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Oriented immobilization of antibodies through different surface regions containing amino groups: Selective immobilization through the bottom of the Fc region

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ABSTRACT

Amino groups on the antibody surface (amino terminus and Lys) are very interesting conjugation targets due to their substantial quantities and selectivity toward various reactive groups. Oriented immobilization of antibodies via amino moieties on the Fc region instead of the antigen-binding fragment (Fab) is highly appreciated to conserve antigen-binding capacity. In this paper, targeting amino moieties on distinct regions, three antibody immobilization strategies were compared with the recognition ability of corresponding adsorbents. Our results demonstrate that oriented immobilization of antibodies onto heterofunctional chelate-epoxy support selectively involving Lys residues placed at the bottom of the Fc region, thus preserved the highest antigen recognition capacity (over 75% functionality). For homofunctional aldehyde support, immobilization at pH 10 demonstrates 50% remaining functionality due to the random orientation of tethered antibodies; while only 10% functionality remained when N-terminus were specifically conjugated at pH 8.5. With the rationalization of moieties density onto heterofunctional support, 2-fold recognition capacity was exhibited over randomly immobilization for antigens with higher size (β-galactosidase, 425 kDa vs. horseradish peroxidase, 40 kDa).

Meanwhile, at least 97% of antigens with a varied concentration in diluted human serum were efficiently captured by the optimized chelate-epoxy support. Therefore, our antibody immobilization protocol proved the potential to be utilized as a promising candidate to capture voluminous antigens (large proteins and cells) in real samples.

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1. Introduction

Specifically recognize and capture analytes with high sensitivity are required in biomedical applications, such as immunodiagnostics [1], biosensing [2–4] and bioimaging [5,6]. Antibodies, an asymmetric macromolecule with (Y)-shape, can be immobilized with four possible orientations (head-on, side-on, bottom-on and flat-on) which may demonstrate distinct antigen-binding accessibilities [7,8]. Regardless of distinct supporting matrices, for an ideal immunosensor, covalently immobilized antibodies with highly controllable orientation and tunable coverage density are crucial [3,9–12]. Numerous coupling strategies have been attempted to link antibodies with high biological activity [7,8,13–17]. Yet, disordered antibody orientation through direct attachment is likely to cause lower signal-to-noise ratio, reduced antigens binding activity and decreased loading capacity [17,18],

Various chemistries have been performed to conjugate immunoglobulin G (IgG) onto supports with uniform orientation, three of the available moieties on the antibody surface have garnered great attention, which are sulfhydryl moieties, polysaccharides chains and amino moieties [19,20]. It was reported that the biological activity of conjugated antibodies was highly maintained when sulfhydryl groups and aldehyde groups were utilized due to the non-participation of antigen-binding regions [21,22]. In addition, the strong affinity of thiolated antibodies toward the Au surface facilitate the quantitation of antibody orientation via surface plasmon resonance (SPR) techniques [22]. However, to introduce such reactive moieties, chemical treatment of native antibody or protein engineering of recombinant antibody fragment is a mandatory procedure [19,23]. In contrast, amino groups on the surface of IgG may be the most suitable for covalent immobilization on activated support surfaces. When amino groups are unprotonated, they easily react, without activation, with a high number of reactive groups on the support surface: aldehyde groups, carboxylic groups activated with *N*-hydroxysuccinimide (NHS), tosyl groups, etc. [24–26]. By controlling the conjugated amino groups during covalent interaction,

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orientations of the IgG on the support surface can be modulated easily. In this paper, three interesting orientations were compared, which are:

A. - By performing the IgG immobilization at slightly alkaline pH (8.5), only the amino terminus (N-terminus) of the four chains of IgG can react with reactive groups on the supports (Fig. 1A).
B. - By performing the IgG immobilization at alkaline pH (10), all amino groups (Lys residues and N-termini) present in all regions of the IgG surface can be attached to the support (Fig. 1B).
C. - By performing a two-step immobilization, the IgG can be immobilized through amino groups (Lys residues) placed on the bottom of the Fc region which would not deteriorate the recognition activity of immobilized antibodies [15,16] (Fig. 1C).

Previously, we demonstrated that metal chelate-glyoxyl activated matrix is a useful tool to specifically bind antigens from a crude protein extract [15]. Taking advantage of fast affinity adsorption between chelate groups and His clusters placed on the bottom of the Fc region of the IgG, the following conjugation allows the intramolecular reaction between glyoxyl groups on the support surface with Lys residues present in the vicinity of this His-rich region. In this way, only Lys residues placed on the bottom of the Fc region can be covalently coupled to the activated support [15]. However, this protocol requires a final step of borohydride reduction and this reduction may undermine the intra-disulfide bonds of the IgG, thus decrease antigen-binding capacity. Now, in this work, we propose another strategy, the selective immobilization of antibodies on heterofunctional chelate-epoxy supports. Epoxy groups have a lower reactivity than glyoxyl groups, but they directly form secondary amino bonds, and hence the final reduction process is not necessary [27].

Herein, anti-horseradish peroxidase antibody (anti-HRP) and anti-β-galactosidase antibody (anti-BGL) were immobilized on functionalized agarose gels according to the three protocols described above. The immobilization rates and subunits involved in the immobilization of IgG were evaluated. The rate and capacity of adsorption of horseradish peroxidase (HRP) and β-galactosidase (BGL) by different immobilized IgGs were also tested. The heterofunctional supports were the more promising supports mainly for the capture of large antigens. Chelate-epoxy supports were further optimized: the density of epoxy groups was modulated, and the optimal ratio between epoxy groups and metal chelate groups was obtained. Random immobilization provided an intermediate capacity of antigen capture. In contrast, IgG immobilization through amino terminus (the most popular and easiest immobilization protocol) gave very poor results.

2. Materials and methods

2.1. Chemicals and reagents

Agarose 6% crosslinked beads (BCL) was purchased from Agarose Bead Technologies (Madrid, Spain). All the other activated matrices were based on agarose beads. Glyoxyl-activated agarose was synthesized as described in the literature [21].

o-Nitro-phenyl-β-D-galactopyranoside (*o*-NPG), sodium borohydride, iminodiacetic acid (IDA), 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), sodium phosphate, glycine, sodium bicarbonate, sodium periodate, horseradish peroxidase (HRP), β-galactosidase (BGL), anti-horseradish peroxidase (anti-HRP), anti-β-galactosidase (anti-BGL) and human serum (from male AB clotted whole blood) were obtained from Sigma (St. Louis, USA). Nickel sulfate, sulfuric acid and hydrogen peroxide were purchased from PanReac AppliChem (Barcelona, Spain). Dithiothreitol (DTT) was obtained from Carbosynth (Berkshire, UK). PEG 6000 was purchased from Merck (Darmstadt, Germany). Coomassie (Bradford) protein assay kit was purchased from Thermo Fisher Scientific Inc. (Rockford, USA). All other reagents were of analytical grade.

2.2. Assay of enzyme activity

The enzyme activity of HRP was determined by monitoring the substrate oxidation (ABTS) to its cation radical ($ABTS^+$) using H_2O_2 as the oxidation reagent (molar extinction coefficient of $ABTS^+$ was 36,000 M^{-1} cm^{-1}) [21]. 0.001 M ABTS and 0.001 M H_2O_2 in sodium phosphate buffer (0.05 M, pH 6.0) mixed with the enzyme to compose the reaction mixture. The absorbance increase at 420 nm was measured at 25 °C by Jasco V-730 UV–Visible spectrophotometer (Tsukuba, Japan). One enzyme unit was defined as the generated 1 μmol of $ABTS^+$ per min/mg enzyme under the conditions described. Each set

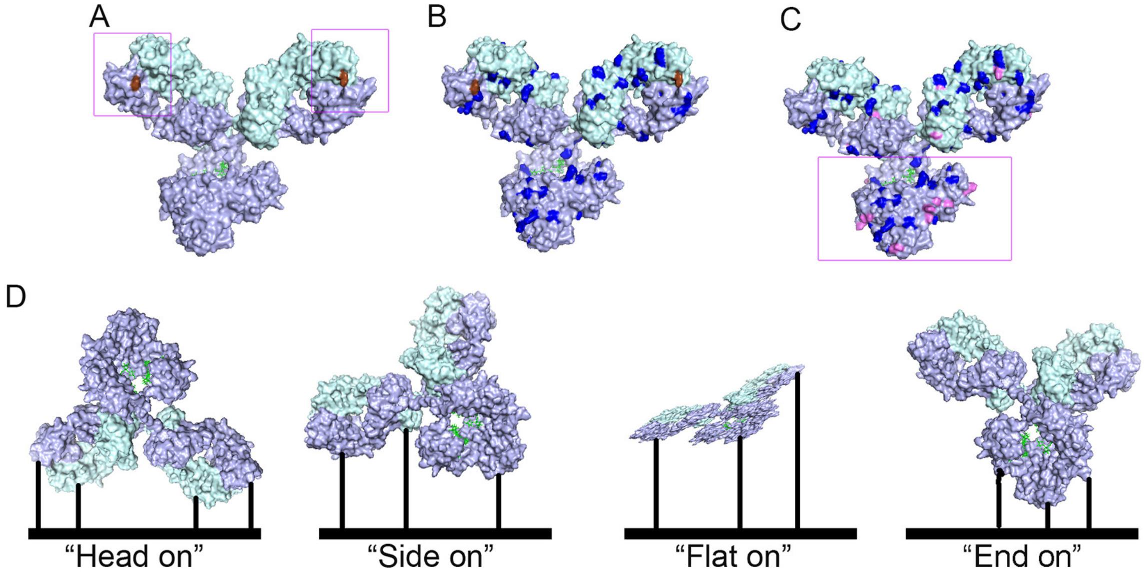

Fig. 1. 3D structure of IgG. (A) For protocol A, N-terminal groups are the only sites involved in the immobilization; (B) For protocol B, antibodies will be immobilized on the support surface through N-terminal groups and lysine residues; (C) For protocol C, the immobilization sites was limited on the Fc region of antibody. The immobilization sites with the most possibility are indicated by the red boxes. (D) Four possible orientations when immobilize antibody onto solid supports. The light chains are represent in cyan, and the heavy chains are light blue. N-terminal amino groups are in brown, and polysaccharide moieties are presented in green. Lysine residues (blue color) are distributed on the whole area of IgG surface, and histidine clusters (violet color) are located on the Fc fragment of antibody. The figure was created with PyMol 3.3 (DeLano, USA) using the PDB ID: 1IGY.

of experiments was performed in triplicate, and mean values were calculated.

The enzyme activity of BGL was determined by monitoring the absorbance increase at 405 nm using *o*-NPG as the substrate [28]. The reaction mixture consisted of 13 mmol/L *o*-NPG in sodium phosphate buffer (0.05 M, pH 7.0) with the enzyme. The absorbance was also measured at 25 °C by Jasco V-730 UV–Visible spectrophotometer (Tsukuba, Japan). Under such condition, the molar extinction coefficient of liberated *o*-nitrophenol (*o*-NP) was 3100 M^{-1} cm^{-1}. The enzyme activity was given in μmol of *o*-NP per min/mg enzyme under the conditions described. Each set of experiments was performed in triplicate, and the mean values were calculated.

2.3. Antibody immobilization on the different agarose-based supports

2.3.1. Antibody immobilization on glyoxyl-activated agarose support

Protocol A (immobilization of antibodies via terminal amino groups): 10 mL anti-HRP (0.4 mg/mL) in 0.025 M sodium bicarbonate pH 8.5 were incubated with 1 g of glyoxyl-agarose wet support at 25 °C for 24 h.

Protocol B (random immobilization of antibodies): 1 g of glyoxyl-agarose was added to 10 mL of 0.025 M sodium bicarbonate pH 10 containing 4 mg anti-HRP, 20% (*w*/*v*) PEG 6000 and 0.05 M DTT. The mixture was incubated at 4 °C for 6 h. The residual protein in the supernatant was checked at different time intervals [29]. After immobilization finished, 1 mg/mL $NaBH_4$ was added into the solution and reacted for 30 min. The preparation was washed with an excess of distilled water to get rid of undesired components.

2.3.2. Antibody immobilization on chelate-epoxy support

Protocol C: The process to prepare chelate-epoxy activated matrix is illustrated in supplementary materials (Fig. S1). 4 mg anti-HRP in 0.025 M sodium phosphate pH 7 were incubated with 1 g of chelate-epoxy wet support at 25 °C. To check the immobilization rate, the remaining protein in the supernatant was checked at different time intervals [29]. After the affinity interaction finished, the formed bio-complex was incubated at alkaline buffer (0.025 M sodium bicarbonate, pH 10) for another 24 h. The covalent attachment was promoted under such conditions. Once the fully covalent attachment was verified, derivatives were incubated with the EDTA buffer (0.5 M EDTA, 0.5 M NaCl, and pH 8) for 1 h. Thus, only covalent attached antibodies were used for antigen capture. The remaining epoxy groups were blocked by glycine solution (3 M, pH 8.5) for 16 h.

2.4. Determination of the non-covalent attached antibody from the different supports

During incubation under alkaline solution, bioconjugate was withdrawn at different time intervals and then incubated with EDTA buffer (0.5 M EDTA, 0.5 M NaCl, and pH 8). The evaluation of fully covalent binding was finished until no leached antibody.

2.5. Immunoassay with different antigens

To calculate the captured antigens per μmol immobilized antibody, a certain amount of protein (HRP or BGL) in sodium phosphate (0.1 M, pH 7) was incubated with 1 g of chelate-epoxy wet support at 4 °C. After incubated for 4 h, the captured antigens were measured by the enzyme activity of bioconjugate.

2.6. Quantification of epoxy groups

Supports that functionalized with varied density of epoxy groups were obtained by controlling the modification time between epoxy groups and IDA. 1 g epoxy functionalized support was incubated with 10 mL H_2SO_4 (0.5 M) for 2 h, epoxy groups were hydrolyzed to vicinal diols [30]. Moreover, it was further oxidized to aldehyde groups with the help of $NaIO_4$ [31]. After functionalization with IDA, the decreased consumption of $NaIO_4$ equals to the density of activated epoxy groups.

2.7. Detection of HRP with different concentrations in aqueous buffer and diluted human serum

To verify the efficiency of optimized chelate-epoxy (4 h), 1 mL HRP solution with different concentrations were prepared in aqueous buffer (0.1 M sodium phosphate, pH 7) and were incubated with 0.1 g anti-HRP conjugated chelate-epoxy (4 h) support at 4 °C. To determine the performance of our proposed strategy under more realistic conditions, samples were prepared using 1% human serum (diluted with 0.1 M sodium phosphate, pH 7) instead of an aqueous buffer. After 4 h of incubation, the captured antigens were measured by the enzyme activity of bioconjugates as described above.

2.8. SDS-PAGE analysis

Samples were boiled with the same volume of lysis buffer (which contains mercaptoethanol and SDS) for 5 min at 100 °C. 12% (*w*/*v*) separating polyacrylamide gel was used to separate the heavy chain and light chain in the supernatants. After gels visualization with Coomassie blue, GelQuant NET (1.7.8) was utilized to analyze the gray intensity of each band.

3. Results and discussion

3.1. Mechanisms of different immobilization strategies

When immobilizing antibodies on the support surface, antibodies may be adsorbed with various orientations (Fig. 1D). To improve the availability of antigen recognition sites, immobilize antibodies on “tail-on” manner on the sensor surface is necessary.

To conjugate one specific group of antibodies onto glyoxyl-activated support, the nucleophilicity of moieties to accomplish covalent reaction is pH-dependent [27]. Deprotonation of various groups is greatly affected by the pH values, and among abundant moieties on the antibody surface, cysteine residues demonstrate strong nucleophiles under acidic pH while deprotonated primary NH_2 demonstrates the highest nucleophilicity under alkaline pH [28]. Taking the relatively large number of Lys residues into consideration, the reactivity of amino groups (α- and ε-amines) are the most available moieties under alkaline conditions.

In this study, the highest binding affinity toward antigens was achieved by the two-step immobilization strategy. Due to the broadly distributed His moieties on the Fc portion, coordination complexes can be formed with metal ions on the matrix by multivalent points of interaction [15,32,33]. Given that epoxides have a relatively high reactivity toward amine groups [28,34], epoxy-activated matrices can easily react with amino groups of an antibody via Lys-rich regions in suitable conditions. Followed by the incubation under alkaline buffer, epoxy groups can interact with protonated Lys residues adjacent to the attached His residues by covalent manner. To find the optimal strategy to achieve antibody oriented immobilized, the other two methods were also evaluated (Fig. 2).

Protocol A: site-specific immobilization under pH 8.5. Under this weak alkaline condition, glyoxyl groups are prone to react with N-terminus (non-ionized amino residues) [27,35]. Thus, the antigen-binding sites can be blocked because of the steric hindrance. This immobilization strategy is considered as a negative control.

Protocol B: random immobilization of antibodies on glyoxyl matrix in the presence of PEG 6000 and DTT. Most protein immobilization protocols involve amino groups that are able to react at pH 7–8 efficiently with glutaraldehyde [36], cyanogen bromide [37], and tosyl chloride [24] activated carriers. Under these mild conditions, immobilization of a protein is expected to proceed mainly by the more reactive amino

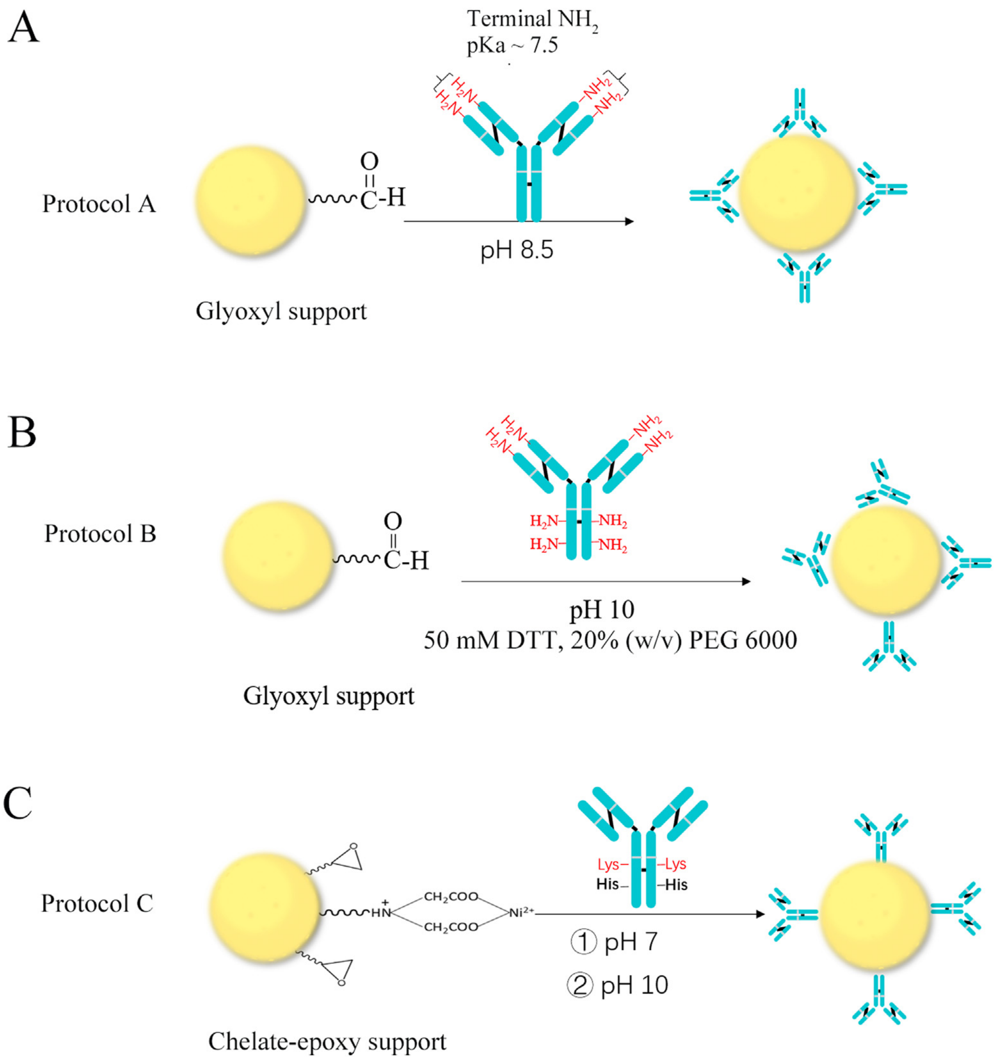

Fig. 2. Scheme of one-step and two-steps immobilization mechanism.

group (N-terminus) through one-point attachment, while Lys residues are hardly involved. Besides, the reactive groups of these carriers are also not very stable at alkaline pH which would hinder their incubation with proteins at this pH where Lys residues are more reactive. When antibodies were incubated at pH 10, highly activated glyoxyl supports quickly immobilized the antibodies since the Lys residues on the antibody surface are highly reactive (Fig. S2) and directing the immobilization of the antibody through the Lys-rich regions. Thus, a multipunctual covalent attachment was established between the antibody and carrier. Using protocol B, a first randomly one-point attachment is favored in the presence of PEG 6000 and DTT. Thus, it may involve more different enzyme regions than the conventional immobilization at pH 10. PEG 6000 works as a stabilizer to maintain the structure and functionality of native antibodies during several hours of incubation under alkaline pH [38]. In the presence of this polymer, the immobilization rate hugely decreased (Fig. S2). To ensure the immobilization of glyoxyl support, a small concentration of thiolated compounds was added to stabilize the imino bonds formed between antibody-support [39]. Under room temperature, the cleavage of disulfide bonds by DTT denatured antibodies. However, DTT did not affect the functionality of antibodies when incubated under 4 °C (data not shown). Besides, DTT is not capable of reducing the imino bonds to stable amino bonds and glyoxyl groups to inert hydroxyl groups [39]. Long term incubation at pH 10 promoted the covalent multiinteraction between the antibody and support. The final reduction step with $NaBH_4$ transforms the remaining glyoxyl groups of the support into hydroxyl groups and the Schiff's bases formed into stable secondary amine bonds.

3.2. Immobilization rate of the antibodies on different matrices

The immobilization rate of anti-HRP ($pI = 5.2–6.55$) using different methods was evaluated (Fig. 3). Antibodies can be immobilized on the monofunctional support directly, and the immobilization rate depends significantly on the incubation condition used and the density of functional groups located on the support and proteins [40,41]. When incubated under lower alkaline buffer (pH 8.5), extreme N-termini are preferentially attached to glyoxyl-activated resin because of the low pK_a of the α-amino group [27]. However, this immobilization process takes 16 h to attach almost half of the amount of input antibodies owing to the small quantity of extreme N-termini (four N-terminal amino acids per antibody). In such conditions, antibodies were immobilized on the matrix with “head-on” mode. Therefore, antigen recognition activity of this conjugated was suppressed due to the steric hindrance.

Under pH 10, covalent attachment between deprotonated amino residues (Lys ε-amino groups and N-terminus) and glyoxyl-activated resin will be obtained [35]. Compare with the limited number of N-terminal of antibodies, a much faster immobilization rate is understandable due to the much increased number of reactive NH_2 groups (over 20 Lys residues are exposed and highly accessible) [42]. Site-directed

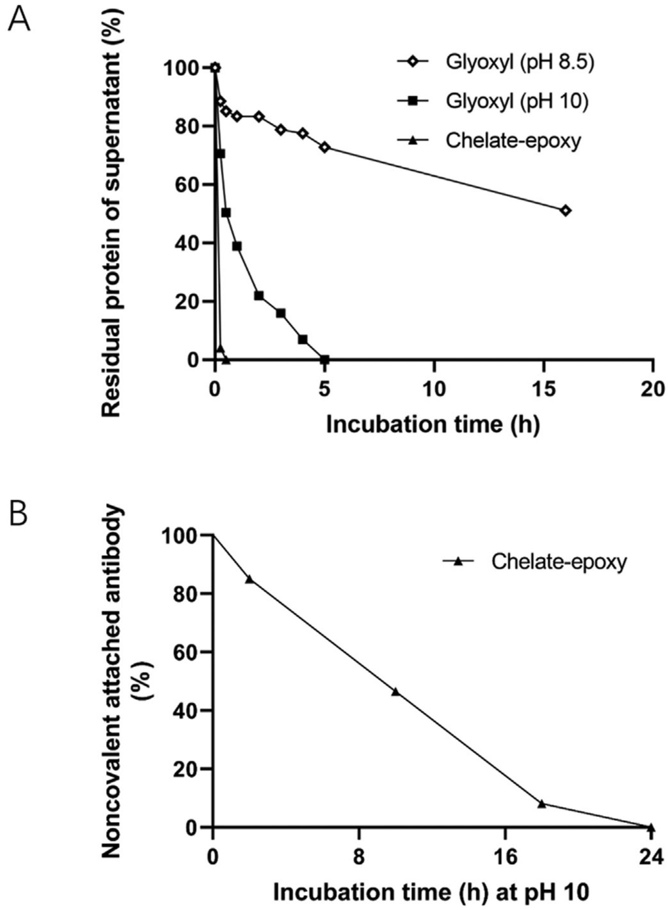

Fig. 3. Immobilization rate of anti-HRP IgG. (A) Immobilization rate of anti-HRP IgG on different supports. (B) Antibodies were adsorbed on the chelate-epoxy support first, then the bioconjugate was incubated under alkaline buffer, covalent attachment ratio was evaluated.

covalent attachment of antibodies targeting Lys residues is hard to achieve because of their prevalence throughout the antibody surface, but some tendency is likely to occur during the immobilization process. Glyoxyl-activated support is prone to react with the surface region of the antibody where the highest density of Lys groups is located [27,35]. When immobilizing antibodies under 4 °C in the presence of PEG 6000 and DTT, abundant Lys residues are likely to randomly immobilize in a slower way. Heterogeneous mixtures and decreased antigen recognition capacity can be expected from this "random" mode.

For the two-step immobilization methods, non-covalent immobilization of enzymes is much faster than covalent immobilization. Under mild pH, specific and rapid interaction was promoted between His residues and metal ions that fixed to the solid support. However, due to the epoxy groups are less reactive than glyoxyl groups, incubation under pH 10 for 24 h is necessary to transform non-specific binding into irreversible conjugate.

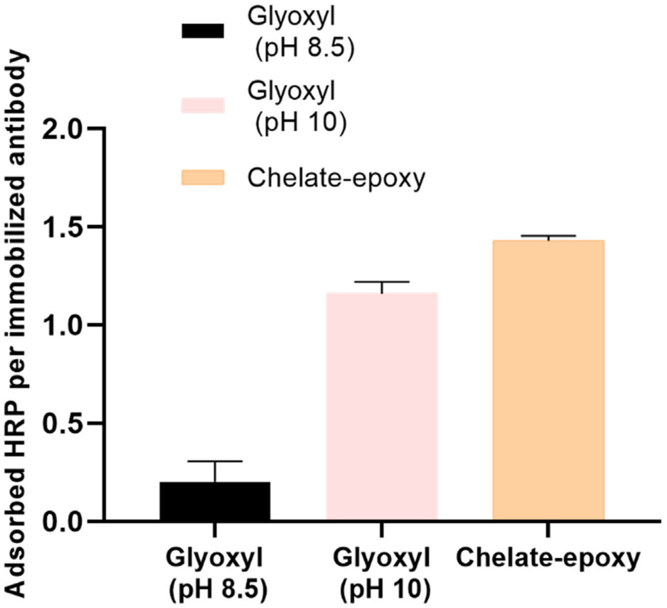

Fig. 4. The capture capacity of free HRP with immobilized anti-HRP IgG by different immobilized antibodies. Mole ratios between HRP and anti-HRP IgG were evaluated after incubated for 4 h at 4 °C.

3.3. Binding capacity of immobilized antibodies on different support

Anti-HRP was utilized as a model antibody to calculate the capacity of bioconjugate to capture HRP [26] (Fig. 4). Owing to the homodimeric structure of this antibody, the soluble antibody is capable of adsorbing two antigen molecules per one antibody molecule. The remaining antigen-binding capacity after antibodies immobilized on mono- and heterofunctional supports may be partly deactivated. The loss of antigen-recognition activity is obvious for antibodies immobilized on glyoxyl support at pH 8.5; immobilized antibodies only preserve less than 10% of the functionality to capture antigens. SDS-PAGE shows that antibody fragments were undetectable after attached to glyoxyl support at pH 8.5 (Fig. 5). It indicates that two Fab portions and two Fc portions were participated in the covalent interaction by multipoint and multi-subunit way. This unappropriated antibody attaching position blocks the majority of antigen recognition sites.

When incubating antibodies with glyoxyl-activated support at pH 10 without the additives, the capture capacity of this strategy is 1.16 μmols per immobilized anti-HRP IgG (Fig. S2B). Our groups previously demonstrate that the conjugation sites were Lys-rich region via this strategy, and the established multiple interactions of antibodies-glyoxyl support involve all four subunits during covalent conjugation [35]. The established multipoint and multisubunit can increase the stability of covalently tethered proteins due to the rigid structure between protein and support, but the Lys residue preferred orientation during conjugation may not contribute the "real" random immobilization. To promote the conformational flexibility of immobilized antibodies under alkaline conditions, we intend to establish less point interaction between antibodies and support via adding additives, so this Lys-rich perfection can be decreased. When immobilizing antibodies on the same glyoxyl support with additives, the steric hindrance caused by high molecular polymer and DTT makes the covalent interaction into a one-point mode, resulting in a much slower immobilization rate, therefore N-terminus have increased possibility to achieve covalent bonds with glyoxyl groups. The capture efficiency of randomly immobilized antibody was one μmol HRP per μmol immobilized antibody. The lower capture ability after adding additives proved that this strategy increases the opportunity of N-terminus to react with glyoxyl moieties and is applicable to be utilized as the reference "random immobilization" methods. The involvement of four antibody fragments during immobilization was proved by SDS-PAGE (Fig. 5). Although antibody functionality may fully be preserved with this methodology [35], the participation of Fab fragments and disordered antibody orientation results in lower affinity toward antigens in the buffer.

On the contrary, the amount of captured HRP per each immobilized anti-HRP was the highest for antibodies immobilized on chelate-epoxy support, near 1.6 μmols of captured HRP per μmol of anti-HRP. Given that the amount of immobilized anti-HRP was the same for glyoxyl (pH 10) and chelate-epoxy matrices, the increased antigen-binding efficiency could be since the immobilization sites would be away from the antibody binding sites. Results indicate that most of the immobilized

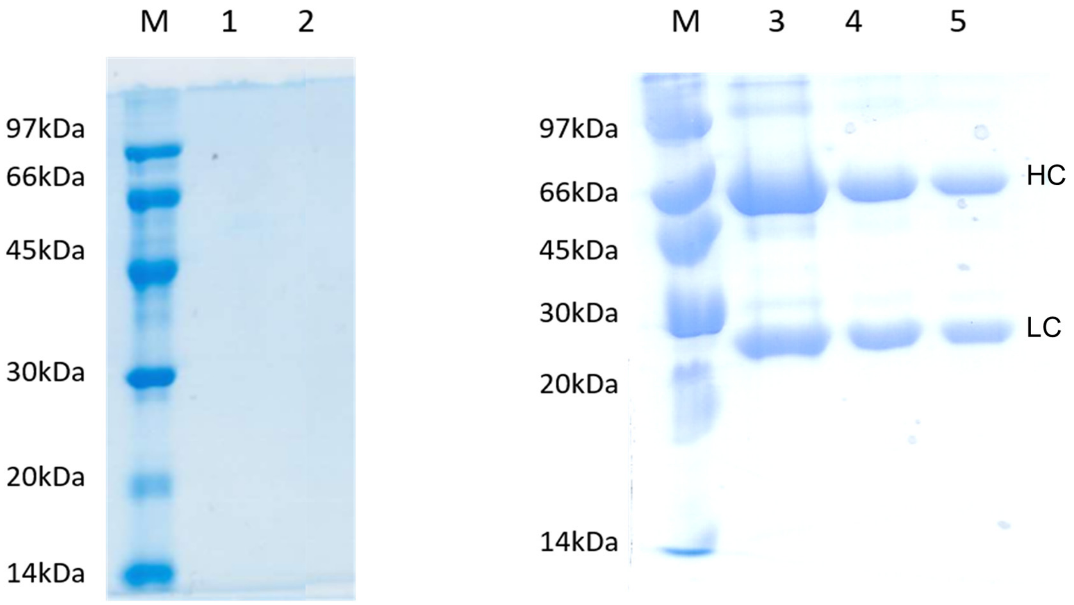

Fig. 5. SDS-PAGE analysis after antibodies immobilized on the supports. (M) Molecular weight markers; (1) supernatant after anti-HRP immobilized on glyoxyl (pH 8.5); (2) supernatant after anti-HRP immobilized on glyoxyl (pH 10); (3) 0.4 mg/mL soluble anti-HRP; (4) supernatant after anti-HRP immobilized on chelate-epoxy support, incubation under pH 10 at 25 °C for 24 h; (5) supernatant after anti-HRP immobilized on chelate-epoxy support, incubation under pH 10 at 4 °C for 7 days.

antibodies had a more uniform orientation, which leads to better accessibility to the antigens.

However, unlike chelate-glyoxyl support, in which heavy chain of antibodies disappeared from the supernatant after incubating the bioconjugate under denaturing conditions [15], the heavy chain of immobilized antibodies still exists when chelate-epoxy was applied (Fig. 5). However, the ratio of gray intensity between heavy chains and light chains decreases from 1.56 (soluble Abs) to 1.32 (immobilized Abs). Some heavy chains were not covalently bound to chelate-epoxy support. It could be explained by the "chain delivery" effect [43] due to the lower relative activity of the epoxy groups toward amino groups compared to glyoxyl groups. Thus, the flexible macromolecules may be immobilized through one heavy chain instead of two heavy chains. When incubation under 4 °C, the float movement of adsorbed antibodies is suppressed, thus antibodies are covalently coupled on the support with initial adsorbed morphology. This explanation has been proven by the further decreased [heavy chain]/[light chain] ratio. After long time incubation (7 days) under alkaline pH at 4 °C, the ratio decreased to 1.11 (Fig. 5). These data demonstrate that heavy chains are likely to be involved with this bifunctional reactive moiety instead of light chains. The results shown here reveal the importance of controlling the antibody orientation. Thus, immobilizing antibodies on a "tail-on" manner would facilitate antibody-antigen recognition. Theoretically, a potential ratio of 2:1 or very close to 2:1 of captured antigens per bioconjugate could be achieved if the immobilization strategy is carefully chosen.

3.4. Effect of the density of functional moieties on the immobilized antibodies

In this study, we propose a site-specific antibody immobilization strategy based on the cooperation between metal chelate and epoxy groups. Owing to the high hydrophilicity of agarose beads and the slow immobilization rate of epoxy support toward proteins, the direct immobilize of antibodies on the support is not appreciated [34]. Adsorbing the Fc fragment of the antibodies on the support as the first step is the prerequisite for oriented immobilization. After the first adsorption, an intensive multipoint epoxide-Lys (ε-amino groups) attachment can happen on the Fc portion and leave antigen recognition sites unoccupied.

To achieve an appropriate and more uniform orientation of the antibodies, the ratio between the metal chelate group and epoxy groups must be regulated. To obtain the heterofunctional matrix, epoxy-activated agarose beads were modified with the chelating group iminodiacetic acid (Fig. S1). In addition, the density of metal chelate groups on the support was controlled by the incubation time of epoxy support-iminodiacetic acid. As shown in Table 1, the increase of modification time exhibited a lower density of epoxy groups. Besides, the ratio [Chelate]/[Epoxy] was increased obviously in the first 8 h; after

Table 1
Density of two functional groups on chelate-epoxy resin after different modification time.

		Epoxy groups (μmol/mL support)	Chelate groups [b] (μmol/mL support)	[Chelate] /[Epoxy] [c]	[HRP]/[Anti-HRP] [d]
Density of epoxy groups before modification		35.3	–	–	–
Modification time[a]	2 h	31.1	4.2	0.13	1.46
	4 h	24.5	10.7	0.43	1.53
	8 h	13.1	22.2	1.68	1.41
	24 h	12.5	22.8	1.82	1.43

[a] The represented values are the average of triplicate experiments (error < 5%), 100 μl of supernatant from each assay was taken to quantify the amount of epoxy groups as described in the methods section.

[b] The density of metal chelate groups increased with the modification time. The whole process to prepare chelate-epoxy resin was illustrated in Supplementary materials.

[c] The represented dates are the density ratio between chelate groups and epoxy groups.

[d] The antigen binding capacity of immobilized antibodies are expressed as mole capture HRP per immobilized antibody.

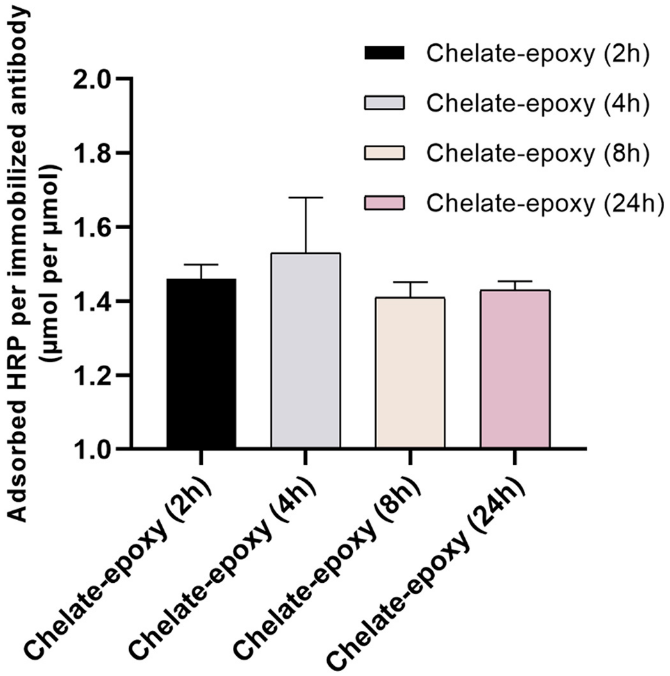

Fig. 6. Capacity of capture HRP with immobilized anti-HRP IgG by chelate-epoxy support. Chelate-epoxy (2 h) represents that functionalization time is 2 h, the same meaning with 4 h, 8 h and 24 h. Mole ratios between HRP and anti-HRP IgG were evaluated after incubated for 4 h at 4 °C.

modified for 24 h, the density of metal chelate groups has little improvement. Anti-HRP antibody was utilized as a model to study the effect of the epoxy group density on the capture efficacy of the antigen (HRP) with the immobilized antibody (Fig. 6). The results show that the decreased density of chelate groups has little effect on the antibody adsorption rate. Antibody molecules can be adsorbed in very short time even modify for 2 h (Fig. S3). The highest captured HRP per immobilized antibody was obtained after the modification of the epoxy support for 4 h, at least 76% of antigen-binding capacity was guaranteed. This optimal result is corresponding to 24.5 μmole/mL epoxy groups and 10.7 μmole/mL chelate groups, which is 0.43 chelate groups per epoxy group (Table 1). The results indicated that controlling the epoxy group density was helpful to achieve immobilization with uniform orientation.

3.5. Evaluation of immobilized antibodies to capture antigens with higher molecular weight

To verify the availability of site-specific immobilized antibodies toward different sizes of antigens, BGL with 10 times higher molecular size than HRP was used (Fig. 7A). BGL is consists of four identical subunits and makes it has a higher possibility to form bioconjugate with antibody due to more antibody recognition sites per μmoles of BGL [28]. However, the amount of captured BGL was lower when compared with a smaller size antigen (such as HRP) for all the immobilization methods. Compared with the other two strategies, chelate-epoxy immobilization strategy has the best antigens capture capacity. Metal chelate groups can adsorb antibodies in a very short time. This high adsorption rate caused the heterogeneous distribution of antibodies and most of the antibodies were immobilized on the agarose surface or pores close to the surface [15]. Once BGL was captured by immobilized antibody, the antigen-binding sites of close antibodies could be blocked because of the steric hindrance caused by its high molecular weight. Besides, an enzyme with a relatively bulky mass would be harder to get access to antibodies inside pores of agarose beads (Fig. 7B). It means only part of immobilized antibodies would have the accessibility toward antigens.

3.6. Recovery of HRP with different concentrations in diluted human serum.

To determine the versatility of our proposed heterofunctional support, a fixed amount of chelate-epoxy (4 h) support was utilized to capture HRP with different concentrations. Simple aqueous buffer and complex diluted human serum were tested and compared (Table 2). In aqueous buffer, the capture efficiencies were around 100% for serial concentrations of HRP which are highly consistent with the amount of added antigens. In diluted human serum, although the recovery capacity slightly decreased. Although, the capture efficiencies were high than 97% due to the inertness of agarose support against unspecific proteins.

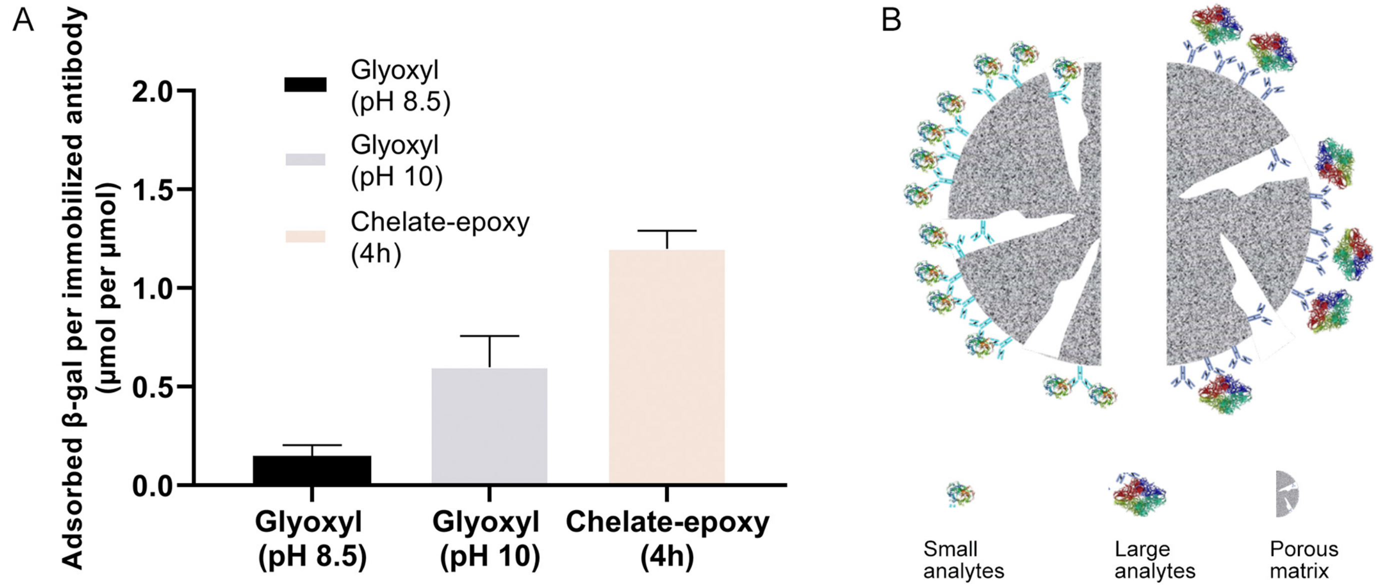

Fig. 7. (A) Capacity of capture β-galactosidase with immobilized anti-β-galactosidase IgG by different support. Chelate-epoxy (4 h) represents that functionalization time is 4 h. Mole ratios between β-galactosidase and anti-β-galactosidase IgG were evaluated after incubated for 4 h at 4 °C. (B) Illustration of possible mechanism when use immobilized antibodies to capture antigens with different molecular weight.

Table 2
Recovery of HRP with different concentration in diluted human serum using the optimized chelate-epoxy (4 h) support.

Entry[a]	Solvent	Added (mM)	Detected (mM)	Recovery (%)
1	Aqueous buffer	0.125	0.124 ± 0.011	99.4
2		0.25	0.251 ± 0.018	100.6
3		0.5	0.512 ± 0.031	102.3
4	Human serum	0.125	0.121 ± 0.006	96.8
5		0.25	0.246 ± 0.051	98.5
6		0.5	0.544 ± 0.023	108.7

[a] The represented values are the average of triplicate experiments (error < 5%). 50 μl of sample from each assay was taken to check the enzyme activity as described in the methods section.

Our results demonstrate the high possibility of our developed support in future applications with real samples.

4. Discussion

We attached two different IgG antibodies onto two different matrices functionalized with homofunctional or heterofunctional groups, respectively. In both cases, directionally oriented antibodies onto bifunctional support outperformed the randomly tethered or Fab directed bioreceptors, but different binding capacity was observed differ with analytes size.

Glyoxyl groups, an aliphatic aldehyde group, react exclusively with non-ionized primary amino moieties very rapidly [31]. Under neutral or slightly alkaline conditions, N-terminus can demonstrate over 300 times reactivity than Lys residues due to its lower pK_a, less than 1% of Lys residues are reactive [27]. Under high alkaline pH (pH 10 in this case), well-activated glyoxyl supports are likely to conjugate with antibody regions that containing the highest density of Lys residues (largely distributed on the Fc region) to achieve intensive multipoint attachment [15,35]. However, four subunits of IgG molecules are involved during its interaction with glyoxyl support [35], thus obtaining mixed IgG orientations which “flat-on” direction may result in altered recognition ability.

Tailor-made heterofunctional support, bearing distinct functionalities simultaneously, has been proven with great success in attaching multimeric protein with well-defined orientation [28,34]. Starting from the affinity between the His-rich metal-binding sites in the Fc region toward chelate groups, the following crosslinking mediated by different chemistries can fix its well-orientated morphology [15]. To achieve the following irreversible conjugation, simple H_2O_2 oxidizes IDA—Co^{2+} to generate “exchange inert” IDA—Co^{3+} resin was first reported by Hale [32]. However, chelated metal ions need at least two accessible His residues simultaneously to achieve stable coordination [33], and IgG-chelate affinity interaction is less stable than His-tag protein-chelate interaction (Fig. S4). Therefore, for nonmodified antibodies, the dissociation of affinity adsorbed IgGs during the oxidation process is likely to be inevitable. Though His-tag engineered antibody fragments (nanobody and scFv) have been developed to enhance the interaction strength and overcome such limitation [44,45], our proposed heterofunctional support is a better option with cost-effective and universal for native antibodies because His clusters are conserved distributed among a wide range of IgG types [33].

Accessibility of immobilized IgGs has easily quantified via HRP that its colorimetric reactivity is quite straightforward. Ruiz et al. revealed that the orientation of physically adsorbed anti-HRP is pH-dependent, while even at optimized pH (pH 7.5) only preserved 33% of its capture activity toward HRP [17]. In contrast, anti-HRP conjugated onto chelate-glyoxyl support via a correctly-oriented manner can bind 1.5 moles HRP per mole of immobilized antibody under the optimized condition [15]. Puertas et al. further demonstrated that directional covalent attachment is applicable employing step-wise interaction, antigen recognition ability of such low-density bound anti-HRP is highly close to the theoretical maximum, but about 1.8 times lower biological activity was observed when antibody coverage is 5 times higher [26]. However, even the orientation of immobilized antibodies has been directional oriented, its capture ability may have differential performance when the properties of the analyte are different [8]. Our initial hypothesis was that the adsorption capacity of larger antigens contain several epitopes should outperform smaller monomeric antigens. BGL, a multimeric enzyme that has a 10 times higher molecular size than HRP, was investigated as another model analyst (Fig. 7A). Lower binding properties upon larger antigens can be explained by the limited diffusion of antibodies and antigens in the agarose pores [15,46]. Smaller antigens possess higher diffusion ability into the inner regions of pores that most of the capture molecules are accessible; however, larger analytes are limited superficial regions of pores and they are likely to shield the neighboring antibody after adsorption [15]. The different molecular size of analytes is not the only properties that contributed to the varied recognition capacity of uniform immobilized IgGs [7]. Trilling et al. revealed that although 7-fold higher sensitivity for small antigen (3 kDa vs 8200 kDa), interaction strength is the conclusive factor that antigens with lower-affinity demonstrated much enhanced detection sensitivity [7].

5. Conclusion

Classic site-specific antibody immobilization strategies have been utilized broadly both in laboratory research and commercial products. However, when cost-effectiveness is taken into account, oriented immobilization of unmodified antibody is still very anticipated. Therefore, the utilization of amino groups distributed on the outer surface of the antibody via simply regulate incubation pH demonstrate great advantage.

In this work, the easiest immobilization protocols involve the N-terminus of IgG antibodies., which very highly reactive at neutral or slightly alkaline pH, this Fab-oriented strategy strongly reduces the adsorption capacity of bound IgG, less than 10% biological activity preserved Random immobilization of IgG antibodies employed the reactivity of accessed Lys residues. Glyoxyl support was conjugate with unprotonated amino groups in a random manner under high alkaline pH, moderate binding ability decrease was obtained and 50% biological activity was maintained. Oriented immobilization of IgGs through Lys residues place on the bottom of the Fc region provides the best adsorption results. This “end-on” strategy guaranteed at least 75% reactivity of bound anti-HRP, and 2-fold higher reactivity over random immobilized anti-BGL was observed. The selective recognition of analytes spiked in the diluted human serum further proved its applicability in complex biological conditions. Without the need to modify antibody structure and preserved antigen recognition capacity, this two-step immobilization strategy has the potential as a universal methodology to fabricate immunosensor for biological systems.

CRediT authorship contribution statement

Shipeng Gao: Investigation, Formal analysis, Writing- Original draft preparation. **Fran Rojas-Vega:** Investigation, Validation, Data curation. **Javier Rocha-Martin**: Conceptualization, Supervision, Visualization Writing- Original draft preparation, Writing- Reviewing and Editing.

José M. Guisan: Methodology, Conceptualization, Supervision, Writing-Reviewing and Editing, Funding acquisition.

Declaration of competing interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2021.02.103.

References

[1] E.A. Bastos-Soares, R.M.O. Sousa, A.F. Gómez, J. Alfonso, A.M. Kayano, F.B. Zanchi, M.E. Funes-Huacca, R.G. Stábeli, A.M. Soares, S.S. Pereira, C.F.C. Fernandes, Single domain antibodies in the development of immunosensors for diagnostics, Int. J. Biol. Macromol. 165 (2020) 2244–2252.

[2] A. Minopoli, B. Della Ventura, B. Lenyk, F. Gentile, J.A. Tanner, A. Offenhäusser, D. Mayer, R. Velotta, Ultrasensitive antibody-aptamer plasmonic biosensor for malaria biomarker detection in whole blood, Nat. Commun. 11(1) (2020) 6134.

[3] D. Quaglio, L. Mangiardi, G. Venditti, C. Del Plato, F. Polli, F. Ghirga, G. Favero, M. Pierini, B. Botta, F. Mazzei, Site-directed antibody immobilization by resorc[4] arene-based immunosensors, Chem. Eur. J.. 26(38) (2020) 8400–8406.

[4] M. Jafari, M. Hasanzadeh, E. Solhi, S. Hassanpour, N. Shadjou, A. Mokhtarzadeh, A. Jouyban, S. Mahboob, Ultrasensitive bioassay of epitope of Mucin-16 protein (CA 125) in human plasma samples using a novel immunoassay based on silver conductive nano-ink: a new platform in early stage diagnosis of ovarian cancer and efficient management, Int. J. Biol. Macromol. 126 (2019) 1255–1265.

[5] K.S. Butler, C.J. Pearce, E.A. Nail, G.A. Vincent, D.F. Sava Gallis, Antibody targeted metal–organic frameworks for bioimaging applications, ACS Appl. Mater. Interfaces 12 (28) (2020) 31217–31224.

[6] C.P. Santana, A.A.P. Mansur, S.M. Carvalho, A. da Silva-Cunha, H.S. Mansur, Bi-functional quantum dot-polysaccharide-antibody immunoconjugates for bioimaging and killing brain cancer cells in vitro, Mater. Lett. 252 (2019) 333–337.

[7] A.K. Trilling, M.M. Harmsen, V.J.B. Ruigrok, H. Zuilhof, J. Beekwilder, The effect of uniform capture molecule orientation on biosensor sensitivity: dependence on analyte properties, Biosens. Bioelectron. 40 (2013) 219–226.

[8] N. Tajima, M. Takai, K. Ishihara, Significance of antibody orientation unraveled: well-oriented antibodies recorded high binding affinity, Anal. Chem. 83 (2011) 1969–1976.

[9] J.P. Oliveira, A.R. Prado, W.J. Keijok, P.W.P. Antunes, E.R. Yapuchura, M.C.C. Guimarães, Impact of conjugation strategies for targeting of antibodies in gold nanoparticles for ultrasensitive detection of 17β-estradiol, Sci. Rep. 9 (1) (2019), 13859, .

[10] J. Zorea, R.P. Shukla, M. Elkabets, H. Ben-Yoav, Probing antibody surface density and analyte antigen incubation time as dominant parameters influencing the antibody-antigen recognition events of a non-faradaic and diffusion-restricted electrochemical immunosensor, Anal. Bioanal. Chem. 412 (7) (2020) 1709–1717.

[11] J. Moon, J. Byun, H. Kim, J. Jeong, E.-K. Lim, J. Jung, S. Cho, W.K. Cho, T. Kang, Surface-independent and oriented immobilization of antibody via one-step polydopamine/protein G coating: application to influenza virus immunoassay, Macromol. Biosci. 19(6) (2019) 1800486.

[12] Z. Li, G. Chen, Current conjugation methods for immunosensors, Nanomaterials 8 (5) (2018) 278.

[13] A. Leidner, J. Bauer, M. Ebrahimi Khonachah, M. Takamiya, U. Strähle, T. Dickmeis, K.S. Rabe, C.M. Niemeyer, Oriented immobilization of a delicate glucose-sensing protein on silica nanoparticles, Biomaterials 190-191 (2019) 76–85.

[14] J. Yang, L. Sun, R. Guo, H. Yang, X. Feng, X. Zhang, A facile route for oriented covalent immobilization of recombinant protein A on epoxy agarose gels: in situ generation of heterofunctional amino-epoxy supports, ChemistrySelect 3 (2018) 10320–10324.

[15] P. Batalla, J.M. Bolívar, F. Lopez-Gallego, J.M. Guisan, Oriented covalent immobilization of antibodies onto heterofunctional agarose supports: a highly efficient immuno-affinity chromatography platform, J. Chromatogr. A 1262 (2012) 56–63.

[16] A.K. Adak, B.Y. Li, L.D. Huang, T.W. Lin, T.C. Chang, K.C. Hwang, C.C. Lin, Fabrication of antibody microarrays by light-induced covalent and oriented immobilization, ACS Appl. Mater. Interfaces 6 (13) (2014) 10452–10460.

[17] G. Ruiz, K. Tripathi, S. Okyem, J.D. Driskell, pH impacts the orientation of antibody adsorbed onto gold nanoparticles, Bioconjug. Chem. 30 (4) (2019) 1182–1191.

[18] J.M. Montenegro, V. Grazu, A. Sukhanova, S. Agarwal, J.M. de la Fuente, I. Nabiev, A. Greiner, W.J. Parak, Controlled antibody/(bio-) conjugation of inorganic nanoparticles for targeted delivery, Adv. Drug Deliv. Rev. 65 (2013) 677–688.

[19] M. Iijima, S.i. Kuroda, Scaffolds for oriented and close-packed immobilization of immunoglobulins, Biosens. Bioelectron. 89 (2017) 810–821.

[20] N.G. Welch, J.A. Scoble, B.W. Muir, P.J. Pigram, Orientation and characterization of immobilized antibodies for improved immunoassays (review), Biointerphases 12 (2017) 02D301–02D313.

[21] M. Fuentes, C. Mateo, J.M. Guisán, R. Fernández-Lafuente, Preparation of inert magnetic nano-particles for the directed immobilization of antibodies, Biosens. Bioelectron. 20 (2005) 1380–1387.

[22] Z. Mai, J. Zhang, Y. Chen, J. Wang, X. Hong, Q. Su, X. Li, A disposable fiber optic SPR probe for immunoassay, Biosens. Bioelectron. 144 (2019), 111621, .

[23] B. Prieto-Simón, C. Saint, N.H. Voelcker, Electrochemical biosensors featuring oriented antibody immobilization via electrografted and self-assembled hydrazide chemistry, Anal. Chem. 86 (3) (2014) 1422–1429.

[24] G. Schlosser, P. Kacer, M. Kuzma, Z. Szilagyi, A. Sorrentino, C. Manzo, R. Pizzano, L. Malorni, G. Pocsfalvi, Coupling immunomagnetic separation on magnetic beads with matrix-assisted laser desorption ionization-time of flight mass spectrometry for detection of staphylococcal enterotoxin B, Appl. Environ. Microbiol. 73(21) (2007) 6945–6952.

[25] M. Aydın, E.B. Aydın, M.K. Sezgintürk, Electrochemical immunosensor for CDH22 biomarker based on benzaldehyde substituted poly(phosphazene) modified disposable ITO electrode: a new fabrication strategy for biosensors, Biosens. Bioelectron. 126 (2019) 230–239.

[26] S. Puertas, P. Batalla, M. Moros, E. Polo, P. Del Pino, J.M. Guisán, V. Grazú, J.M. De La Fuente, Taking advantage of unspecific interactions to produce highly active magnetic nanoparticle/antibody conjugates, ACS Nano 5 (2011) 4521–4528.

[27] K. Hernandez, R. Fernandez-Lafuente, Control of protein immobilization: coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance, Enzym. Microb. Technol. 48(2) (2011) 107–122.

[28] J.M. Bolivar, C. Mateo, V. Grazu, A.V. Carrascosa, B.C. Pessela, J.M. Guisan, Heterofunctional supports for the one-step purification, immobilization and stabilization of large multimeric enzymes: amino-glyoxyl versus amino-epoxy supports, Process Biochem. 45 (10) (2010) 1692–1698.

[29] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[30] *C. Mateo*, G. Fernandez-Lorente, J. Rocha-Martin, J.M. Bolivar, J.M. Guisan, Oriented covalent immobilization of enzymes on heterofunctional-glyoxyl supports, in: J.M. Guisan (Ed.), Immobilization of Enzymes and Cells. Methods in Molecular Biology (Methods and Protocols), Humana Press, Totowa, 2013, pp. 73–88.

[31] J.M. Guisán, Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes, Enzym. Microb. Technol. 10 (6) (1988) 375–382.

[32] J.E. Hale, Irreversible, oriented immobilization of antibodies to cobalt-iminodiacetate resin for use as immunoaffinity media, Anal. Biochem. 231 (1995) 46–49.

[33] D. Todorova-Balvay, O. Pitiot, M. Bourhim, T. Srikrishnan, M. Vijayalakshmi, Immobilized metal-ion affinity chromatography of human antibodies and their proteolytic fragments, J. Chromatogr. B 808 (1) (2004) 57–62.

[34] O. Barbosa, R. Torres, C. Ortiz, A. Berenguer-Murcia, R.C. Rodrigues, R. Fernandez-Lafuente, Heterofunctional supports in enzyme immobilization: from traditional immobilization protocols to opportunities in tuning enzyme properties, Biomacromolecules 14 (8) (2013) 2433–2462.

[35] P. Batalla, C. Mateo, V. Grazu, R. Fernandez-Lafuente, J.M. Guisan, Immobilization of antibodies through the surface regions having the highest density in lysine groups on finally inert support surfaces, Process Biochem. 44 (3) (2009) 365–368.

[36] Y. Wang, X. Zhang, N. Han, Y. Wu, D. Wei, Oriented covalent immobilization of recombinant protein A on the glutaraldehyde activated agarose support, Int. J. Biol. Macromol. 120 (2018) 100–108.

[37] M. Bruni, M.S. Robescu, D. Ubiali, G. Marrubini, R. Vanna, C. Morasso, I. Benucci, G. Speranza, T. Bavaro, Immobilization of γ-glutamyl transpeptidase from equine kidney for the synthesis of kokumi compounds, ChemCatChem 12 (1) (2020) 210–218.

[38] J.M. Guisan, F. López-Gallego, J.M. Bolivar, J. Rocha-Martín, G. Fernandez-Lorente, One-point covalent immobilization of enzymes on glyoxyl agarose with minimal physico-chemical modification: Immobilized "native enzymes", in: J.M. Guisan, J.M. Bolivar, F. López-Gallego, J. Rocha-Martín (Eds.), Immobilization of Enzymes and Cells, Humana Press, New York, 2020, pp. 83–92.

[39] J.M. Bolivar, F. López-Gallego, C. Godoy, D.S. Rodrigues, R.C. Rodrigues, P. Batalla, J. Rocha-Martín, C. Mateo, R.L.C. Giordano, J.M. Guisán, The presence of thiolated compounds allows the immobilization of enzymes on glyoxyl agarose at mild pH values: new strategies of stabilization by multipoint covalent attachment, Enzym. Microb. Technol. 45(6–7) (2009) 477–483.

[40] Y. Zhang, G. Zhang, J. Feng, Y. Kong, J. Gao, Y. Li, Z. Su, Site-specific immobilization of lysozyme upon affinity chromatography resin by forecasting lysine activity and controlling pH and epoxy group density, J. Chromatogr. A 1592 (2019) 192–196.

[41] G. Tsekenis, M. Chatzipetrou, M. Massaouti, I. Zergioti, Comparative assessment of affinity-based techniques for oriented antibody immobilization towards immunosensor performance optimization, J. Sensors 2019 (2019) 1–10.

[42] M. Abdollahpour-Alitappeh, M. Lotfinia, T. Gharibi, J. Mardaneh, B. Farhadihosseinabadi, P. Larki, B. Faghfourian, K.S. Sepehr, K. Abbaszadeh-Goudarzi, G. Abbaszadeh-Goudarzi, B. Johari, M.R. Zali, N. Bagheri, Antibody–drug conjugates (ADCs) for cancer therapy: strategies, challenges, and successes, J. Cell. Physiol. 234 (5) (2019) 5628–5642.

[43] T. Liu, D.Q. Lin, H.L. Lu, S.J. Yao, Preparation and evaluation of dextran-grafted agarose resin for hydrophobic charge-induction chromatography, J. Chromatogr. A 1369 (2014) 116–124.
[44] J. Qu, S. Horta, F. Delport, M. Sillen, N. Geukens, D.-W. Sun, K. Vanhoorelbeke, P. Declerck, J. Lammertyn, D. Spasic, Expanding a portfolio of (FO-) SPR surface chemistries with the Co(III)-NTA oriented immobilization of His6-tagged bioreceptors for applications in complex matrices, ACS Sensors 5 (4) (2020) 960–969.
[45] J. Di Russo, J.L. Young, A. Balakrishnan, A.S. Benk, J.P. Spatz, NTA-Co3+-His6 versus NTA-Ni2+-His6 mediated E-Cadherin surface immobilization enhances cellular traction, Biomaterials 192 (2019) 171–178.
[46] F. Hagemann, P. Adametz, M. Wessling, V. Thom, Modeling hindered diffusion of antibodies in agarose beads considering pore size reduction due to adsorption, J. Chromatogr. A 1626 (2020), 461319, .