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A Study of Selectivity of Artificial Gel Antibodies for Protein

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Summary

Artificial gel antibodies against proteins (haemoglobin and human growth hormone) in the form of granules of polyacrylamide were synthesized using a molecular-imprinting approach. The selectivity of artificial gel antibodies was studied by spectrophotometric and electrophoretic methods.

When artificial gel antibodies against haemoglobin were incubated with haemoglobin, some haemoglobin was adsorbed. The selectivity of the gel antibodies was determined by comparing the amount of haemoglobin adsorbed by the gel antibodies and blank gels. In theory, the gel antibodies should have a higher selectivity than the blank gels, but the results were inconclusive. However, electrophoretic analysis (free zone electrophoresis with gel antibodies) indicated that the gel antibodies interacted strongly with haemoglobin and migrated faster than blank gels in the capillary.

Artificial gel antibodies against human growth hormone (HGH, dimer and monomer) were also synthesized by a molecular-imprinting approach. When the gel antibodies against dimer were incubated with dimer and monomer, the relative migration velocity of the gel with dimer was higher than that of the gel with monomer by free zone electrophoresis. It indicated that the dimer-specific gel antibodies interacted with more dimer than monomer. The monomer-specific gel antibodies incubated with monomer migrated faster than the same gel incubated with dimer, which indicated that the monomer-specific gel antibodies adsorbed more monomer than dimer. The above results demonstrate that the artificial gel antibodies have a high selectivity for template proteins.

Introduction

Molecular imprinting technique

In the biological field, molecular recognition is a ubiquitous and extremely important process usually involving proteins. Proteins are macromolecules that often contain recognition sites that can specifically bind target molecules. Molecular recognition in proteins, especially antibodies, promotes the development of useful biological tools. Expanding the natural process by development of artificial recognition materials that imitate the natural binding process is also an interesting and attractive research subject. One approach is to synthesize artificial recognition agents using a process of molecular imprinting [36].

The molecular imprinting is defined by Alexander as: ‘The construction of ligand selective recognition sites in synthetic polymers where a template (atom, ion, molecule, complex or a molecular, ionic or macromolecular assembly, including micro-organisms) is employed in order to facilitate recognition site formation during the covalent assembly of the bulk phase by a polymerization or polycondensation process, with subsequent removal of some or all of the template being necessary for recognition to occur in the spaces vacated by the templating species’ [1]. The molecular imprinting process is shown in Figure 1.

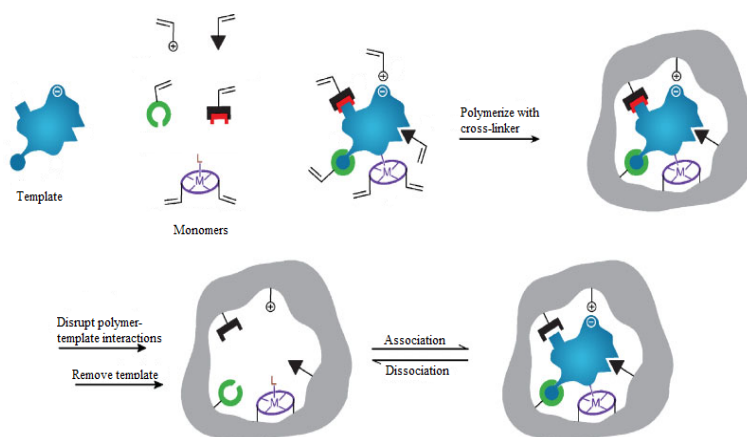


Figure 1. Schematic representation of the molecular imprinting process (reproduced with permission from the copyright owner) [1]. Polymerization is carried out in the presence of monomers, cross-linker and template, which results in the formation of polymer in which template recognition sites are created. The template is then removed from the polymer by disruption of template-polymer interactions. The template may then be selectively reabsorbed by the polymer.

In 1972, Wulff and Sarhan first used the molecular imprinting technique to synthesize artificial antibodies against low-molecular-mass compounds [39]. Molecular imprinting is well developed in the field of preparation of molecular recognition materials and has enormous potential applications because of easy preparation, mechanical robustness and cost

effectiveness [21]. This technique has many potential applications in the pharmaceutical and biotechnological field [1]. Imprinted polymers can be used as vehicles of drug delivery to control release of drug and as screening tools to discover new drugs [1, 4, 11, 15, 18, 23, 28-30].

Artificial gel antibodies

In general, small molecules ($MW < 1000$) as templates can lead to high affinity and specificity of imprinting polymers [3, 36]. However, the affinity and specificity may decrease with an increase of the template size [36]. Therefore the imprinting of macromolecules, in particular proteins, is a challenging research field [36]. Antibodies are widely used recognition elements in the lab practice and diagnosis of diseases [36]. Although antibodies are used as essential lab tools, they are expensive to produce and easily denatured [36]. Therefore, there is a strong need to develop alternatives with improved characteristics, such as cheapness, stability, high selectivity, accuracy and reusage. By using the molecular imprinting technique, Wulff [38] attempted to synthesize gel antibodies against biomolecules [34, 35]. However, since he didn't find the appropriate monomers and synthesis conditions, the selectivity of the gel antibodies was very low [38].

Some issues that aren't shown during small molecule imprinting need to be addressed when protein imprinting is attempted, for instance solvent, biomolecule size and biomolecule denaturation [36]. Solvent: it is the major factor and plays a very important role in protein imprinting. In order to strengthen the hydrogen bonding on which the imprinting polymer depends for recognition, the molecular imprinting mostly proceeds in organic solvents [36]. Since proteins mainly work in aqueous solutions in biological systems, their instability and insolubility in organic solvents affect the choice of solvent [36]. Biomolecule size: in general, traditional molecular imprinting technique (small molecule imprinting) is used to prepare polymers with narrow pores in which the biomolecule is too large to reach any formed recognition sites [36]. Denaturation: imprinting procedures are mostly carried out in an environment where the organic solvents or monomers may denature the biomolecules or change their conformations; consequently specific imprinting sites can't be formed [36].

To solve the above issues, many researchers (e.g. Wulff [38] and Venton & Guidipati [37]) have attempted to find the optimal conditions in protein imprinting. Finally, Stellan Hjertén successfully synthesized highly selective artificial gel antibodies specific against proteins using acrylamide and methylenebisacrylamide as monomers and aqueous buffer as a solvent by the molecular imprinting technique in 1996 [19]. The method was to prepare the gel granules in the presence of a protein of interest [14, 25], where the protein (antigen) was mixed and polymerized with a monomer solution (a buffer containing acrylamide, methylenebisacrylamide, TEMED and ammonium persulfate). After the polymerization, the gel was granulated [2, 6, 7, 9, 25, 31-33]. Removal of the protein by sodium dodecyl sulphate

(SDS) or by a proteolytic enzyme created cavities in the gel particles with shapes complementary to the protein present during the preparation [2, 6, 7, 9, 25, 31-33]. Upon application of a sample containing this protein and other proteins, only the protein used in the polymerization step will be adsorbed selectively by the artificial gel antibodies [2, 6, 7, 9, 25, 31-33]. The interactions between protein and polymer depend on non-covalent forces such as hydrogen bonds, van der Waals forces and dipole-dipole interactions. The artificial gel antibodies were synthesized by a molecular imprinting approach, and the bonds between proteins and gel granules were so strong that SDS and enzyme were used to break the interactions and remove the proteins [7]. In general, different proteins require different degradation treatments, for example, both SDS and trypsin were used to remove the haemoglobin from the gel granules, whereas growth hormone could be degraded by trypsin, but not by SDS [7].

The selectivity of the artificial gel antibodies is quite high because of the strong interactions between gel antibodies and protein [7]. Most native antibodies only bind part of the antigen; however, the gel antibodies interact with the whole antigen, which indicates that they may possess higher selectivity than native antibodies [7]. In the living organisms, the interactions between functional proteins influence all life activities, metabolism, and regulation and so on. Hence, the complex of gel antibodies-protein could be envisaged to be used as a tool to regulate and control biological systems in future. Furthermore, this material can play an important part in diagnosis of diseases by detecting the level of proteins in body fluids.

Target proteins

In medicine, biomarkers play an essential role in early diagnosis, disease prevention, drug target identification and so on. A biomarker is a substance in blood or tissue whose concentration can indicate the presence of a disease state or measure the progress of disease or the effects of treatment. A biomarker can be a cell, a gene, but most commonly a protein, e.g. both haemoglobin and human growth hormone are very important biomarkers.

Haemoglobin contains four subunits, two α and two β subunits, and its total molecular weight is about 68 000. Haemoglobin concentration is an important indicator for human health. For example, low concentration can be caused by nutritional deficiency, kidney failure or bone marrow disease. Moreover, tumors and dehydration may cause too high concentration of haemoglobin. Human growth hormone (HGH) monomer is a 191 amino acid peptide hormone that stimulates cell growth and reproduction. Sometimes HGH is in the form of dimer consisting of two monomers non-covalently bound to each other, but the functional differences between dimer and monomer haven't been identified. In medicine, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are often used to detect the HGH based on the interaction between antibody and antigen [8]. However, the antibodies usually only reflect the concentration of HGH and don't discriminate the structural

differences between HGH dimer and monomer [8]. So it's difficult to purify the dimer and monomer from the HGH. Recently Ghasemzadeh *et al.* [8, 9] used a molecular imprinting approach to synthesize artificial gel antibodies against glycosylated HGH, non-glycosylated growth hormone, HGH dimer and monomer. The gel antibodies could specifically adsorb this growth hormone used for their preparation, and also be used to determine the concentration of the protein in a body fluid in combination with free zone electrophoresis technique [8, 9]. Moreover, the structural differences between HGH dimer and monomer could be discriminated by artificial gel antibodies with the analysis of high performance liquid chromatography (HPLC) [8]. Although HPLC is an efficient method for protein analysis, it is solvent-consuming.

Methods for protein purification

Protein purification involves a series of steps to separate a particular protein from a mixture containing many constituents [26]. To obtain a pure protein is important for studies of the structure and function of proteins [26]. Purification steps are mainly dependent on the differences in protein properties, for example, size, molecular weight, isoelectric points and polarity [26]. According to the amount of protein separated, the methods of protein separation can be largely classified into preparative and analytical methods [26]. Preparative methods are used to prepare and isolate the protein of interest [26]. Typical methods are ion exchange chromatography, affinity chromatography, immunoaffinity chromatography and HPLC [26]. Analytical methods are employed for the detection and identification of proteins, e.g. gel electrophoresis, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry [26].

Preparative methods

Chromatography is a widely used tool for protein purification. In principle a mixture containing various proteins flows through a column packed with some material [26]. Due to different interactions between the proteins and the column material, the various proteins will be retained differently in the column [26]. Ion exchange chromatography is based on the type and strength of the charge of the proteins to be separated [26]. In the column, anionic exchange resins with positive charges are used to separate negatively charged proteins, whereas cationic exchange resins containing negative charges are used to separate positively charged proteins [26]. Affinity chromatography separates proteins by resins containing ligands that are used to specifically interact with the desired protein [26]. Immunoaffinity chromatography uses a specific antibody to bind and purify the desired protein [26]. The antibody immobilized to a column material selectively interacts with the target protein which is then eluted by a strong buffer solution [26]. HPLC is a popular separation technique using high pressure to move the solution through the column [26]. Reversed-phase chromatography is the most common HPLC, where the proteins interact with a hydrophobic column material and are eluted through a gradient of increasing amounts of an organic solvent [26]. Although

these preparative methods are very useful to purify large amount of proteins, they cannot treat the extremely low of concentrations of proteins [26].

Analytical methods

SDS-PAGE is a common laboratory technique based on the protein size [26]. The proteins are first denatured by an SDS solution and coated with negative charges [26]. They are then separated in an electric field according to their charges, which are proportional to their molecular weights [26]. Both chromatography and electrophoresis are very popular and widely used to separate proteins, which are based on the bioaffinity to separate a particular protein from a mixture containing many constituents [35]. There is a great need for novel analysis techniques, which permit quantitative determination, of extremely low concentrations of proteins rapidly and with high sensitivity and reproducibility. In two papers it was demonstrated that the absorption spectrum of proteins adsorbed to artificial antibodies in the form of gel granules could be used to evaluate the selectivity of artificial gel antibodies for the proteins [6, 7]. An alternative, previously employed technique was also useful to study the selectivity of the artificial gel antibodies [8, 9, 31-33]. This method was based on an electrophoretic separation technique, free zone electrophoresis in a rotating glass tube. The rotation prevented the gel granules from sedimentation towards the tube wall [8, 9, 31-33]. The higher the concentration of the protein in the gel granules, the higher the electrophoretic migration velocity of the granules.

Free zone electrophoresis

The apparatus for the free zone electrophoresis (Figure 2) was designed in 1958 [31] and employed in molecular studies of proteins, viruses and bacteria [2, 8, 9, 31-33]. Free zone electrophoresis is in some aspects similar to capillary electrophoresis. Capillary electrophoresis is a technique in which electrophoresis is performed under high electric fields in a small diameter glass capillary where the ionic compound or charged molecule can be separated [27]. The free zone electrophoresis is used to separate small molecules, large molecules and particles [12]. It can separate inorganic ions, organic ions, nucleic acid bases, nucleosides, nucleotides, proteins, nucleic acids, subcellular particles, viruses, and erythrocytes [12]. In combination with the artificial gel antibodies, free zone electrophoresis can be used not only to separate proteins, but also to differentiate minute structural differences between related viruses, bacteria and proteins [2, 8, 9, 31-33].

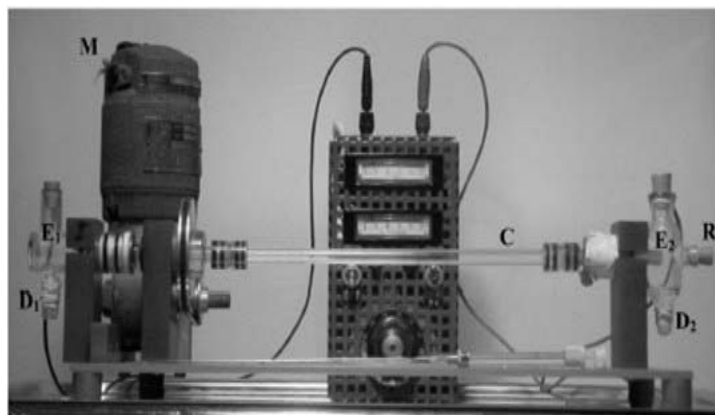


Figure 2. Apparatus of capillary free zone electrophoresis (reproduced with permission from the copyright owner) [31]. C is a glass tube (OD: 9.6 mm, ID: 2.5 mm, length: 245 mm) which rotates at a speed of 40 rpm by motor (M). The electrolyte vessels are covered by dialysis membranes (D1, D2) in order to prevent the flow of buffer into the tube. The samples are injected by a syringe into the glass tube. E1 and E2 are electrode vessels. Samples are run in a rotating 245 mm long glass tube which is coated with covalently attached polyacrylamide at room temperature. The coating is used to reduce adsorption and EOF. In the experiment, samples contain negative charges and run toward the anode at the current of 0.2 mA and voltage of 500 V.

Electroosmotic flow (EOF)

The inner surface of a fused-silica glass capillary contains negatively-charged groups that can arrest positively charged ions [17, 27]. When the positively charged ions move towards the negative electrode, the solvent molecules migrate in the same direction [17, 27]. The migration of solvent molecules is called electroosmotic flow (EOF) (Figure 3) [17, 27]. When separation is carried out in the glass capillary, the negatively-charged molecules migrate more slowly, the positively-charged molecules migrate faster, and uncharged molecules migrate with the same speed as the electroosmotic flow [17, 27]. Coating can cover the inner surface of glass capillary, and reduce the effect of EOF.

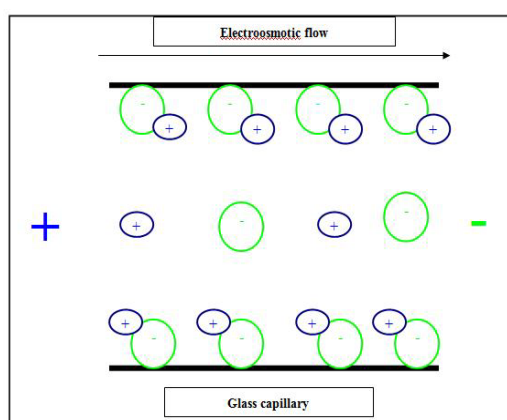


Figure 3. The schematic of electroosmotic flow in the glass capillary. Big green circle means negatively-charged molecule, small blue circle means positively-charged molecule. “+” means positive electrode, “-” means negative electrode, the arrow indicates the direction of electroosmotic flow.

Aims:

The aim of this study was to determine the selectivity of artificial gel antibodies against haemoglobin using both spectrophotometric and electrophoretic methods, and to study the selectivity of artificial gel antibodies against human growth hormone for the template and template isoform by free zone electrophoresis technique.

Results

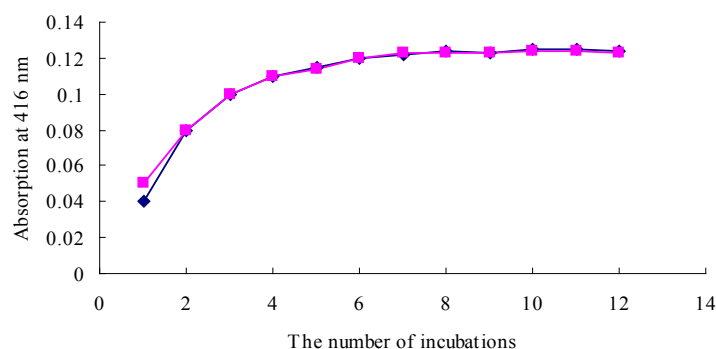
Spectrophotometric analysis to study the selectivity of artificial gel antibodies

To detect the selectivity of artificial gel antibodies, gel antibodies against haemoglobin were synthesized from the N, N'-methylenebisacrylamide and acrylamide in the presence of haemoglobin using a molecularly imprinting technique. Blank gels were prepared under the same condition but without the addition of haemoglobin. After the removal of haemoglobin by SDS and the protease trypsin, the gel antibodies and blank gels (240 mg) were separately incubated with 1 mL of haemoglobin solution. During the incubation, the gel antibodies against haemoglobin interacted with haemoglobin and reached the equilibration, whereas the non-adsorbed haemoglobin was left in the supernatant. After incubation and settlement of the granules by gravity, the absorption of haemoglobin in the supernatant was measured at the wavelength of 416 nm by spectrophotometry. After measurement, another 1 mL of haemoglobin solution was incubated with the two gels and the absorption in the supernatant measured again after settling. The procedure was repeated until the twelfth addition of haemoglobin where the absorption curve (Figure 4 A) was constant.

In another experiment, the gel antibodies and blank gel were incubated with 3 mL of a haemoglobin solution. After every 5 minutes' incubation, the absorption of haemoglobin supernatant above the settled gels was measured and the supernatant was then put back to the gel and incubated again. The steps were repeated until the absorption of the supernatant no longer decreased as shown in Figure 4 B.

As shown in Figure 4 A, the supernatant from the gel antibodies as well as from blank gels contained very similar amounts of haemoglobin. In Figure 4 B, there was no significant difference in the amounts haemoglobin in two gels' supernatants. These results indicate that the concentration of haemoglobin was so high that the gel antibodies and blank gels already became saturated with the first addition of haemoglobin. In addition, lower concentrations of haemoglobin were also attempted, but no difference in selectivity between two gels was observed. It indicates that spectrophotometry was not a good method to determine the selectivity of the artificial gel antibodies against haemoglobin.

A



B

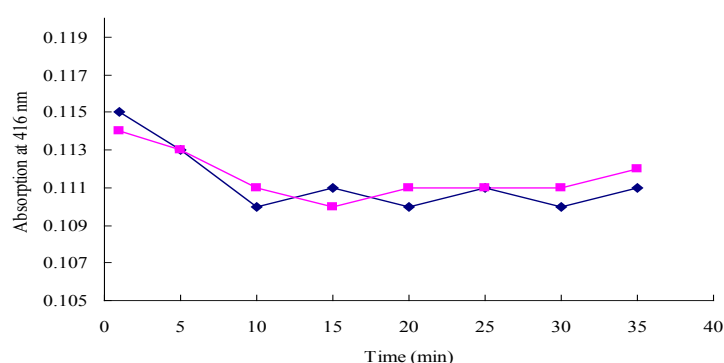


Figure 4. Absorption of haemoglobin supernatant above the artificial gel antibodies (■) and blank gels (◆) after incubation. A: the two gels were incubated continuously with twelve 1 mL portions of haemoglobin solution. B: the two gels were incubated continuously with a 3 mL of haemoglobin solution. The concentration of the haemoglobin solution was 0.375 g/L. The light absorption of the supernatant solution was measured at 416 nm.

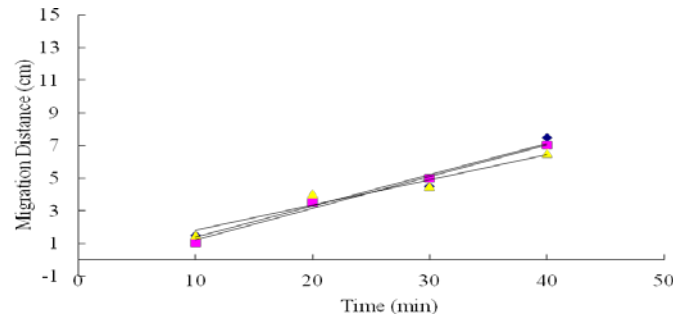
Electrophoretic analysis to study the selectivity of the gel antibodies

Artificial gel antibodies against growth hormone dimer (MW: 45 000) and monomer (MW: 22 000) were prepared by the molecular imprinting approach and tested for selective recognition of the antigens by free zone electrophoresis. The two gel antibodies were incubated with HGH dimer and monomer. After incubation, non-bound HGH was removed by washing. They were then injected and run in the free zone electrophoresis equipment for 40 minutes. Free zone electrophoresis was also an important approach to detect if the imprinted protein in the gel antibodies was removed completely, i.e., protein-depleted gel antibodies and blank gels were run simultaneously in two separate zones of the glass capillary. Figure 5 shows that the removal was efficient; the migration velocities of both granules were quite similar. The migration of control and blank gels may be caused by EOF or buffer leakage.

In Figure 5, the imprinted but protein-depleted gels ran with the same velocity as non-imprinted gels in the same run, but different experimental runs differed. This might be

caused by fluctuations in actual experimental conditions that might lead to differences in either EOF or leakage or both. Thus, to obtain a relative migration distance, the samples and control gels were always run at the same time. The relative migration distance of the samples was calculated by subtracting the distance migrated by control gel from the distance migrated at the same time by sample gels.

A



B

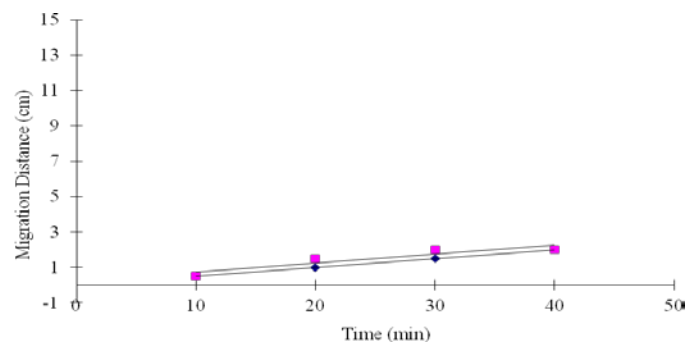
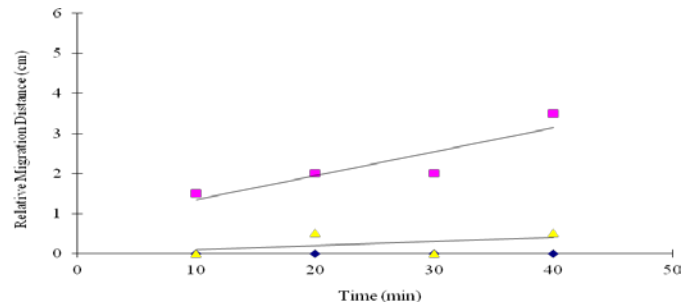


Figure 5. Free zone electrophoresis of protein-depleted gel antibodies and blank gels. A: human growth hormone (HGH) dimer-depleted (■), monomer-depleted (■) gel antibodies and corresponding blank gels (●) were run at the same time. B: haemoglobin-depleted gel antibodies (■) and corresponding blank gels (●) were run at the same time. The rotating narrow bore tube (capillary) was filled with 50 mM Tris-HCl, pH 8.5. Voltage: 500 V. The charged particles moved towards the anode, current: 0.2 mA. Length of the narrow bore tube: 24.5 cm, id: 2.5 mm, od: 9.6 mm.

Figure 6 A shows that the relative migration velocity of the dimer-charged dimer-specific granules was higher than that of the monomer-charged dimer-specific granules and control (gel antibodies depleted of the dimer). Thus, the dimer-specific granules adsorbed dimer selectively. Furthermore, the monomer-charged dimer-specific granules and the control showed no significant migration differences indicating that this gel didn't bind monomer. Figure 6 B shows that the relative migration velocity of the monomer-charged monomer-specific granules was higher than that of the dimer-charged monomer-specific granules and control (gel antibodies depleted of the monomer), which indicates that the monomer-specific granules bound more monomer compared to dimer.

A



B

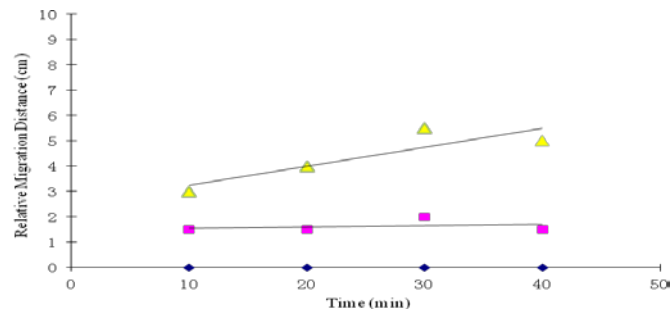


Figure 6. Free zone electrophoresis of HGH-charged gel antibodies. A: dimer-charged dimer-specific granules (■), monomer-charged dimer-specific granules (▲) and control (dimer-depleted dimer-specific granules) (●) were run at the same time. B: dimer-charged monomer-specific granules (■), monomer-charged monomer-specific granules (▲) and control (monomer-depleted monomer-specific granules) (●) were run at the same time. The experimental conditions were the same as for Figure 5.

Free zone electrophoresis was also used to determine the selective binding of the gel antibodies against haemoglobin to this protein. The gel antibodies were incubated with haemoglobin, after incubation, the haemoglobin-charged gel antibodies were then run in the free zone electrophoresis equipment. Figure 7 shows a significant migration difference between the gel antibodies and blank gels, and indicates that the gel antibodies selectively adsorbed haemoglobin.

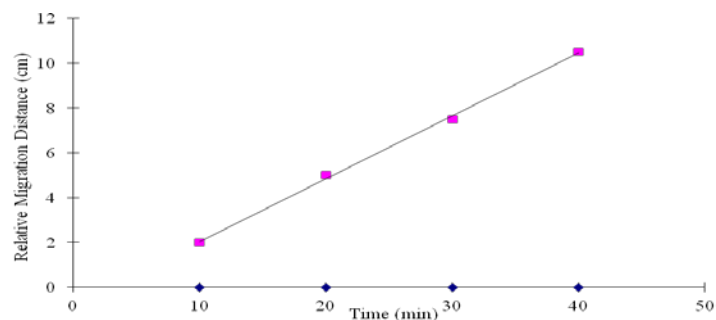


Figure 7. Free zone electrophoresis of gel antibodies against haemoglobin saturated with this protein (■) and blank gels (●). The experimental conditions were the same as for Figure 5, 6.

Discussion

Spectrophotometric analysis to study the selectivity of gel antibodies

Although spectrophotometry is a simple and efficient technique to measure the concentration of proteins, it couldn't detect any selectivity of the gel antibodies in this study. This may be caused by two factors. First, the capacity of the gel antibodies was not so high that the gel granules became saturated already at low protein concentration. Second, the total volume of the protein solution between the settled gel granules was relatively large, i.e. a change in the sample amount would only slightly change the protein concentration in this interstitial volume and thereby also slightly change the concentration in the supernatant following sedimentation. In addition, there might be some gel granules in the supernatant. Thus, when the supernatant was measured, the haemoglobin content might have been overestimated due to the presence of gel granules. To avoid this, the granules can be settled by centrifugation. Some other measuring errors may also have affected the result, for instance, loss of the supernatant when the pipettes and cuvettes were used and so on. In order to detect the selectivity by spectrophotometry, more precise instruments should be used to minimize errors.

Electrophoretic analysis of the gel antibodies

The selectivity of artificial gel antibodies against HGH dimer and monomer was studied by free zone electrophoresis. The dimer-specific granules interacted with more dimer than monomer, which indicates that the dimer-specific granules could selectively adsorb dimer. Since the monomer-charged dimer-specific granules and the control granules migrated at the same speed, any monomer binding to the gel granules was likely unspecific. The monomer-specific granules adsorbed more monomer than dimer, which indicates that the binding of monomer to the gel granules was specific. The haemoglobin-charged haemoglobin-specific granules adsorbed more haemoglobin than blank gels, which indicates that the gel granules could selectively recognize the haemoglobin.

For future study of artificial gel antibodies, they can be used to detect the concentration of proteins (growth hormone, haemoglobin) in the solution by free zone electrophoresis. The higher the protein concentration in this solution, the higher its concentration in the gel granules and, thereby, also the higher the electrical charges of the granules. Therefore, when analyzed by free zone electrophoresis the migration velocity should be proportional to the concentration of the protein in the sample solution. Accordingly, from a plot of the migration velocity against the concentration of the protein in a sample solution with known concentration (calibration curve), the concentration of the protein in any solution, for instance serum, can be determined. This concentration is of a great interest in many analytical experiments, for instance when biomarkers in serum or cerebrospinal fluid are used for

diagnosis and prognosis, for detection of hepatitis viruses and HIV in blood, and for rapid detection of air-borne anthrax and bacteria in urine [31]. In addition, artificial gel antibodies have more potential applications: they may be used as sensing elements in biosensors, or injected into the blood circulation system to interact with virus particles and so on [31].

Artificial gel antibodies are a successful example of protein imprinting, and used to specifically recognize and separate the template protein in combination with free zone electrophoresis [2, 8, 9, 31, 33]. Recently, researchers have also developed some novel methods of protein imprinting [5, 10, 42]. Herrero *et al.* [10] used bovine serum albumin as a template to prepare molecular imprinting capsules by ionic gelation of sodium alginate and calcium chloride. The capsules could recognize higher quantities of protein than the existing technologies developed until now. Cai *et al.* [5] reported a carbon-nanotube nanosensor coated with an imprinted non-conducting polymer which could specifically adsorb human ferritin and human papillomavirus-derived E7 proteins with subpicograms per litre using electrochemical impedance spectroscopy. The protein-imprinting nanosensor could also differentiate between Ca^{2+} -induced conformational changes in calmodulin. Zayats *et al.* [42] used acrylamide, N-isopropylacrylamide, N-[3-(dimethylamino)propyl]methacrylamide, methacrylic acid and N,N-methylenebisacrylamide as functional monomers to synthesize molecularly imprinted polymer films which could specifically recognize maltose binding protein. The films not only had high selectivity, but could also discriminate the differences between maltose binding protein and reference proteins with similar molecular weight, dimensions and isoelectric point. Although the researchers make great progress in the protein imprinting, there are still many challenges for future study of this technique. Due to the different properties of proteins, it is very difficult to develop a common preparation method of protein imprinting suitable for every template protein.

Materials and methods

Preparation of human haemoglobin from fresh blood

Haemoglobin was prepared as described by Takátsy *et al.* [16, 33]. Fresh human blood (5 mL) was obtained from the University Hospital in Uppsala and centrifuged at $1300 \times g$ for 10 min at room temperature. After the removal of the supernatant, the pellet containing the red blood cells was suspended in 5 mL of 0.9% NaCl solution and centrifuged at $1300 \times g$ for 5 min. This washing step with NaCl solution was repeated 3 times. A 200 μL suspension of red blood cells was then mixed with 800 μL of sterile water in a 1.5 mL Eppendorff tube and centrifuged at $12000 \times g$ for 3 min at room temperature. The supernatant was then stored in Eppendorff tubes in a freezer at $-20\text{ }^{\circ}\text{C}$. The concentration of haemoglobin was about 300 g/L, determined by spectrophotometry at 416 nm and using Beer-Lambert Law Calculator, $c=A/b \cdot \epsilon$ (A: absorbance, ϵ : molar absorptivity ($1.8\text{ L mol}^{-1}\text{ cm}^{-1}$), b: path length of the sample (1 cm), c: concentration of the compound in solution (mol L^{-1})).

Preparation of the gel antibodies

Synthesis of artificial gel antibodies against haemoglobin

Ammonium persulfate, Tris, TEMED, N, N'-methylenebisacrylamide, acrylamide, and SDS were purchased from Bio-Rad Laboratories (Hercules, USA) [6]. Acrylamide (568 mg) and N, N'-methylenebisacrylamide (30 mg) were dissolved in 8.8 ml of 20 mM sodium phosphate buffer (pH 6.8) in a 10 mL test tube. Template molecule, haemoglobin (800 μL , 300 g/L) and 200 μL of 5% (w/v) TEMED were then added to the acrylamide solution. After deaeration for 3 min, the polymerization was initiated with 200 μL of a 10% (w/v) solution of ammonium persulfate and allowed to proceed for 16 h at room temperature. For comparison, blank gels were prepared under the same condition except that no haemoglobin was added. After polymerization, both gels were granulated by manually pressing them through a 60 mesh net three times, followed by a 100 mesh net twice. The non-imprinted haemoglobin was removed by repeated wash with a total of 50 mL of 50 mM Tris-HCl buffer, pH 8.5. For removal of the imprinted haemoglobin, the gel granules were first washed for 2 h using a washing solution (50 mM sodium dodecyl sulphate (SDS) in 50 mM Tris-HCl, pH 8.5) and then with this Tris-HCl buffer alone 12 times (10 mL, 1 h for each washing) to remove the SDS [7].

To test whether the SDS had been removed from the gel granules, all the granules were settled by gravity for 10 min. 17 mg of KCl was then added to 500 μL of the supernatant above the gel granules (K^+ ions precipitate dodecyl sulphate). If there was SDS, the clear supernatant turned cloudy due to the precipitate, and the washing was continued until no precipitate appeared. To completely remove the template, the gel granules were then treated by trypsin (Sigma, Stockholm, Sweden [7]). Trypsin (3 mg) dissolved in 10 mL of enzyme buffer (25

mM Tris-HCl containing 20 mM CaCl₂, pH 8.0) was then added to 10 mL of settled (by gravity for 10 min), haemoglobin-imprinted gel granules and the treatment proceeded at 37 °C in a water bath for 4 h. The enzyme was then removed by repeated washing with 50 mM Tris-HCl, pH 8.5 (total: 50 mL).

Synthesis of artificial gel antibodies against HGH dimer and monomer

The procedure was similar to that used for the preparation of haemoglobin-imprinted gel antibodies, but acrylamide (288 mg) and N,N'-methylenebisacrylamide (12 mg) were dissolved in 5 mL of 0.1 M sodium phosphate, pH 6.2, and 2.5 mg of HGH (dimer or monomer) was then added [9, 14]. After the granulation, the imprinted HGH was removed by trypsin. Blank gels were also prepared under the same condition except that no template was added.

Determination of selectivity of the gel antibodies by spectrophotometric method

Spectrophotometry was used to determine the selectivity of the gel antibodies for haemoglobin which has a maximum absorption at 416 nm. Both blank gels and gel antibodies suspension were settled by gravity and the supernatant was removed. The two gels were settled for about 10 min. 4 mL of settled gel was withdrawn by pipette and separately mixed with 1 mL of diluted haemoglobin solution (0.375 g/L) in 10 mM sodium phosphate buffer, pH 6.8. The suspensions were incubated for 30 min at room temperature. After incubation, the gel granules were settled for 2 min and the optical density of the supernatant (900 µL) above the gel was measured at 416 nm in a spectrophotometer (Lambda Polynom, Tokyo, Japan [7]). After the removal of the supernatant, another 1 mL of the diluted haemoglobin solution was incubated the same way with the granules for another 30 min and the absorption measured after settling of the gel. These steps were repeated 12 times.

To further determine the capacity of the gel antibodies, 3 mL of diluted haemoglobin solution was incubated with another 4 mL of settled blank gels and gel antibodies respectively for 1 min and the absorption of supernatant above the gel was then measured at 416 nm. After measurement the supernatant was put back to the gel, incubated for another 4 minutes, and the absorption measured again. These steps were repeated 8 times, and each time the gel suspension was incubated for 5 minutes.

Electrophoretic analysis of the gel antibodies

Polyacrylamide coating of glass capillaries

Glass capillaries were coated with polyacrylamide to eliminate adsorption and EOF based on a procedure by Hjertén and collaborators [13, 22]. To obtain a clean inner capillary surface, the capillary tube was first washed with 1 M NaOH, 1M HCl and finally water and dried with

an air stream. The capillary was then filled with a pH 3.5 silane solution containing 80 μL of γ -methacryloxypropyltrimethoxysilane (Sigma, Stockholm, Sweden), 20 μL of 99% (w/v) acetic acid and 20 mL of sterile water, left for 20 h at room temperature, then rinsed with sterile water and dried in vacuum for 24 h. The inner capillary surface was then coated with 150 μL of acrylamide, 3 μL of ammonium persulfate and 3 μL of TEMED, all three components being 5% aqueous solutions (v/v for TEMED and w/v for the other two compounds) which was allowed to polymerize for 20 h at room temperature. After 20 h, the polymer was removed from the capillary by rinsing with water, and the coated capillary was dried in an oven at 35 °C.

Preparation of samples

800 μL of the settled (by gravity for 10 min) gel antibodies against HGH dimer or monomer were mixed separately with 800 μL of 0.5 g/L HGH dimer or monomer solution in 50 mM Tris-HCl, pH 8.5. The mixture was incubated with agitation at room temperature for 2 hours. The gel granules were then washed with 50 mM Tris-HCl, pH 8.5 (total volume: 30 mL) to remove the non-adsorbed growth hormone. Finally, the gel antibodies were settled by gravity for 10 min and the supernatant was removed by pipette. The gel antibodies against haemoglobin and the corresponding blank gels were treated using the same conditions except for the use of haemoglobin instead of HGH. The samples prepared were used to run the free zone electrophoresis.

Electrophoretic conditions and measurement of samples

Zone electrophoresis of gel antibodies was carried out in a rotating 245 mm long polyacrylamide-coated glass capillary with the inner diameter 2.5 mm and the outer diameter 9.6 mm. To avoid settling of the granules, the capillary was rotated at a speed of 40 rpm by a motor and filled with 50 mM Tris-HCl buffer, pH 8.5. The granules were analyzed as follows [32]: A suspension (25-30 μL) of the gel antibodies (10-15 gel granules) in 50 mM Tris-HCl buffer, pH 8.5 was sucked into a 25 cm long metal tube (inner diameter 0.5 mm, outer diameter 1.5 mm) by a 2 mL syringe and then injected into the rotating glass capillary. In general, two or three separate zones were applied to place the granules in this capillary. A pen was used to mark the position of the starting zone of the granules that was 3-4 mm long. When a 500 V voltage was applied, a current of 0.2 mA was obtained and the migration of the charged granules towards the anode could be measured. The whole running procedure proceeded for 40 min at ambient temperature. The migration distance of granule zones was measured at 10 min intervals by a ruler. The migration velocity was the slope of a straight line obtained from plotting migration distance against time.

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