presence of allergens

It is very frequent to observe allergenic intolerance towards more than one type of foods in a person. Proteins, seldom other chemicals, are found to be antigens in foods. It creates an enormous potential for detection and analysis of known allergens and allows to monitor changes in their concentration during various technological processes that food is exposed to. Gained knowledge can be used to modify diet of the sufferers so that the specific peptides that naturally occur as food ingredients will be eradicated and the control over production process in regard of allergens presence will improve.

The content of protein food allergens can be determined at the level of a protein or a gene that encodes it. Amongst the laboratory techniques for that kind of research, immunometric assays stand as the most significant ones – ELISA, and Western Blot (immunoblotting) capable of direct detection of allergenic proteins, as well as indirect method – polymerase chain reaction (PCR) with proper modifications for quantitative analysis [1, 5, 6, 8-10]. Application of the aforementioned analytical methods in determining content of allergens in foods are widely described in science papers and give a good idea about their credibility. For example, these two independent ways were combined to have an insight in both concentration of proteins from peanut extract as well as Ara h 2 that was amplified during PCR. The results obtained for low-processed foods were similar and complementary for both techniques (with PCR being slightly little more sensitive than immunometric method), as well as specific after exclusion of cross-reactivity [1, 11]. However, it can be misleading to support the research just by the results received from DNA analysis. It can generate a serious fault as most of the food products are highly processed; some of the ingredients were exposed to denaturating conditions. Besides, specific amount of DNA does not translate proportionally into proteins due to gene expression control mechanisms. Except already described techniques, it is also possible to engage spectroscopy in allergens detection (for example mass spectroscopy) which is far more specific and unambiguous giving qualitative results but rather used as a confirmation tool [5, 8, 11].

Most allergen biosensors base on ELISA as a primary allergen capture method. It is nearly perfect for analyses conducted in laboratory conditions and allows to measure concentration of any given target analyte [12]. With a good limit of detection (0.05 – 10 ppm) and high specificity for allergen binding it stands as reliable mechanism for further discussion about how and to what extent a biosensor can be simplified for a regular customer. Animal and human borne antibodies are used to bind a specific protein allergen and, since an antibody can be attached to other chemicals, notably enzymes, analytical signal is obtained clearly. ELISA itself is a basis for biosensor mechanism design and although different variations of the method exist, none is good enough to call it a final solution. It is a long procedure, which requires technical knowledge and expensive equipment to complete it.

Moreover it is not approachable enough to become a commercial product [13]. However, its immunometric nature is to be mimicked by a rapid counterpart.

In case of protein-detecting techniques it is essential to use specific antibodies against allergenic peptide. Antibodies can be raised against a single allergizing agent or target a number of proteins. It depends on whether an animal was sensitized to a given allergen or food extract as a whole [10]. To run the qRT-PCR test, however, it is enough to have necessary equipment and to know nucleotide sequence of the analyzed genes, but we are still confined to laboratory environment. When analyzing food of known little protein amount such technique appears useful [8]. It allows to bypass problems associated with distortions from chemically complex food matrix and avoid false positive results that occur when antibodies bind wrong target [11].

Biosensor is a functional system for detection. It engages biological components such as cells, enzymes, antibodies etc. immobilized on solid matrix. In order to detect protein antigens, antibodies are used as they bind specifically to the epitope in allergenic peptide. Often they are coupled to the analytical factor that functions as a converter – exposed to given physical-chemical interactions (for example when the energy state changes during conformational alterations in the peptide) it generates a visual signal or one that is measurable with the use of instrumental methods of analysis. Altogether, combination of these elements makes up a detection tool that finds practical reflection, for example, in the form of qualitative/semi-quantitative lateral flow assay devices where examined extract must only be placed in the pad with already-supplied antibodies. Macroscopic results of the test are read fairly quickly as colored bands [13]. There is one problem, though, when considering application of labeled antibodies immobilized on solid plate for specific binding to the defined allergenic proteins from food extract – namely, how to achieve lasting and effective immobilization on a small surface so that the biosensoric system we get would show larger scale of bands intensity for more accurate reading. Obviously it must be easy to use and compact.

Were we able to design a biosensor with an antibody chemically immobilized on solid surface, capable of giving repeated results, we would introduce an easy and commonly available specific test to the commercial market. Such products are already on sale (companies such as Neogen, Crystal Chem, R-Biopharm) with examples presented in Table 1, however it is unknown how they work. Also, prices are high, and the range of detected allergens is still limited [14]. It is therefore scientifically and economically justified to proceed with research that aims at creating similar, generic specimen.

Lasting chemical bond between specific antibody and a fluorescent or radioactive dye [15] (for example $^{125}I$) generating analytical signal allows for successful antigen detection on the molecular level. Alternatively, analytical signal can come in the form of colorful reaction detectable macroscopically and measurable spectrophotometrically if fluorochrome is replaced by specific enzymatic system (horseradish peroxidase, glucose oxidase, alkaline phosphatase etc.). Such solution underlines mechanism of the most popular immunometric technique for allergens

detection – ELISA method. It is popular mainly due to its simplicity, ease of results interpretation and significant precision of quantitative measurement. However, it also has flaws like little potential for automatisation, consumption of work and time [15-18]. Fluorescent labeling, contrary to the enzymatic one does not require addition of substrates disintegrating to form colorful product.

**Table 1**

Examples of commercially available tests for allergens detection

| Detected allergen | Sensitivity [ppm] | Product | Source |
|---|---|---|---|
| Almonds, Eggs, Giadins, Peanuts, Milk, Soy | > 5 | Alert® (Neogen) | (A) |
| Almonds, Eggs, Hazelnuts, Mustard, Peanuts, Milk | 2.5 – 25 | Veratox ® (Neogen) | (B) |
| Gliadins | 5.0 – 50 | | |
| Soy | 10 – 100 | | |
| Eggs | > 0.078 | Egg Protein ELISA Kit (Crystal Chem Inc.) | (C) |
| Milk | > 0.078 | Milk Protein ELISA Kit (Crystal Chem Inc.) | |
| Peanuts | > 0.078 | Peanut Protein ELISA Kit (Crystal Chem Inc.) | |
| Gliadins | > 0.078 | Wheat Protein ELISA Kit (Crystal Chem Inc.) | |
| Coco nuts | > 1 | Lateral Flow Coconut (R-Biopharm) | (D) |
| Pistachio nuts | > 1 | Lateral Flow Pistachio (R-Biopharm) | |
| Sesame | > 1 | Lateral Flow Sesame (R-Biopharm) | |

(A): http://www.neogeneurope.com/En/FoodSafety/A_Product_List.asp?Test_Kit_Cat=203
(B): http://www.neogeneurope.com/En/FoodSafety/V_Product_List.asp?Test_Kit_Cat=203
(C): http://www.crystalchem.com/products/FoodProteinELISAKits.html
(D): http://www.rbiopharm.com/product_site.php?product_range=Food%20and%20Feed%20Analysis&&product_class_one=QWxsZXJnZW5z

When maximal emission spectrum exceeds the frequency of visible light it is necessary, though, to apply special equipment – fluorescent microscope with z suitable filters which limits opportunities for spreading such sensors as commonly available product. Choice of dyes can be done freely based on desired range of emission and absorption. It is possible to visualize bound allergens by combining the fluorochrome molecule with an antibody of our choice, and thus prove the presence or the lack of protein in a product. Such molecular detectors have been used for a long time up till now by numerous manufacturers [19, 20].

What is challenging is to design repetitive experiment with labeled antibodies, so that the waiting time is possibly short, and the whole concept of antibodies use for diagnostic examination – as little complicated and intuitively approachable for an average consumer.

## Matrices for antibody immobilization

Antibodies can be immobilized on solid surface, which allows to separate ligand pool that is bound to the protein from the remaining unbound components of an extract in question.

Matrix surfaces can be divided into three main types:

- plastics that bind protein with hydrophobic bonds (polystyrene) or covalent bonds (modified polystyrene),
- hydrophilic, cross-linked carbohydrates covalently binding the proteins (agarose),
- porous membranes (nitrocellulose, nylon, polyvinylidene fluoride) that create hydrophobic bonds with the protein.

The type of matrix directly influences antibody binding density. It is also important to characterize spatial orientation of the protein [18]. Features to be considered when choosing matrix for a biosensor are: surface charge, hydrophilicity /hydrophobicity, physical and chemical stability, easy activation, interactions with an analyte and the capacity to bind a protein on the matrix without denaturing effect [21].

Much attention is given to biopolymers that partially satisfy criteria required from a functional biosensor matrix. Chitin is a good example. It is similar to cellulose, but far more functional and open for further modifications. It is abundant in nature and has a range of favourable properties like stability, biodegradability and biocompatibility [22] which are desired for biosensor mass production.

## Modified matrices

Following the observation of a significant denaturating effect combined with quite chaotic immobilization on hydrophobic polystyrene and partial desorption of the molecules, the search was started to develop more efficient matrices. Modified, i.e. exposed to radiation, styrene acquires functionality thanks to the release of free,

reactive chemical groups. Other way to improve the performance of a standard matrix is to anchor a layer of long hydrophilic 'arms' on a polystyrene plate. Those arms are made of polyacrylamide and finished with hydrazine groups that interact with proteins' aldehyde groups. Such a modified matrix tested in the form of polystyrene balls allowed for the improvement in immobilized antibody performance by 38% compared to antibodies immobilized on styrene with no such modifications [23]. This example confirms that covalent binding is much more effective than passive adsorption.

Porous fibers like nitrocellulose or nylon are considered one of the best matrices for antibody immobilization. Their structure is complex and their surface – vast which allows them to surpass competitive plastics as regards adsorption efficacy, even despite loss resulting from denaturation associated with hydrophobic adsorption, which is the main mechanism of binding for both these materials. Unfortunately, they are less universal – their efficiency of binding depends on many additional factors like the properties of the specific, immobilized protein, the type of polymer, and the way it was manufactured [24]. Porous fibers have been applied to antibody immobilization for a long time. The efficiency of IgG antibody binding to nylon is 30-fold higher than on conventional polystyrene [25]. Despite this fact, research is done to further improve performance in antibody binding with as few imperfections as possible that come from conformational changes in proteins when subject to hydrophobic binding. It is illustrated by the case in which modified polycaprolactam was used as an alternative for plastic ELISA wells when constructing a biosensor to test for the presence of a number of specific protein virulence factors in physiological environment of pathogens. Biosensor bears resemblance to litmus paper for pH testing – it is easy and intuitive. Positive result is marked by a colorful reaction obtained by labeling secondary antibody with alkaline phosphatase. Nylon, exposed to maleic anhydride methylvinyl ether copolymer was able to covalently bind proteins with substantial specificity. The procedure is quite elaborate, though (1h) [26].

Another way to modify porous nylon is to use SAM layer (*Self-assembled layer*) comprised of thioctic acid particles anchored to the gold-coated nylon surface and terminated with carboxyl group. Strong spontaneous adsorption of the acid to gold is mediated by two atoms of sulphur. Carboxyl group of thioctic acid is additionally activated by carbodiimide so that amid bond can be generated between anchored acid and a part of a protein containing amine group. Protein immobilization density is substantial (thanks to vast surface of nylon) and stable while biological functions of an antibody are preserved. With 95% efficiency of immobilization it is almost 3-fold higher than when unmodified nylon membrane is used [21].

Polycarbonate nanoporous membranes were also used for antibody capture. Deposited with a gold layer for effective binding, they were able to specifically immobilize peanut allergenic protein Ara h1. Such a material was tested with regard to conductivity changes. This parameter was decreasing as peanut protein concentration was rising [27].

Gold is relatively popular when it comes to protein immobilization as it interacts well with such biomolecules. Gold nanoparticle surface ensures that the antibody is bound well with a matrix that normally would have had much less affinity for protein immobilization. In a related example, polycarbonate membranes covered in gold turned out to succeed in an assay where anti- Staphylococcal Enterotoxin B antibody was adapted for the use of biosensoric detection. For signal transduction enhanced chemiluminescence reaction was used where horseradish peroxidase catalyzed the oxidation of light-emitting luminol. As a result, a reliable biosensor with detection range of 0.01 to10 ng/mL was built [28].

## Ways of immobilizing the antibodies

There are four main mechanisms for macromolecule immobilization on solid surface:

- spontaneous and non-specific passive adsorption of a protein to synthetic surface (ELISA),
- covalent binding to the functional group of a surface,
- immunochemical immobilization,
- other techniques, i.e. non-adsorptive and non-covalent binding.

Matrix has a large impact on immobilized antibodies and their behavior [24]. It is important to orientate the antibody on the plate surface. When it is immobilized on solid material, it shows less activity compared to the soluble form. It is known that the direction in which active center is exhibited influences binding constant. Naturally it would be best if it was in direct contact with the analyte layer spread over the plate, while the steric effect is minimal. It is beneficial to place the antibody on 'jibs', which makes the antigen more available for paratope fragments. Such a solution is, however, impaired by loss in density of immobilized antibody. Traditional approach is to adsorb proteins on reactive, polylysine surface. However it does not favor spatial structure of an antibody and results in randomness of binding. Instead, it is possible to run a controlled (binding places known in advance) synthesis of antibodies with biotin and immobilize them on a surface coated with streptavidin layer. Biotinylated antibodies as well as biotinylated single Fab fragments whose two opposite poles bind, respectively, antigen and streptavidin. Laboratory research was done with the use of two matrices, both modified so that the distance between an antibody and a plate is maximal. First one, PLL-PEG (*poly(L-lysine)-[g]-poly(ethylene glycol)*), is a titanium (IV) dioxide plate coated with polylysine layer with long ethylene glycol chains bound to biotin and thus ensuring the right distance between antibody and a matrix. The other matrix is – b-SAM (*biotinylated self-assembled monolayer*) – gold plate bound with surfhul bond to a SAM layer. SAM structure resembles surfactants. Alkanethiols account for its hydrophilic group, while poly(ethylene glycol) is a 'tail' that is bound to biotin. The type of surface has an influence on capability to bind antigens was clear (increase in antibody binding efficiency from 0.3-fold to

even 10-fold depending on conditions). Whole antibodies as well as Fab fragments tend to reach higher efficacy after they prior get specifically orientated. As the Fab fragments are smaller they cover biosensor surface more densely, which increases the potential for successful binding [29]. Coupling with biotin allows to eliminate randomness in spatial orientation on a matrix surface, while keeping the antibody far from the plate increases its reactivity and reduces denaturation associated with adsorption to plastics.

## Signal detection

In simple biochemical detectors, analytical signal does not have to come from a dye that is 'artificially' coupled to the protein. It is interesting to utilize genetic engineering techniques for biosynthesis of already labeled biosensors in recombinant single cells. This method is used when analyzing expression and functions of enzymatic proteins *in vitro*. In such case it is the fusion protein that acts like a biosensor. Fluorescent protein is its integral part, responsible for analytical signal emission. It can be ex. GFP (*Green fluorescent protein*), CFP (*Cyan fluorescent protein*), or YFP (*Yellow fluorescent protein*) [30]. It is also possible to insert a secretory sequence, which allow biosynthesis to run automatically and give easy to purify products. That kind of solution entails necessity to design a biosensor as early as on the nucleic acid stage. In case of allergenic proteins only antibody-equipped biosensors will yield enough specificity for binding. It would be ideal to transfect DNA into cell and overproduce recombinant labeled antibodies. However, it is complex task due to the complicity of many different factors during antibody biosynthesis.

It is possible to give up on coupling antibodies with elements generating analytical signal. New concept for a biosensor assumes no converter to know the binding occurred. Successful attempt was made by using a biosensor made of a monoclonal antibody with high-tech equippment that engages surface plasmon resonance phenomenon (SPR). Biosensoric system was simplified and time necessary for automatic analysis was reduced to just 10 minutes for a number of simultaneous assays [15, 13]. The results were obtained with sensitivity of 1pg molecules per square millimeter [14]. Test is done by measuring refractive index on the surface where antibody was immobilized and results and generated in the real time as equilibrium constant for reaction epitope-paratope stabilizes [15, 30]. Electromagnetic wave spreads over and scans the surface between biosensor metal plate and air phase which allows for accurate detection of any changes made by binding antigen to immobilized specific antibodies. This novel technique lets us conduct a test in just a few minutes, instead of waiting 1-3h (ELISA) [14, 17], and improves sensitivity of examination with a few picograms of analyte per square millimeter [32].

The other technique available for determination of allergenic proteins is imaging ellipsometry. It does not require incorporating a dye to the protein. Optimized method assumed immobilization of an oriented and indirectly bound to matrix antibody. Antibody was coupled to A protein, which strongly binds immunoglobulins of a certain type. It was immobilized on hydrophobic surface covered with silicon and exposed to instrumental analysis as the secondary antibody was being added [33]. Changes in thickness of a biosensor surface layer allowed to speed up a test and obtain results in the real time in high definition, without work-consuming necessity to label with chromophores. Test is based on a measurement of the state of light polarization reflected by the analyzed surface and it can be combined with aforementioned SPR [34]. Unfortunately, instrumental methods require expensive equipment which narrows their use significantly.

Electrochemical means of analysis exist that allow to follow reaction of complexation between antigen and antibody (ex. electrochemical impedance spectroscopy). In this case a glassy carbon electrode serves as a matrix for the reagents and changes in its properties are monitored. When deposited with gold nanoparticles, it enables higher density of bound antibodies/antigens as the nanogold modified electrodes create larger surface for agent capture. By examining impedance response on the electrode in the form of impedance spectra reaction progress could be observed. Immunoassay was conducted with the use of recombinant dust mite allergen Der f2 and its specific antibodies. The technique proved successful and viable for future analyses [35].

## Conclusion

Mass-produced antibodies are used as a precise tool for detection in immunometric assays. As the products are commonly available, it is essential to use them not only in laboratory work. Primitive isotopic signal converters as well as the heavy macromolecular enzymes with colorful reaction in a specific environment are being superseded by fluorescent molecules. As the new companies emerge that do services in the field of instrumental techniques, it becomes possible to scan the probes with electromagnetic waves instead of labeling the antibodies. Analysis of a sample for the presence of a number of antigens at the same time and with high sensitivity is still a challenge.

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# IMOBILIZOWANE PRZECIWCIAŁA JAKO BIOSENSORY DO DETEKCJI BIAŁKOWYCH ALERGENÓW POKARMOWYCH W PRODUKTACH ŻYWNOŚCIOWYCH

## Streszczenie

W pracy przedstawiono teoretyczne podstawy tematu immobilizacji przeciwciał oraz opisano rozwiązania, które mogą zostać zastosowane przy projektowaniu testu na obecność alergenów w produktach żywnościowych. Objawy alergii są konsekwencją spożycia produktów zawierających elementy o własnościach alergennych. Oznaczanie alergennych białek wykonuje się głównie z użyciem metod immunometrycznych. Pierwsze biosensory składały się ze specyficznych przeciwciał unieruchomionych na płytce polistyrenowej. Później podejmowano

kroki w celu uwolnienia chemicznych grup funkcyjnych w polimerze matrycowym tak, aby wiązanie przeciwciała odbywało się z mniejszą przypadkowością (nitroceluloza) i było bardziej stabilne. Następnie opracowano złożone matryce zdolne do wiązania z wyższą gęstością i ograniczonym efektem denaturacyjnym (polikaprolaktam) oraz skomplikowane struktury znane jako warstwy SAM (self-assembled layers – ang.). Duży dystans pomiędzy przeciwciałem a matrycą jest korzystny, gdyż pozwala zminimalizować straty z tytułu efektów sterycznych. Transducer łączony na stałe do przeciwciała pozwala rejestrować wyniki reakcji wiązania.