Analytical Biochemistry 609 (2020) 113909

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Mechanism of antibodies purification by protein A

Mahshid Zarrineh [a], Ilnaz Soleimani Mashhadi [a], Mohsen Farhadpour [b], Alireza Ghassempour [a,*]

[a] Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, G.C, Evin, P.O.Box: 19835-389, Tehran, Iran
[b] National Institute for Genetic Engineering and Biotechnology, Tehran, Iran

ARTICLE INFO

Keywords:
Protein A
Antibody
Purification mechanism
Protein engineering
Sorbent
Affinity

ABSTRACT

Protein A, a major cell wall component of *Staphylococcus aureus*, is one of the first immunoglobulin-binding proteins that is discovered about 80 years ago. However, a great deal of development in both purification methods and application of antibodies in treatment have been done. There are many publications based on the untargeted (size exclusion, ion exchange and hydrophobic interactions) and targeted (affinity) methods by scientists in academic/industry groups. In this review, we have focused on the study of both native and engineered Protein A to understand its mechanism in the purification of antibodies. What domain of Protein A dose interact with antibody? Where are contact regions? What is the non-covalent interaction mechanism of Protein A and antibody? Does alkaline condition, in the washing step, influence on antibody structure and activity? On the other hand, the immobilization of Protein A on various sorbents such as agarose, silica, polysaccharide, polymers, and magnetic nanoparticles have investigated. Also, the application of Protein A as biosensor for detection of the antibody is discussed. We have tried to find interesting and stimulating answers to all these questions.

1. Introduction

Due to the therapeutic effects of antibodies on viral diseases, cancer, rheumatic diseases, and other nervous system, their production and purification is exponentially increased. In 2019, the USA Food and Drug Administration (FDA) approved 90 mAbs (monoclonal antibodies) such as PD-1 antibody and anti-EGFR mAb while more than 50 mAbs are still under the clinical trial phase. Further, in therapeutic, it could directly attach to activate solid supports and use as a rapping agent for detection of antigens [1,2].

What functions in antibodies do cause these properties? Immunoglobulins, as known Igs, have molecular weight about 150 kDa, two heavy chains with 450–650 amino acids and two light chains with 214 amino acids, a Stokes' radius of 4 nm and an isoelectric point in the range of 4.0–8.6. They consist of four polypeptide chains. Two identical 50 kDa γ heavy chains and two identical 25 kDa k or λ light chains that generate a protein of about 150 KDa (146 KDa for IgG1, IgG2, IgG4 and 170 KDa for IgG3). The two heavy chains are connected in the hinge by disulfide bonds: two bonds for IgG1 and IgG4, 4 bonds for IgG2 and 11 bonds for IgG3. Moreover, the last cysteine residue of the light chain and the fifth cysteine residue of the heavy chain produce the disulfide band. The five classes of Igs in human based on glycoproteins composed of 82–96% protein and 4–18% carbohydrate are IgM, IgD, IgG, IgA, and IgE. Based on the structural differences of IgG, it is divided to four subclasses; IgG1, IgG2, IgG3 and IgG4, which are identical more than 90% on the amino acid level while these less than 10% differences (Fig. 1-A) that play a fundamental role on their function and response to various type of antigens. It is noteworthy that almost all antibodies used or underdevelopment step utilize an IgG1 subclass backbone.

Based on Igs structures, Igs divided to two heavy chains and two light chains. Heavy chain includes N-terminal variable domain (VH) and three constant domains (CH1, CH2 and CH3) while the light chains consist of N-terminal domain (VL) and a constant domain (CL). Based on binding sites, Igs have to two parts; two fragment antigen binding site (Fab) regions and a fragment crystallizable (Fc) region (Fig. 1-B).

The Fab and Fc regions are linked together by disulfide bonds that known as the flexible hinge region [3,4]. Fab as a high selective site binds to antigens while Fc region is binding to many different proteins e.g. cell surface receptors containing neonatal Fc-receptors and bacterial proteins such as Protein A and Protein G [5].

IgG3 has 11 disulfide bonds in comparison with the 2–4 disulfide bonds in the other IgG subclasses (IgG1, IgG2 and IgG4). It results in 13 different polymorphisms for IgG3 that known as allotypes, in comparison to 4, 1, and 0 allotypes for IgG1, IgG2 and IgG4, respectively. This difference causes the interaction of IgG3 only with Protein G or Protein A/G and no interaction with Protein A.

* Corresponding author. Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, G.C., P.O. Box 19835, Evin, Tehran, Iran.
E-mail address: a-ghassempour@sbu.ac.ir (A. Ghassempour).

https://doi.org/10.1016/j.ab.2020.113909
Received 24 February 2020; Received in revised form 2 August 2020; Accepted 11 August 2020
Available online 20 August 2020
0003-2697/© 2020 Elsevier Inc. All rights reserved.

Up to now, several published reviews have studied the different kinds of Protein A for affinity purification of Igs, such as Protein A mimetic peptides and engineered proteins and compared different types of commercially available Protein A matrices and their properties [6–10]. Nanobodies smaller alternative antibody formats such as the antigen binding fragments (Fab) or single-chain variable fragments (scFv) with a terminal hexa-histidine tag are easily purified via immobilized metal affinity chromatography, but previous studies revealed a negative impact of the His-tag on the nanobodies biodistribution. Here, we have focused on the non-covalent interaction molecular mechanism of antibody, Protein A alongside Protein A covalent bond to different sorbents and new applications of this purification.

2. Protein A

Protein A, as an important protein extracted from pathogenic bacteria, *Staphylococcus aureus* (*S. aurous*), is bonded to the cell wall of bacteria and in another form is secreted to the culture supernatant [6]. In the 1970s, the first attempt for Protein A affinity purification method was represented by Hjelm and coworkers and they found out that Protein A binds strongly to the Fc region of IgG and weakly to its Fab region [7]. Today, it is known that these tendencies vary with respect to animal species and subclasses (for instance, Protein A will not bind to human IgG3). Another binding protein of *Streptococcal* is Protein G that binds to all subclasses of majority mammalian species' IgG. However, Protein A has commercially important domination because of its more advantageous binding properties like weaker IgG binding and milder elution conditions [8].

For purification of IgG, purified protein A is required to covalently bond to sorbent and capture Igs. Protein A can be purified by means of different methods based on the non-specific and specific interactions. Non-specific techniques were first applied in 1966 based on anion exchange and gel filtration chromatography [6] and they continued to grow, due to the high cost of specific purification methods. As reported recently, researchers have used a simple cost-effective chromatography column through Per Aqueous Liquid Chromatography (PALC) technique by which the separated Protein A retains its IgG binding ability [9]. Furthermore, Denizli and coworkers reported the purification of Protein A by an imprinted polymer on the surface of crygel beads. In this work, 18.1 mg of Protein A/G of wet cryogel beads was obtained with high selectivity [11]. The specific techniques were introduced by Hjelm and coworkers which Protein A was purified through immobilized-metal affinity chromatography (IMAC) utilizing six histidine residues as affinity fusion tag on Protein A chain [12,13].

2.1. Interaction of native protein a with IgG

Due to the acceptable resistance of Protein A toward harsh acidic and basic cleaning condition, it is the best candidate to use to remove IgG from serum and plasma matrices in order to facilitate the separation of other immunoglobulin classes such as IgA, IgM, IgD, and IgE. On the other hand, there are different types of antibody-binding proteins: Protein A, G, L, Z and recombinant (fusion proteins), which have different affinity to IgGs, Table 1 [13,14].

Protein A, a 42-kDa protein contains five homologous domains proceeding from N to the C terminus: E, D, A, B and C and its amino acid sequence is determined by hydrolysis and enzymatic digestion [10,15]. The investigation on protein A has proved that the B domain is more stable than the other ones toward solvent and temperature denaturation experiments [16].

It is known that genes responsible for Protein A expression sequenced are composed of three different regions:

- One of them is responsible for the secretion process, named S region,
- The second region consists of five individual IgG binding domains,
- The last one is cell-wall an anchoring region XM (Fig. 2-A) [12].

The IgG binding domains: E, D, A, B, and C (58 amino acid residues in each domain) settle in an anti-parallel three α-helices, all domains show

Table 1
General properties of the most used bacterial (fusion) proteins.

Proteins	Size (kDa)	Target	Elution pH
Protein A	42	Fc, Fab	3.5
Protein G	30	Fc, Fab, ScFv	3.2
Protein L	76–106	Fab, ScFv, κ-light chain	2
Protein Z	6.7	Fc	3.6
Protein LG	50	Fc, κ-light chain	2
Protein LA	60	Fc, κ-light chain	2
Protein AG	47.3	Fc	3

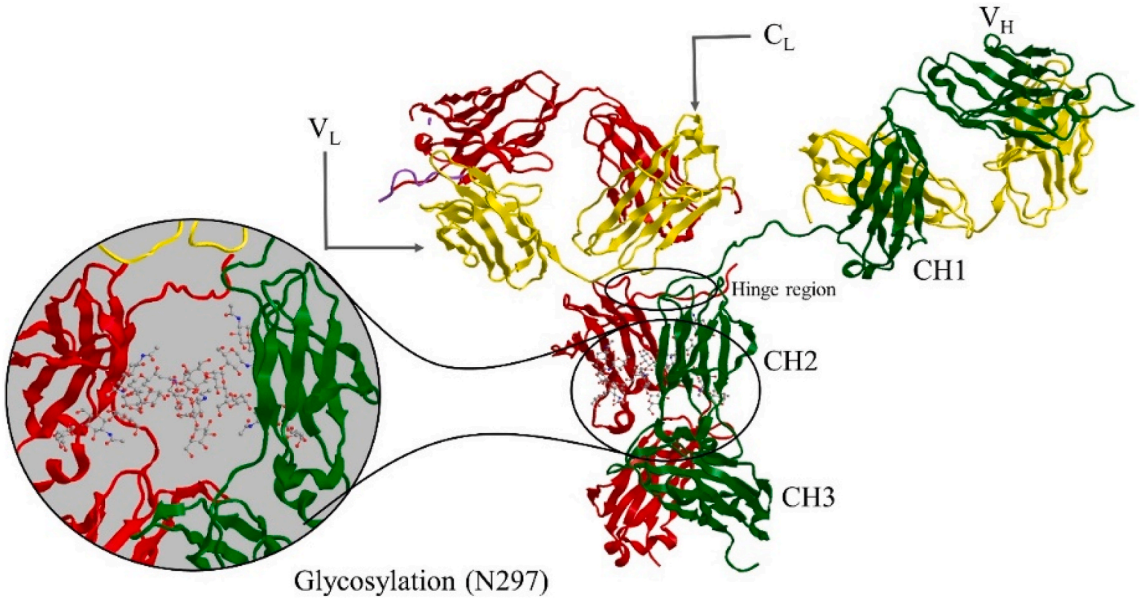

Fig. 1. A) Four subclasses, B) Tetra-peptide chain of IgG and its fragments (Heavy Chains; Red and Green, Light chains; Yellow). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

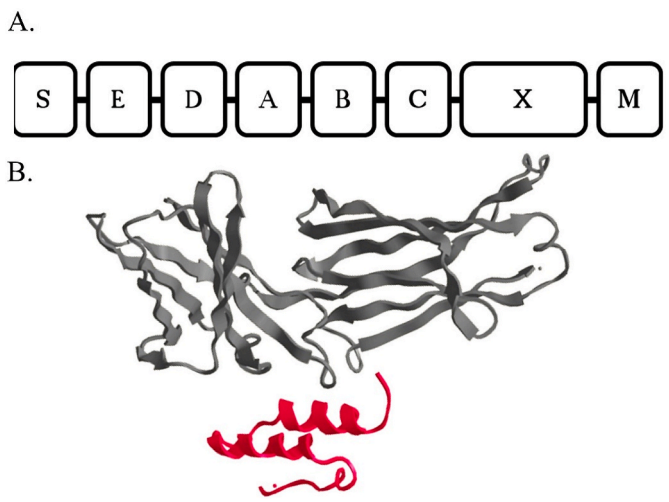

Fig. 2. A) Protein A genes consisting of signal sequence (S), five IgG binding domains (D, E, A, B and C), the cell wall attachment region (XM), B) The interaction of three beta-turns of Fc region of IgG (gray fragment,the upper protein) and two alpha-helices of B domain of Protein A (red fragment, the lower protein). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

individual interaction to the Fc region subclasses of IgG (IgG1, IgG2, and IgG4) with an affinity constant, K_A of 10^8 (M^{-1}) and a very weak affinity to IgG3.

The second structure study of Protein A from *S. aureus* by Circular Dichroism (CD) has been published by Lindmark. He made a new study based on the last CD report, done by Sjöholm, for study of native Protein A. He declared that the content of α-helical of native Protein A is 48% which is much higher than the sum of the α-helical content of the fragments constituting the native Protein A. Lindmark performed experiments of this study again and found 31% α-helix, 13% β-structure and 56% random coil in structure of native Protein A [17].

Robertson and coworkers believed that the protein aggregation is also a good marker for several neurodegenerative diseases such as Alzheimer, Parkinson, prion and the polyglutamine (polyQ) diseases and it can determine the conformational changes and understanding the pathogenic mechanism. They followed the interaction of Protein A-polyO proteins and the NMR study of this interaction led to the structure of Protein A in PDB. They revealed that the intra-domain polyQ repeat structurally perturbs conformation of native Protein A [18].

According to the Igs interactions, Protein A divided in two distinct Ig-binding activities: the distinct of Protein A can bind to Fc fragment of IgG like B Domain and a distinct can bind to Fab fragment of Igs like D Domain. The equilibrium constant of Protein A and IgG is about 10^8–10^{12} mol^{-1}. For purification of IgG, CH2 and CH3 regions of Fc participate in the interaction with B domain of Protein A and produce a non-covalent interaction. Like Fc-B domain interaction, the helices of the D domain of Protein A interacts with the variable regions of Fab in Igs. The crystal structure of the complex between D domain of native Protein A and Fab region of IgM antibody with 2.7 °A resolution was studied by Graille and coworkers [19]. In 1978 and 1981 Johann Deisenhofer (winner Nobel Prize, 1988) conducted crystallography studies on the structure of B domain (from membrane protein A of bacteria) and found that it binds to the Fc domain of IgG (Fig. 2-B). They showed B domain of Protein A has three parallel helices arranged in triangular array and two of these helices attached to the C-terminal regions of γ-heavy chain of IgG (Fc region). Thus, the α1 and α2 helices of B domain of Protein A interact with CH2 and CH3 regions of IgG while α3 helix is not involved in this interaction [16]. The interaction between β-sheet of CH2 and CH3 in Fc fragment of IgG and helices of B domain of Protein A occurs in two contact regions. Deisenhofer and coworkers divided the observed interactions of Protein A into two parts, the first one contains interactions with a hydrophobic nature contributed in the solution state of physiological conditions of IgG, and the second one is a polar interaction that is more involved in the crystallography situation and not produced in the solution [20,21].

The main interaction occurs through 23 amino acid residues and these amino acids are located on three beta-turns of IgG and two α-helices of each Protein A domain. The basis of the interaction of B domain and CH2 and CH3 regions is known to be hydrophobic and is followed by some hydrogen bonds and salt bridges located on the first and the second α-helix of protein A. Amino acids like Phe-6, Phe-14, Tyr-15, Leu-18, Ile-32, Lys-36 contribute for the hydrophobic interaction and Gln-10, Asn-12, Tyr-15, Asn-29 involve in polar part of the interaction and Arg-28 participates in an ion pair with Asp-315 on Fc region of IgG. The amino acids that cause the interaction are listed in Table 2 and their spatial location are shown in Fig. 3 [22,23].

Torigeo and coworkers used ^{15}N NMR and hypothesized when each domain interacts with Fc region of IgG, helix III would disrupt in the interaction [24]. In the same study, Gouda and coworkers reported a three-dimensional solution structure of B domain in both free and attached status by ^{1}H-^{15}N HSQC spectrum of [^{15}N] B domain of Protein A. The amide proton resonances and stereospecific assignment were evaluated by NOESY and $^{3}J_{N\text{-}H}$ cross-peaks. They incubated [^{15}N] B domain of Protein A in the solution of Fc fragment (at the molar ratio of 1:1) and it was diluted and incubated in D_2O for the amide proton exchange. They indicated that all three helices are anti-parallel and helix III remains intact. They found several amide protons within helix III which are protected from rapid hydrogen/deuterium (H/D) exchange (both in free and bonded forms) and it is attributed to the existence of intramolecular hydrogen bonds in its intact form [25].

These studies, based on NMR, CD, and hydrogen/deuterium exchange data, have been practical in advance of genetic engineering and protein modification surveys, which is aimed to create stronger interaction, more stable protein, or easier protein immobilization under milder chemical conditions.

Bolton and coworkers reported that the discontinuity of Protein A to IgG 3 is due to the lack of histidine in IgG3 structure and proved that the important residues in this affinity are two conserved histidines, His435 on the Fc fragment of other IgGs (IgG1, 2 and 4) and His137 on Protein A. The interaction occurs when both amino acids are uncharged at neutral pH, while the purification process happens under acidic pH that imposes positive charges on both histidines, so the electrostatic repulsion will desorb IgG from Protein A [13,14].

Shah and coworkers predicted that R435 by Nη2 atom in IgG3 can

Table 2
List of amino acids involve in interaction between Protein A and IgG, contact 1 includes hydrophobic interaction and four hydrogen bonds to stabilize this part while contact 2 is naturally polar and includes a sulfate ion (Sequences base on 1bdc PDB).

Contact 1	**Contact 2**
Hydrophobic contact	**Polar contact**
PHE 6	GLU 26
GLN 11	ASN 29
PHE 14	GLY 30
TYR 15	PHE 31
LEU 18	GLN 33
HIS 19	SER 34
ARG 28	LYS 36
ILE 32	ASP 37
GLN 33	ASP 38
LYS 36	PRO 39
Hydrogen bonds	SER 40
GLN 10	GLN 41
ASN 12	**Salt bridge**
TYR 15	Lys 36
ASN 29	

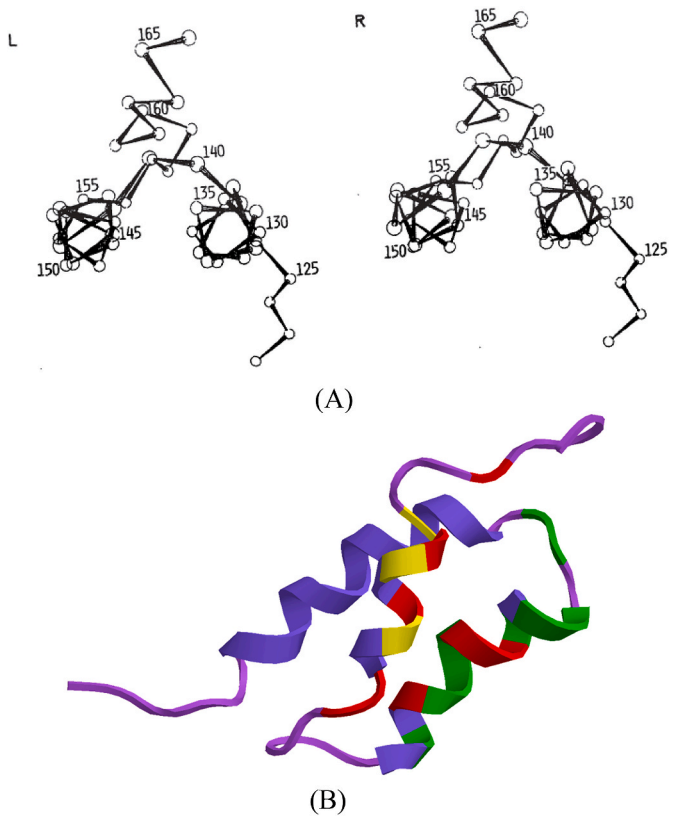

Fig. 3. A) Two stereo view of partly B domain of Protein A, the whole binding site is located in helix I and II [20], B) the interaction takes place through polar (Green), hydrophobic (Red), and hydrogen bonds (Yellow) (PDB: 1bdc). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

interact with Oε2 atom of E27 in protein G by a salt bridge. According to their report, IgG3 has a 3-fold higher affinity for binding to Protein G in comparison with IgG1 which is resulted from the longer R435 side chain in its structure to form extra contact with protein G [26].

2.2. Interaction of engineered protein A with IgG

The first study in the field of engineered Protein A is the elimination of unnecessary parts like S and XM regions from native structure of Protein A. The first recombinant Protein A was designed as a protein without the XM region, which has no affinity to IgG. This recombinant protein is widely used in commercial products and decreases non-specific adsorption of proteins in comparison with the wild-type ligand [27]. Wang and coworkers designed a hexapeptide (FYEILH) ligand by biomimicking the sequence of binding portion of B domain and minimizing the IgG binding portion from a native Protein A. To find the active sites of IgG, hexapeptide could be mapped on IgG by saturation transfer difference (STD) nuclear magnetic resonance (NMR) spectroscopy that is a powerful method for studying protein-ligand interactions. The Results showed that in spite of the undesirable yield of hIgG (73%), the dissociation constant (1.8 μmol/L); binding capacity (49.7 mg/mL drained wet beads) and the purity of hIgG isolated from human serum (94.02%) were outstanding [2].

Recently, Kangwa and coworkers introduced a new *Staphylococcal* Protein A (AviPure thereafter) based on a synthetic ligand analogue like native *S. aureus* protein A, containing a B domain, with a molecular weight of approximately 14 kDa. Due to its lower molecular weight, subsequently reduced steric hindrance, two B domain for interaction with Ig and two cysteine – histidine at the C - terminal for the immobilization on the stationary phase, this new ligand (AviPure) increases the access possibility of IgG to the active sites of Protein A [28].

In addition, most of the engineering efforts are focused on improving the higher binding capacities, prolonged media lifetime, temperature resistance and stability of sorbents to increase their tolerance after exposure to the harsh conditions like alkali pH in Clean in Place (CIP) elution and acidic pH in IgG purification steps. The strong binding of Protein A to antibodies causes a harsh elution condition such as < pH 3.0 and results in aggregation of IgG that will discuss in the next sections.

2.2.1. Engineered protein a and alkaline stability

CIP is a necessary step in affinity chromatography processes to remove contaminants such as aggregated proteins, nucleic acids and lipids from the purification equipment. CIP is commonly employed in an easy and simple way using sodium hydroxide (NaOH) > 0.1 M concentration as a most effective cleaning agent to remove tightly bound denatured and aggregated species and inactivates most microorganisms such as bacteria, viruses and endotoxins [29].

Although this method can easily be used and achieved in low-cost, all equipment, proteins and matrices must be stable toward high pH. In order to increase Protein A stability in alkali pH, the protein engineering has the successful results of which the most famous is the study on Z domain which is derived from the B domain [12].

Nilsson suggested the first concept of Z domain in 1987 based on the second structure of B domain of Protein A. According to the articles, amino acids directly involved in the interaction of the Fc region and Protein A are located on two helices of each domain [21,22,30,31]. Nevertheless, the studies proved that methionine residues existing in the domains E, D and A, increase the sensitivity of Protein A. In addition, the existence of asparagine-glycine (Asn-Gly) at position 28–29 is sensitive to high pH and deaminates under CIP harsh conditions (Fig. 4). This reaction is spontaneous, water dependent, and is accelerated by hydroxide ions. Thus, the lack of Asn-Gly and the reduction of water content is desirable [32].

According to additional informational of crystallography, Asn-28 plays an important role in the interaction, thus the amino acid substitution of Gly-29 was chosen as the best mutation. Intensive changes in the location of glycine probably cause α-helix structure unfolding, so alanine (Ala), because of its resemblance to glycine, was chosen as the amino acid substitution candidate. The head-to-tail structure of the Z domains (by A1V mutation) demonstrates as molar IgG binding capacity as native Protein A with five domains. Nowadays, Z domain lacks the Asn-Gly dipeptide and is mutated to Asn-Ala. These changes cause some disturbances in the binding of Protein A like missing the ability of Fab affinity [32].

Jendeberg and coworkers determined the chain fold of Z domain backbone by CD. They calculated α-helical content by using this strategy that collecting and analyzing the CD data over the wavelength range from 250 to 200 nm and choosing 222 nm as a distinct wavelength for α-helices in which other secondary structure elements such as β-sheet turn, or random coil display only few signals. They compared spectra in the free and bonded states and the data revealed the structural changes in helices while binding to Fc. They proposed a mechanism of binding in which a small tilt of the helix I and II is involving while the helical structure of helix III is maintained intact [33]. Nine amino acid residues in the Fc domain of IgG and 11 amino acid residues in B domain of Protein A contribute to the binding between the two molecules while helix III is intact in the structure of this complex. The angle change of the helix I to the helix II and III before and after complexation of IgG and Protein A and this reorientation in Z domain was investigated [34].

By the discovery the effects of asparagine residues on the Protein A stability in the alkaline condition, asparagine modifications are widely investigated in Protein A. Z domain contains eight asparagine residues (N3, N6, N11, N21, N23, N28, N43 and N52) and each of them can have different influence on the deactivation rate depending to their environment and flexibility of the residue. In order to determine sensitive amino acids, Linhult and coworkers set a goal to exchange asparagine

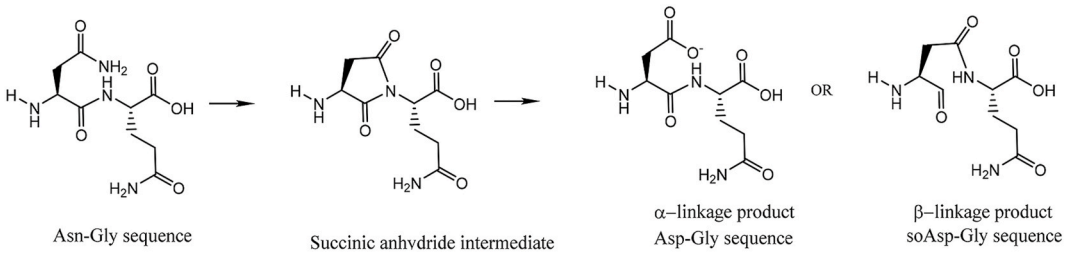

Fig. 4. Deamination reaction in Asn-Gly sequence.

and assess the domain resistance. Due to the significant stability of Z domain, they used a mutated version of Z domain as a framework. This mutation was in line to decrease the structural stability without affecting the domain affinity to IgG. Although phenylalanine in residue 30 (F30) is not participated in the Fc-binding, it has a dramatic impact on the hydrophobic core and the conformational stability of the domain. Therefore, Z (F30A) was studied as a base ligand.

In this way, eight different ligands (Z (F30A,N6A), Z (F30A,N11S), Z (F30A,N21A), Z (F30A,N23T), Z (F30A,N28A), Z (F30A,N43E), Z (F30A, N52A) and Z (F30A,N23T,N43E)) were designed and characterized by SDS-PAGE and mass spectrometry. The content of analogues' secondary structure and their differences in association and dissociation rates was determined by CD and Surface Plasmon Resonance (SPR), respectively. As the most important stage, their stability against alkaline conditions were measured by exposing analogues to the cleaning agent (0.5 M NaOH) for every 30 min and binding capacities were determined. The column with Z (F30A) as base ligand losses 70% of its binding capacity and data for other ligands indicate that N23 has an important role in the stability of protein under harsh conditions of CIP. On the other hand, the replacement of asparagine by threonine, in position 23, remarkably increases the protein stability. These results were proved by applying parental Z domain and Z (N23T) as affinity ligand coupled to HiTrap™ columns [35].

According to above statements, this idea comes to mind to use C domain as a main platform that has naturally Thr-23 in place of the Asn-23, the reason for the high chemical stability of the C domain. So, in the next step, the possibility of increasing the alkaline stability of C domain was examined. For this goal, the resistance of native Protein A and some engineered domains against alkaline pH and protease in the Chinese hamster ovary (CHO) cell culture were investigated. They exposed the derivatives and the wild type of Protein A domains to the alkaline condition and analyzed by SDS-Page and N-terminal sequencing to determine the cleavage sites. They compared all studied domains including: C domain (C-Wild), B domain (B-Wild), Z domain (B-A1V/G29A, Z-Wild), a G29A mutant of the C domain (C-G29A) and the same mutant of the B domain (B-G29A) in terms of alkaline and thermal stability. The interpretation of results demonstrated that C domain is the most stable ligand both in CIP and protease conditions. Although it was suggested that alanine causes only minimal changes in the secondary and tertiary structure, it is important in the deamidation rate. Therefore, in the last step, they engineered thirteen mutants of C domain with different amino acid substitutions at the Gly-29 position. In this study, the binding capacity of all thirteen mutants was evaluated by using surface plasmon resonance (SPR) and CD techniques. The vast increase in the domain stability observed distinctively for G29Y, G29 M, and G29W [36].

Aside from all studies done on various Z domain models and their sustainability, the Z domain is commercially available and is widespread in the industry, so some ligands of Z domain is created as dimer [37] or tetramer (commercially available as MabSelect SuRe of GE Healthcare).

2.2.2. *Dissociation of protein a sorbents from IgG*

Because of the tight binding of Protein A-IgG, its dissociation would be achieved by harsh conditions, like acidic elution, e.g. acetate, citrate or glycine at pH 2 to 3. On the other hand, the use of these conditions is known to cause IgG aggregation [38]. As a method to solve this problem, Bywater and coworkers benefited from the elution technique of competing-ligand type in neutral pH. They suggested high concentration (because of the slow kinetics dissociation of the IgG from Protein A) and different kinds of competing ligands at pH 7. These ligands were classified in three groups containing; ionic (effective when the interaction is held with a salt bridge), aromatic, and ligands which disrupt hydrophobic interactions (glycine, L-tryptophan, L-lysine, betaine, the derivatives of glycine with ethyl ester, L-tryptophan, L-tyrosine and L-histidine). Two effective elution solutions were attributed to 0.1 M glycine at pH 3 and Gly-His at pH 7 with 100% and 40% IgG elution, respectively. According to these results, the high concentration of these ligands at neutral pH could improve the desorption, but the dissociation ratio could not exceed 40% (listed in Table 3) [39].

The failure to use competing ligands as the elution condition makes low pH the most successful step in desorption of IgG from Protein A sorbents. Due to its therapeutic usage, the aggregation level must be clarified and kept in the minimum. Protein A affinity chromatography itself has the nature of aggregation [40], so the protein engineering as a solution is attracted researchers' attention that can moderate the elution by debilitating the interaction.

For desorption at milder pH, Gülich and coworkers focused on the modification of Z domain having a weaker affinity toward IgG. One approach to decrease the tendency of Protein A-IgG is lengthening the loop II, attaching helix II and III, that causes protein destabilization due to the higher energy level required for its closure. Two types of mutation were studied on this loop: First, the second loop has been lengthened by six glycine residues (Z6G) and in the second mutation, the sequence of the loop has been substituted with four glycine residues (ZL4G). Their properties such as interaction in real time and elution conditions were evaluated and compared with Z domain. They employed three proteins in an affinity system and applied three different pH values for the elution: 4.5, 4.75, and 5. According to their results, the lengthening the loop creates better results in comparison with the substitution of a loop. The results showed the best recovery for purified IgG in Z6G ligand with 97% in pH 4.5 compare to ZL4G and Z with 93% and 70% recovery, respectively. However, the elution in pH 5 had the less recovery but the major part of IgG is still eluted from Z6G ligand with 67% compare to 64% for ZL4G and only 31% eluted IgG for Z [41]. In another work, Pabst and coworkers designed Z domain mutants based on histidine to serine substitution, Z $(H18S)_4$ and Z $(H18S, N28A)_4$. Ligands were immobilized on the support matrix and compared against the

Table 3
Dissociation ratio of different competing ligands used as elution.

solute	concentration	pH	IgG elution ratio in volum 20 ml
Gly	0.1 M	3	100
Gly-Tyr	0.1 M	7	10
Gly-Phe	0.1 M	7	22
Gly-His	0.1 M	7	39
Ethylene Glycol	20%	7	16*

**volume of* 10 ml.

commercial Z_4 stationary phase for purification of IgG and Fc-fusion molecules. They used step-wise elution in a gradient program to find the best pH elution and subsequently dynamic binding capacity was measured at the same time. Z (H18S, N28A)$_4$ showed 30% increase in the yield of eluted IgG compared to Z_4 [42].

About 50 years ago, Nisonoff designed a bispecific antibody (bsAb) by mild re-oxidation to couple rabbit antigen-binding fragments (Fabs) of different specificities. They showed that this new type of antibody mediates the agglutination of two different cell types. They produced the first engineered antibody based on the created dual-antigen targeting concepts. For Purification, the bsAb and wild type parental antibody can be eluted from protein A resin at mild pH – allowing separation from parental antibody [43].

Sato suggested a new mutation of Protein A to carry out the elution of immunoglobulin from a column by means of a temperature change. Based on the fact that acidic pH in elution step of IgGs purification using conventional Protein A resins will cause the loss of the three dimensional structure, he designed a mutant which lose its three dimensional structure at high temperatures and can retain its first structure again in low temperatures. He focused on the hydrophobic core of protein A which does not really involve in the Protein A-IgG interaction, but contribute to the stability of the three dimensional structure of the Protein A [44]. Later, Koguma and coworkers used the mutant protein A-coated porous beads made of cross-linked polyvinyl alcohol as thermo-responsive protein A (TRPA) resin to purify IgG1 from the culture supernatant of CHO cells. The binding between IgG1 and TRPA occurs in 5 °C and releases in 40 °C, and the IgG1 purified with recovery of 88% from the culture supernatant of CHO cells producing AE6F4 hIgG1 [45]. Now the resin is commercially available as Byzen Pro from Nomadic Bioscience Co., Ltd.

Krepper and coworkers investigated the role of temperature on the release of IgG from engineered Protein A. They selected three commercially engineered Protein A media: Byzen Pro from Nomadic Bioscience Co., Ltd., a cross-linked polyvinyl backbone with a mean particle size of 70 μm, MabSelect SuRe (GE Healthcare), an agarose backbone with a mean particle size of 85 μm, and TOYOPEARL AF-rProtein, A HC-650F (Tosoh Bioscience LLC) with polymethacrylate matrix and the mean particle size of 45 μm. Five different temperatures from 4 °C to 40 °C were investigated on the release of IgG1 and IgG 2 from Protein A. The advantages and drawbacks of these engineered Protein A in these selected temperatures were discussed. They kept the conditions for all three chromatography media identical. They put each loop and column in a water bath at 4 °C for 5 min to make sure they had achieved the desired temperature before loading, after washing, the loops and the columns were again placed in a 40 °C water bath for 5 min. All three chromatography media showed temperature-dependent adsorption behavior. however, MabSelect SuRe showed greater binding capacity, but only for Byzen Pro they observed temperature-dependent desorption behavior with a yield of 96% [2,46].

2.2.3. Non-specific bindings

Another important factor in affinity purification processes is non-specific binding of contaminants, the compounds that bind to the matrices or the immobilized protein and are co-eluted with the target protein from the matrix. Engineering methods, as a solution, were suggested by several researchers to increase the selectivity, but since the enhancement of selectivity needs to apply stringent condition to release Monoclonal Antibodies (MAbs), the method became obsolete. Therefore, the second suggestion came to their minds. In this hypothesis, ligand PEGylation was tested as a method for the reduction of non-specific interactions. Hydrophobic chains of PEG provide a steric repulsion that reduces binding of contaminants whereas the influence of ligand PEGylation on the dynamic binding capacity must be negligible. The most important factors in PEGylation include; the molecular weight of PEG, the chain structure, the yield of modification and coupling conditions. On the other hand, PEGylation occurs in lysine residues and as there is the high amount of lysine in the Protein A amino acid sequence, discriminate in PEGylation will be avoided. Gonzalez-Valdez and coworkers conjugated activated PEGs, 5 and 20.7 kDa, at pH 5 to control only N-terminal PEGylation. These ligands immobilized on a matrix, and the columns efficiency was investigated by injection of rabbit serum IgG as a standard and Yeast extract and Fetal bovine serum (FBS) as contaminants. The selectivity of ligand was characterized before and after PEGylation with different concentrations of contaminants spiked in the targeted protein. The calculated Static Binding Capacity (SBC), Dynamic Binding Capacity (DBC) and mass transfer for columns showed an acceptable decrease in non-specific binding while the binding capacity was maintained. The average IgG recovery percentages were 92.861.3% in the unmodified column, 95.56 ± 0.9% and 96.46 ± 1.7% in the 5.0, 20.7 kDa PEGylated columns, respectively. The PEGylated media showed greater selectivity than the unmodified media with significant increase in the FBS spiking experiments. At higher FBS concentrations, which for the 5.0 kDa PEGylated Protein A media was about 50% larger than that of the unmodified media; and for the 20.7 kDa PEGylated Protein A media was two to three times larger selectivity for mass of recovered IgG in comparison to the unmodified media [47].

2.2.4. Other engineered protein A

Alongside Protein A, there are some investigations on Protein G that has affinity to both IgG (all subtypes) and human serum albumin. The C-terminal of protein G contains three homogenous IgG binding domains with a central α-helix and three β-sheets with 11.5 kDa molecular weight [42–44]. Of course, due to its non-specific interaction with albumin, some attempts have been done on structural modification, like using only IgG binding domains as a ligand or their conjugation to Protein A domains. Dong and coworkers designed successfully attachment of D domain of Protein A to C1 domain of protein G with association constant value 12 and 8.4 times higher than protein G and protein A, respectively [48].

According to the content provided, a lot of attempts have been done to obtain the best antibodies purification method based on Protein A ligands (Table 4). Each attempt tried to focus on a specific problem. For example, Z domain, was the result of an attempt to increase the protein's resistance to alkali conditions, which seems to be the most successful ligand offered to the scientific and industrial world. But later researchers led to find a more appropriate ligand, such as the mutant C Domain.

On the other hand, due to the all efforts made in the field of IgG elution using milder conditions, the best elution buffer is still using an acidic buffers (e.g.Glycin-HCl) which uses in the industry and commercial resins. Against the use of competing-ligand type in neutral pH which is failed, again protein engineering methods were successful to introduce alternative methods to researchers instead of using acidic pH like some ligands we discussed about them in this review: Byzen Pro from Nomadic Bioscience Co., Ltd. Which is a thermo-responsive protein A ligand and Z4G ligand (lengthened second loop of Z domain).There are also some attempts to provide a modified Protein A ligand to minimize the non-specific bindings.

So it seems that there are bunch of Protein A ligand that can be use in every condition, but there is still a leakage for a ligand that can fill all the gaps in this approach. A ligand, which, while has a sufficient binding capacity, has other features such as stability against alkali and acidic pHs, the ability to release IgG in milder conditions, the minimum amount of non-specific bindings and the proper structure to immobilize on the sorbents of purification with minimum steric repulsion.

2.3. Protein A sorbents applications

The idea of antibody therapy is started over a century and is now mostly a successful treatment for cancer and immunodeficiency diseases. There are many attempts for identification and characterization of antigens in order to find a way for diagnosis and treatment of cancers and other diseases. The cancer immunologists' routes began by

Table 4
Different kinds of recombinant Protein A.

Recombinant protein	Mutation	Results	Description	Ref.
Protein A without XM region	Elimination of XM region	–	Registered by GE health care	–
Z domain	Mutation on B domain;		Missing the ability of Fab affinity	[32]
	G29A	Elimination of diamination site		
	A1V	Facilitate genetic engineering		
Mutation on Z domain	N23T	Increasing alkaline stability	–	[35]
Mutation on C domain	G29A	Elimination of diamination site	–	[36]
Mutation on C domain	G29W	Increasing alkaline and thermal stability	–	[36]
Mutation on Z domain	Increase length of loop by 6 Glycine	Destabilize the interaction	IgG elution can be occurred in pH 4.5 with 97% yield	[40]
Mutation on Z domain	H18S N28A	Destabilize the interaction	30% Increase in the IgG elution yield compare to Z domain in milder pH	[41]
Protein A/G	D Domain/C1 Domain	Coupling D domain of Protein A and C_1 domain of Protein G	Increase in associated contacts: 12 times more that Protein G 8.4 times more than Protein A	[44]
Protein A- Cys	Addition of Cys to Protein C-terminal	Oriented immobilization	–	[45]

identifying cancer-specific antigens and the tumor-associated cell surface ones. MAbs are chosen in this way because they dramatically bind to antigens and act as a best cell surface detector and cancer killer [49]. FDA has proved the polyclonal IgG as a replacement strategy in the treatment of primarily immunodeficiency diseases (PIDD) [50].

Based on progress in IgG and Fc-fusion proteins therapeutic usage, Protein A affinity chromatography as a well-known, selective and high capacity purification method is rapidly grown in biopharmaceutical industries and is always followed by polishing steps like ion-exchange or hydrophobic interaction chromatography to remove any remained impurities, aggregated IgG and leached Protein A.

Interaction of Protein A and IgG opens another important ability for creating variant kinds of gold and silicon sensors for IgG detection [51,52] and it can be operated by measuring shifts in the spectrum wavelength, UV adsorption, or real-time SPR methods [53].

Protein A kits are designed specifically for binding of immunoglobulins, in both laboratory and process scale applications. Table 5 offers some of the antibody-purifying kits which are different in the type of matrices, structures, and spatially antibody binding capacity per gram of supports.

Retaining the bioactivity of proteins is one of the most important subjects in their immobilization. The random amide bond formed between protein and support can lead to great loss of protein activity, so correct orientation of proteins on the surface of sorbent should be considered. Since Protein A interacts with Fc region of IgG, it can be employed for solving the random immobilization problems which cause binding site blocking and low binding capacity. One-site attachment methods in which Protein A do not interfere with the antigen binding sites, increase the capacity of sorbents, improve surface sensitivity and reduce the risk of antibody partial denaturation caused by surface effects. In addition, the sorbent can be used for different purposes by eluting immobilized MAbs and incubated with another MAb. In this way Protein A modified particles showed great results in enrichment of bacteria like *Vibrio cholera* and abnormal fetal cells, hematopoietic stem cells, chicken red blood cells (CRBC) and sheep red blood cells (SRBC) [54–57]. It is noteworthy that this protein is alone used for purification of cells. Protein A as an antigen can stimulate the peripheral B cell population and employ in different treatments like rheumatoid arthritis and hemophilia [58].

2.3.1. *Biosensors based on protein A and IgG*

Biosensors, a type of immunosensors, are other interesting surfaces considered for immobilization of sensitive proteins such as Protein A since they can detect the low amount of proteins such as IgG with high sensitivity in a short time and they are facile to work. Chen and coworkers suggested that immunosensors coating with hydrophilic polymer could be employed for indirect immobilization of protein. They developed a biosensor based on the poly-L-lysine-modified crystal surface that is coated with periodate-oxidized dexteran as a linker for covalently attachment of Protein A. The results indicate that these types of polymer-coated biosensors take advantage of high capacity and activity along with low non-specific interaction [59].

Shen and coworkers attached hyper branched polymers interesting materials for immobilization of proteins to the surface of immunosensor to detect antibodies (Fig. 5). Since this approach provides the indirect attachment of Protein A to the sensor, the biological activity of protein will be remained [60].

Baltierra-Uribe and coworkers produced a covalent bond of protein A on the silicon surface and oriented adsorption of specific IgG on the surface to detect *Brucella abortus*. They obtained sensitive detection with three-fold higher than the random immobilization of anti-*Brucella* IgG on the biosensor [61].

3. Sorbents and methods for immobilization of protein A

Active functional groups in proteins for covalent bonding are amine group in lysine residues, thiol group in cysteine and carboxyl groups in aspartic and glutamic acid. For IgG purification, activated agarose- and silica-based supports are the most common solid ones for immobilization of Protein A. One of the major problems of the application of bacterial proteins, such as Protein A and G, is the trace number of contaminants accompanied with purified target protein (IgG) in the final formulated drug. As these contaminates can strongly influence the immune response of the human body, several researches have focused on finding highly selective methods for Protein A immobilization to obtain a sorbent without any extra non-specific adsorption.

The most used method is the engineering of Protein A with the cysteine residue in its C-terminal. This modification helps that the protein be immobilized on maleimide-functionalized beads and on a gold surface for IgG sensing purposes. Cysteine residue increases the yield of the Protein A immobilization and its IgG binding capacity according to the thiol-gold strong interaction and the stable thioether bond generated by chemical coupling of thiol and maleimid, (Michael addition to α, β-unsaturated, Fig. 6). Therefore, further binding activity of IgG would be preserved because of its oriented immobilization [8,62].

In addition, due to the outstanding role of lysine in immobilization of proteins, this feature has been used in protein engineering to control oriented immobilization by enriching the number of lysine on helix III [31].

Glutaraldehyde is a common compound used as spacer on the sorbent surface to attach to Protein A. After the attachment of glutaraldehyde, it produces free amine groups (cationic groups that can act as an anion exchange function on the sorbent) and also there are hydrophobic areas on the surface that cause physically adsorption of B domain of Protein A and then the amino group of glutaraldehyde reacts to α3 of B domain Protein A.

Wang and coworkers studied the immobilization mechanism of an

Table 5
Properties of Protein A resins produced by different companies.

Resin name	Ligand	Matrix	Binding capacity	Use for	Company	Ref.
rProteinA Sepharose	rProtein A	Agarose	~27 g/L resin	Monoclonal antibody	Bio-Pharm	[75]
Mab Select SuRe	Alkali-tolerant Protein A	Agarose	~35 g/L resin	Monoclonal antibody	Bio-Pharm	[75]
Mab Select SuRe LX prototype	Alkali-tolerant Protein A	Agarose	~60 g/L resin	Monoclonal antibody	Bio-Pharm	[75]
Dynabeads Magnetic Beads	Protein A	Monosized, super paramagnetic polystyrene beads	25–30 μg human IgG/mg beads	Polyclonal IgG from rabbit, pig, dog, cat serum	ThermoFisher scintific	[76]
Pierce Protein A Agarose	Protein A	Crosslinked 6% beaded agarose	12–19 mg human IgG/mL resin	Polyclonal IgG from rabbit, pig, dog, cat serum	ThermoFisher scintific	[76]
Pierce Protein A Plus Agarose	Protein A	Crosslinked 6% beaded agarose	34 mg human IgG/mL resin	Polyclonal IgG from rabbit, pig, dog, cat serum	ThermoFisher scintific	[76]
Pierce Recombinant Protein A Agarose	Recombinant Protein A	Crosslinked 6% beaded agarose	15–17 mg human IgG/mL resin	Polyclonal IgG from rabbit, pig, dog, cat serum	ThermoFisher scintific	[76]
POROS MabCapture A Select	r Protein A produced in yeast	Polystyrene-divinylbenzene	≥45 mg/mL human IgG/mL resin	Polyclonal IgG from rabbit, pig, dog, cat serum	ThermoFisher scintific	[76]
Immobilized Protein A spin column kit	rSpA	6% highly cross-linked agarose	>40 mg human IgG/ml resin		G- biosciences	[77]
Protein A Magnetic Beads are Fe_3O_4 beads	rSpA	dextran coat to agarose slurries	260 μg human IgG/ml	Chromatin immunoprecipitation (ChIP) Small-scaleIgG Purification and antibody labeling Protein Isolation	G -biosciences	[77]
KPL Protein A Kit	rSPA	6% cross-linked agarose	15 mg/ml of human IgG per ml of resin.	immunoglobulin molecules of many mammalian species without disturbing their binding of antigen	seracare	[78]
High-Affinity Antibody Purification Kit	rSPA	covalently coupling a derivative of iodoacetic acid to 4% cross-linked agarose beads			GenScript	[79]
Proteus Protein A Mini spin Kit	rSPA 35–50 kDa	covalently coupled to agarose resin	1 mg/ml of rabbit IgG per ml of Mini resin.	Human IgG1, IgG2, IgG4, IgD, IgE, IgM, Mouse IgG1, IgG2a, IgG2b, IgG3, IgM, Rat IgG2c, IgM	BIO-RAD	[80]
Proteus Protein A Midi spin Kit	rSPA 35–50 kDa	covalently coupled to agarose resin	20 mg/ml of rabbit IgG per ml of Midi resin.	Human IgG1, IgG2, IgG4, IgD, IgE, IgM, Mouse IgG1, IgG2a, IgG2b, IgG3, IgM, Rat IgG2c, IgM	BIO-RAD	[80]
Montag Kit	Protein A	Porous glass	>20 mg of rabbit IgG		Merck	[81]
PURE1A-1 KT	Protein A		Human IgG 20–25 mg/ml	Human IgG Mouse IgG1 Mouse IgG2a Mouse IgG2b Mouse IgG3 Rabbit IgG Goat IgG Bovine IgG	Sigma	[82]
TCS protein A resin	Protein A	agarose beads	up to 5 mg/ml	IgG fractions from hybridoma supernatants	abcom	[81]
MabSelect PrismA	protein A-derived ligand	agarose beads	~80 mg/mL	Monoclonal antibody	GE life sciences	A
MabSelect SuRe LX	protein A-derived ligand	Superdex 200 highly cross-linked agarose	~60 mg/mL	Monoclonal antibody	GE life sciences	A
MabSelect SuRe pcc	protein A-derived ligand	Superdex 200 agarose beads	~60 mg/mL	human polyclonal IgG	GE life sciences	A
Praesto® APc	Recombinant Protein A	Highly cross linked agarose (4%)	>50 mg hIgG/mL	human IgG	Purolite Life-Sciences	B
Protein A Agarose 50% suspension	Recombinant Protein A	Agarose 50%	0 mg human IgG/ml 1 mg/ml human monoclonal IgG1	human monoclonal IgG1	iba Life-Sciences	C
WorkBeads Protein A	recombinant protein A	Agarose	>40 mg human IgG/ml	monoclonal- and polyclonal antibodies	Bio-Works	D
Protein A-Agarose Beads	recombinant protein A	Agarose	~25 mg human IgG/ml	IgG, some IgA and IgM	abm™ Applied	E
KANEKA KanCapA™	recombinant protein A	highly cross-linked cellulose	>40 mg human IgG/ml	IgG	KANEKA KanCapA™ 3G	F
ProSep® Ultra Plus Media	Recombinant native protein A	Controlled Pore Glass	≥67 mg/mL (hIgG)	monoclonal antibodies	Merck	[81]
Fastback Protein A Sepharose FF	recombinant protein A	Sepharose matrix	30 mg/ml human IgG	human IgG	Protein Ark	G
PurKine™ Protein A Resin	Recombinant protein A	cross-linked 4% agarose	40 mg/mL	human lgG mouse IgA, IgM	Protein Ark	G
PurKine™ Protein A Resin 4FF	Recombinant protein A	cross-linked 4% agarose	40 mg/mL	human lgG	Abbkin	H
HiFliQ Protein A Column	Recombinant protein A	Agarose FF resin	30 mg/mL	human lgG	Protein Ark	I
MAbPurixTM Protein A	Recombinant protein A	Agarose Beads	~33 mgs/ml~ 33 mgs/ml	human lgG	Sepax Technology	J
MabPurix Protein A	Recombinant Protein A	4% Highly cross linked Agarose	>40 mg human IgG/ml resin	human lgG	Sepax Technology	J

* Typical dynamic binding capacities according to resin manufacturer data.
A: https://www.gelifesciences.com.
B: https://www.purolite.com/life-sciences.
C: https://www.ibalifescience.com.
D: https://www.bio-works.com.
E: https://www.abmGood.com.
F: http://www.bioseparation.kaneka.com.
G: https://proteinark.com.
H: https://www.abbkin.com.
I: https://www.sepax-tech.com.
J: https://www.sepax-tech.com.

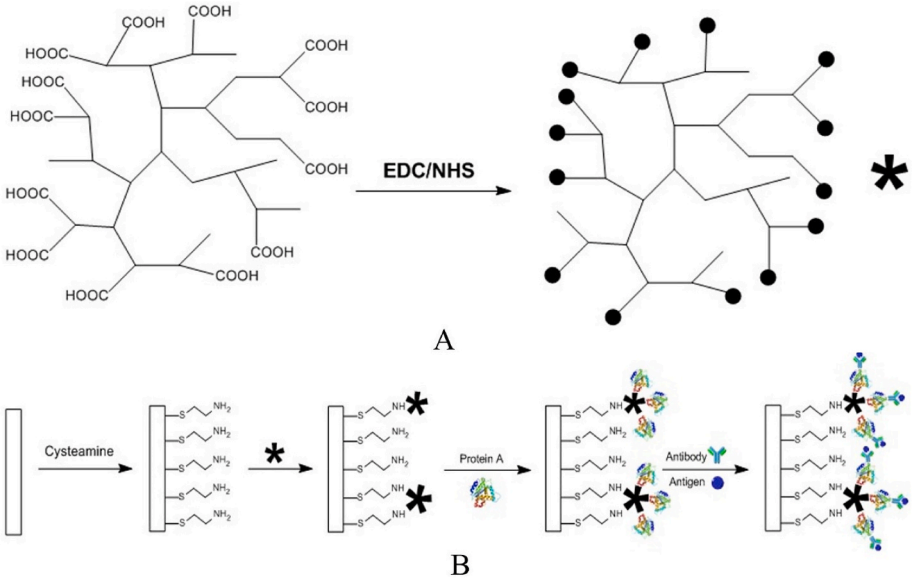

Fig. 5. Hyper branched polymer as an indirect tool for immobilization of Protein A.

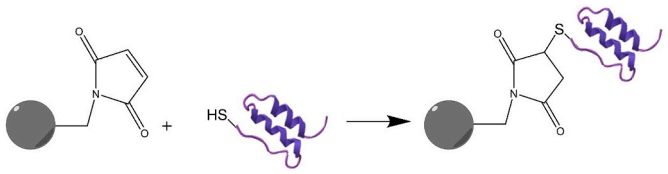

Fig. 6. Chemical reaction of thiol and maleimid (Michael addition to α, β-unsaturated).

engineered B domain of Protein A on both monomeric and dimeric glutaraldehyde activated agarose under different ionic strength conditions. The immobilization of Protein A on the sorbent surface is followed by a two-step mechanism: at the first step, the Protein A is physically adsorb on the surface by adsorptive groups of sorbent and in the second step, a covalent binding binds the adsorbed Protein A group (helix III of B domain) to reactive groups of sorbent. By modeling the 3D surface structure of B domain, they found the distribution of adsorptive and reactive groups and determined the role of the Protein A amino acids on anionic and hydrophobic interactions. B domain contains three α-helices in the polypeptide segments Lys7 to His18 (α1), Glu25 to Asp36 (α2) and Ser41 to Ala54 (α3). The α3 including Lys-35 to Lys-50 and Lys-58. They focused on two scenarios for adsorptive and reactive groups of Lys-35 to Lys-50 and Lys-58. First, the amine groups (as an anionic exchange function) adsorb the B domain and the hydrophobic groups are located near the regions of Lys-35 to Lys-50 and Lys-58. Then this hydrophobic adsorption performs the first immobilization function. B domain could be covalently bonded on the sorbent through the amine group of Lys-58 residue. They followed the static and dynamic binding capacity of glutaraldehyde agarose alongside monomer and dimer spacer's length [16].

Another paper published by this group considered the epoxy-activated sorbents instead of glutaraldehyde-activated ones. They believed that epoxy groups take advantage of more stability at neutral pH conditions, longer life-time, more reaction with different nucleophiles on the Protein A surface, the minimal chemical modification of the protein and resistance to moderate alkaline conditions [63].

In recent years, the production of recombinant proteins has expanded to fuse a sequence of amino acid to the protein as affinity tags. These affinity tags have some features that can be used in different kinds of studies including: increasing the lifetime of protein drugs (Fc-fusion proteins), one-step purification (His-tag), one-step immobilization (cellulose binding domain and the silica tag), etc. In this technique, a specific sequence is fused into the ligand to provide a linking site for its immobilization on sorbents without any chemical reaction or matrix activation. These affinity tags help proteins bind to various matrices according to their affinity. Cellulose-binding domains (CBDs) are polypeptides found in *Trichodermareesei* fungi and *Cellulomonasfimi* bacteria, and have nearly 12 different families from 27 to 189 amino acid residues with the molecular weight of 3–20 kDa. They bind irreversibly to cellulose under the wide range of pH (3.5–9.5) and are released only with urea or guanidine hydrochloride [64]. Bai and coworkers purified interferon α-2b (anti-IFN α-2b) by using magnetic cellulose microspheres functionalized with Protein A and anti-IFN α-2b IgG. They used cellulose-binding domains Protein A (CBD-SPA) as a ligand in their affinity sorbent [65]. This immobilization method provides an effective affinity sorbent to purify different proteins from complex mixtures [64].

In the following, other tags were introduced for this purpose like Si-tag, a derivation of bacterial ribosomal protein L2, to immobilize of

protein on the silica surface. The reaction between Si-tag proteins and silica occurs in a simple condition even in the presence of high concentration of salt and detergents. This method suggests a powerful way for binding of proteins to silica and silicon wafers [52].

Since the comparison of various commercial Protein A media is previously investigated in detail in other reports [12,66–68], here we focus on different kinds of sorbents, linkers and methods which are employed for immobilization of Protein A.

3.1. Polysaccharide

Although cyanogen bromide activated agarose beads are commonly used for coupling amino groups of Protein A to stationary phase, the high toxicity of cyanogen bromide and the harsh activation condition of the reaction is caused other alternative procedures to be recommended. In this regard, several modifications in linkers and stationary phases have been investigated.

Agarose polyacrolein microsphere beads (PAMB) were introduced [69] by Nathan and coworkers who utilized these beads to immobilize Protein A. Due to biocompatibility and suitable physical properties, these sorbents have been introduced as the selective ones for purification of IgG from blood samples (Fig. 7-A) [70].

Recently a fast strategy, which is based on radical cross-linking reactions, for covalent coupling of Protein A on the surface of agarose was introduced by Jia and coworkers. They took advantage of photochemical reactions occurred on the surface of sorbents. According to their procedure, media containing phenol groups have a great potential for participating in photochemical reactions with –NH, –SH and especially phenol groups existing on the structure of proteins. In order to immobilize Protein A, tyramine was placed on the surface of epoxy-activated agarose and visible-light irradiation of the surface led to the generation of phenol radicals and subsequently cross-linking of proteins (Fig. 7-B) [71].

Bao and Zhang reported in two different articles the site-specific immobilization of recombinant Protein A. In these studies, a cys-modified C terminus of zz domain or zz domain of Protein A was respectively used and coupled to agarose bead, which was functionalized with maleimide. The comparison between site-specific conjugation and randomly immobilization of Protein A revealed that the capacity of sorbent for binding to the antibody is increased when Protein A is bound by thiol group at the C terminus of zz-cys or zz-cys than randomize form. Zang and coworkers attributed such high capacity to decrease steric hindrance caused by oriented immobilization of zz-cys [37,72].

Binding capacity is one of the most important factors for selection a proper affinity medium. Although ligand modification is more considered for this purpose, the improvement of matrix can be an effective method too. Zhao and coworkers proposed the dextran-grafted agarose matrix for enhancing binding capacity (Fig. 7-C). They used epoxy-activated agarose grafted with dextran to immobilize site–directed recombinant Protein A on it. These kinds of sorbents take advantage of more stability and less ligand leakage resulted from penetration of dextran molecules inside the matrix and generation a denser gel. The faster binding rate, more rough surface, the improvement in binding performance, reduction in steric hindrance, and having higher binding capacity are other advantages of Dextran-grafted epoxy-activated agarose that makes it a more suitable support for Protein A immobilization in comparison with non-grafted one [73]. GE Healthcare Co. introduced an agarose backbone with a mean particle size of 85 μm as MabSelect SuRe which is suitable for capturing monoclonal antibody. The features of this product are high dynamic binding capacity, tolerance to harsh alkali condition.

3.2. Polymers

Besides agarose and cellulose, as general sorbents for immobilization of Protein A, copolymers like polyhydroxyethylmethacrylate (PHEMA), a hydrophilic polymer, was introduced for this purpose. Piskin and coworkers successfully immobilized up to 3.5 mg Protein A per gram of CNBr-activated PHEMA. According to the results, non-specific adsorption on the surface of this polymer was negligible in the plain form [74]. In order to improve the properties of these kinds of sorbents, Piskin and coworkers reported other microbeads which are possessing smaller particle size, and they are based on copolymers of methylmethacrylate

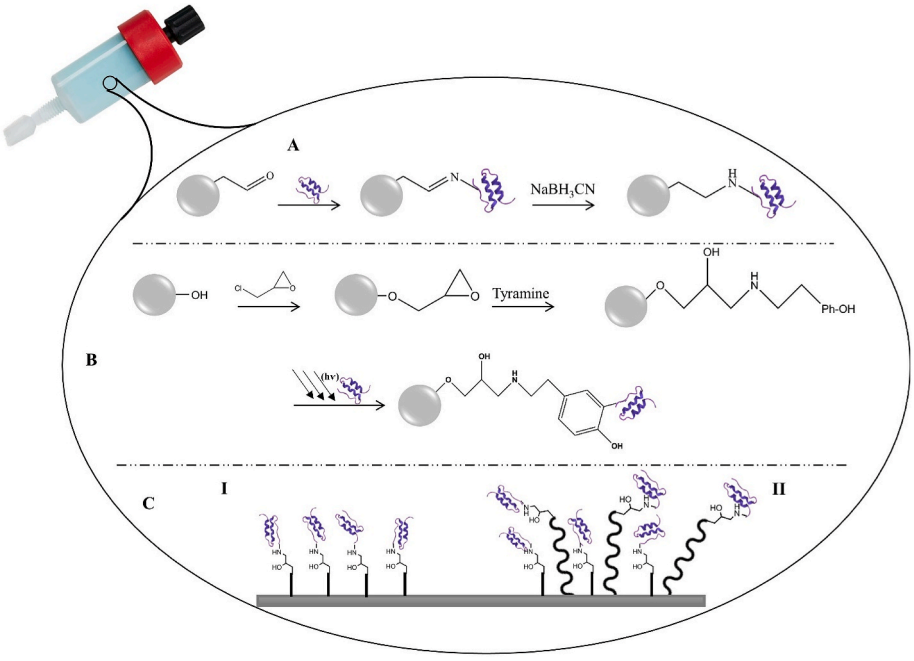

Fig. 7. Chromatography columns based on Polysaccharides: A) Immobilization of Protein A on polyacroline agarose beads by Schiff base reaction and Imine reduction by $NaBH_3CN$, B) Protein A affinity sorbents based on photochemical reaction on epoxy-activated agarose and C) I. Non-grafted and II. Dextran-grafted Protein A affinity chromatographic media.

and 2-hydroxyethylmethacrylate activated by periodate oxidation instead of cyanogen bromide. In this way immobilization of Protein A was achieved by hexamethylenediamine as the linker [75]. Along with employing PHEMA microbeads, Denizli and Arica introduced PHEMA affinity membranes for purification of IgG from human plasma [76].

Copolymer beads based on vinyldimethylazlactone and methylene-bis-acrylamided have been used as affinity supports for purification of IgG. Coleman and coworkers took advantage of the properties of azlactone polymerization to provide a suitable surface for coupling Protein A by a rapid nucleophilic attack, which maintains its biological activity. These kinds of polymeric beads are able to immobilize over 200 mg/g proteins implying their high capacities [77].

The application of monolithic capillary columns for immobilization of Protein A was proposed by Zou and coworkers. This sorbent was achieved by in situ polymerization of glycidyl methacrylate (GMA), cross-linked with trimethylolproane (TRIM), and subsequently Protein A immobilized on this surface. The high flow rate, the minimum amount of sample for analysis and fast mass transfer are attributed to these sorbents [78]. Nomadic Bioscience Co. introduced Byzen Pro (mentioned in section 2.2.2) as a cross-linked polyvinyl backbone with a mean particle size of 70 μm. Tosoh Bioscience LLC Co. suggested polymethacrylate matrix with the mean particle size of 45 μm as support of Protein A. one od the best feature of this sorbent is multipoint attachments of ligand that improves the chemical and thermal stability of the resin.

3.3. Silica

Optimization of silica supports for immobilization of Protein A was introduced by Katoh and coworkers to provide a sorbent with high quality for large-scale purification of IgG [79]. Although silica supports have high mechanical stability, silanol groups existing on their surface cause unwanted non-specific interactions. On the other hand, polysaccharide supports like agarose and dextran have minimum non-specific interaction but suffer from low mechanical properties. Muller and coworkers combined the properties of both types of sorbents to create a support with high physical stability and minimum non-specific adsorption. In their work, silica was coated with (Diethylaminoethyl) DEAE-agarose or DEAE-dextran to reduce cation exchange capacity and then a layer of agarose or dextran was placed on it. For immobilization of Protein A, double-coated silica was activated by 1, 1′-carbonyl diimidazole (CDI) or 1,4-butanediolglycidylether (BDGE) [80].

In another work, Maeno and coworkers introduced new types of silica supports for immobilization of Protein A with low non-specific adsorption. They used phosphorylcholine (PC), a high hydrophilic group owning a neutral structure that decreases non-specific interaction in the surface of sorbent, to modify porous silica and subsequently activated the beads by N-hydroxysuccinimide (NHS) groups to immobilize Protein A [81].

Mesoporous silica (MPS), as a medium with the high surface area and magnificent stability, is a proper sorbent for immobilization of biomaterials. Kato and coworkers studied the effect of pore size and surface characteristics on the synthesized MPS composites for immobilization of Protein A. Their results showed that the pore size of MPS plays an important role in the immobilization of Protein A. In fact, MPSs with the pore size of 2.7 nm can fix and stabilize Protein A and so improve the accessibility of IgG to Protein A. Besides the effect of pore size, the type of chemical adsorption of Protein A on this composite was investigated. According to their results, MPSs functionalized with γ-glycidoxypropyl trimethoxysilane generate a surface for the covalent attachment of Protein A with high stability and consequently more binding activity [82].

3.4. Nanoparticles

Magnetic nanoparticles are other sorbents which are interesting for immobilization of Protein A and it is attributed to their exclusive properties. Jiupengand and coworkers immobilized Protein A on Fe3O4@SiO2 core-shell nanoparticles functionalized with (3-aminopropyl)triethoxysilane (ATPES) and subsequently activated by glutaraldehyde (Glu) and the monoclonal antibody was achieved by a purity of about 95.4% (Fig. 8-A) [83].

In another study, genetically modified Protein A was immobilized on chlorophenylsilane functionalized magnetic nanoparticles (CPTMS@MNPs) and its binding capacity was compared with commercial microparticles (Dynabeads). In this way, Chang and coworkers expressed Protein A with a cysteine residue in *E. coli* so this modified Protein A consists of a –SH group that can bind to CPTMS@MNPs (Fig. 8-B). The binding capacity of these particles is 7-fold higher than commercial ones and the amount of Protein A on the surface of particles is 11.5-fold higher than those of Dynabeads. Therefore, these particles are very suitable for industrial purification of the antibody [84].

Since coated magnetic nanoparticles (MNPs) show an increase in size, in comparison with uncoated ones, MNPs without any coating materials are more desirable for immobilization of proteins. On the other hand, a surface area enhancement observed in uncoated MNPs results in high binding capacity. Lype and coworkers considered the properties of uncoated iron oxide nanoparticles for immobilization of Protein A. They introduced a new method in which epichlorohydrin as a containing epoxy cross-linking agent was used to crosslink proteins through NH, OH and SH groups existing in their structure and OH groups on the surface of MNPs. So MNPs get entrapped in a matrix which is formed by cross-linking and then the proteins immobilize on the surface of particles [85].

Recently an alternative sorbent to the common core-shell magnetic silica nanoparticles based on the magnetic monodisperse SiO_2 microsphere [Mag (SiO_2)] was introduced by Salimi and coworkers for immobilization of Protein A (Fig. 8-C). This microsphere sorbent takes advantage of both macroporous and mesoporous properties. Macropores of this sorbent facilitate the diffusion of Protein A and IgG and mesoporous compartments generate high surface area for binding and adsorption of Protein A and IgG, respectively. The purity achieved by this sorbent is higher than 95% [86].

Salimi and co-workers presented a covalent immobilization method for coupling of Protein A or Protein A/G on the surface of both mesoporous and macroporous SiO2, 6.5 μm in size. Immobilization of Protein G, another bacterial protein that have two IgG binding domains, is introduced for selective IgG purification. They could successfully obtain the faster magnetic response, more purity for IgG (higher than 95%) and considerable isolation yield in comparison with commercial protein A affinity sorbents. To clarify the concept of the method employed for the immobilization, we followed the chemical route for the attachment of chromatographic ligand. The presence of hydroxyl groups on the Mag (SiO2) microspheres allows the covalent attachment of (3-glycidyloxypropyl) trimethoxysilane (GLYMO) via silane coupling. Then, a bifunctional molecule, ethylenediamine is covalently linked onto the GLYMO attached-microspheres via the reaction between epoxypropyl and amine groups. In the next step, amine functionalized magnetic microspheres react with glutaraldehyde (GA) for the generation of aldehyde functionality on the surface. The covalent attachment of chromatographic ligand(s), protein A or protein A/G performs through the Schiff base formation between primary amine groups of protein A and protein G and aldehyde groups. This covalent linking of ligand onto the magnetic microspheres ensures the selectivity of sorbents through IgG purification. In fact the surface hydroxyl groups of Mag (SiO2) microspheres provide hydrophilicity to the base matrix for preventing non-specific interactions during IgG purification and also allow the selection of an efficient binding protocol for protein A attachment onto the base material. Based on these reasons, Mag (SiO2) microspheres derivatize with protein A or (protein A/G) is proposed as new affinity sorbents for IgG purification [86].

Depletion of human IgG (hIGg) in the plasma for detection of disease-

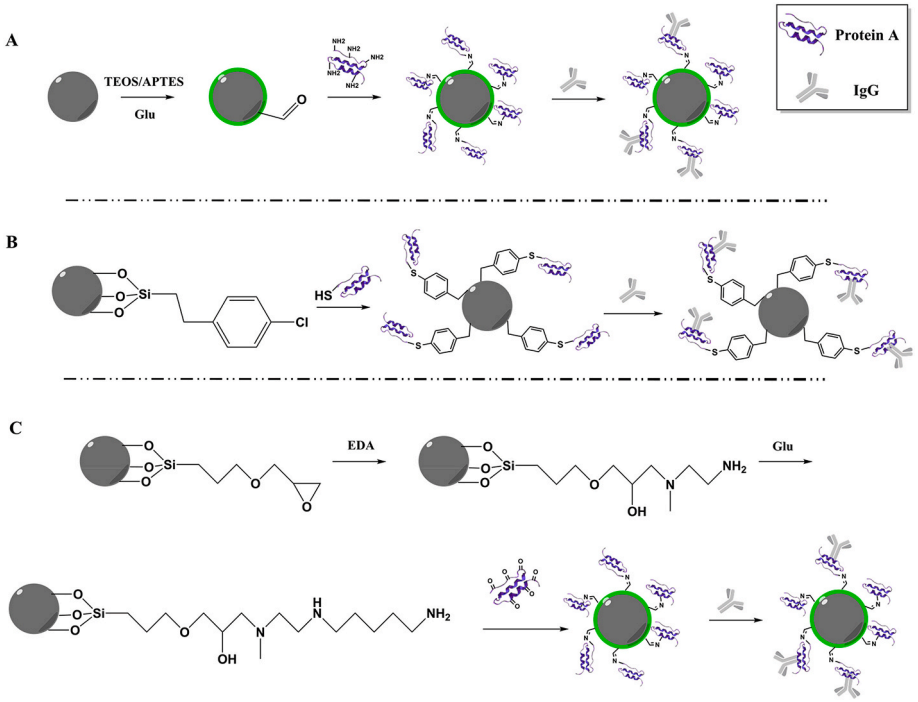

Fig. 8. A) Functionalized Fe3O4@SiO magnetic nanoparticles by (3-aminopropyl)triethoxysilane (APTES) and subsequently glutaraldehyde (Glu), B) chlorophenylsilane-functionalized magnetic nanoparticles for Oriented immobilization of Protein A, C) Magnetic monodisperse SiO2 microsphere activated with (3-glycidyloxypropyl) trimethoxysilane (GLYMO), ethylenediamine (EDA), and subsequently reacted with glutaraldehyde (GA) and Protein A.

related biomarkers was reported by Horak and coworkers. In order to create a flexible platform able to deplete hIgG from human plasma and capture antibody from cell culture supernatants, they applied citrate-capped gold nanoparticles (GNPs) synthesized and conjugated with cysteine-tagged recombinant Protein A (rProtA) and Protein G (ProtG). According to their results, these new design nanoparticles could be a suitable alternative to magnetic beads based on immunoaffinity capturing [86].

Wang and coworkers studied on the oriented immobilization of mono and tetramers of Z domain on Fe3O4 magnetic nanoparticles coated with carboxymethyl dextran (CMD). They focused on the changes in the molecular structure of Protein A on the solid surfaces and mechanism of Protein A surface interaction. This investigation revealed that ligand polymerization and buffer pH had a significant influence on the molecular structure of Z domain immobilized via thiol coupling [87].

4. Concluding remarks

Protein A, with its simple single chain structure, binds reversibly to the one of the most effective and critical proteins, Immunoglobulin G (IgG), with an affinity constant of K_A of 10^8 (M^{-1}). Due to the striking growth of IgG application in most countries, in disease diagnosis and treatment, only seemingly simple ability of Protein A has made that as one of the most lucrative protein in pharmaceutical industries. This manuscript provides a comprehensive overview for researchers to gain better insight into the various recombinant Protein A and sorbents used in Protein A affinity purification techniques.

Although there are many achievements in alternative ligands like small molecules and peptides as Protein A mimetics, they could not find their way to industry until now. Therefore, Protein A affinity purification sorbents, despite the high costs, can be introduced as the only reliable method for IgG and MAbs purification.

Protein engineering strategy makes a successful way by means of elimination of unnecessary regions, amino acid substitutions (like Z domain), and addition of amino acids (like lengthening the loop between helix I and II) for Protein A stability optimization. Alongside these efforts, fused tags are introduced for faster and better immobilization without any complex chemical reaction.

In the field of media used in protein A affinity methods, each sorbent reflects some advantages like good biocompatibility and suitable physicochemical properties of agarose and sepharose, high mechanical stability and high mass transfer of mesoprous silica, and the high surface area and magnificent stability of magnetic nanoparticles. The purification process based on Protein A perfectly communicate between microbiological, genetic engineering and chemistry sciences and we are hopeful more development in ligand and matrices provides better and easier use of Protein A in biopharmaceutical industry.

References

[1] A.M. Ramos-de-la-Peña, J. González-Valdez, O. Aguilar, Protein A chromatography: challenges and progress in the purification of monoclonal antibodies, J. Separ. Sci. 42 (2019) 1816–1827.
[2] W. Wang, D. Hao, J. Ge, L. Zhao, Y. Huang, K. Zhu, X. Wu, Z. Su, R. Yu, G. Ma, A minimalist peptide ligand for IgG by minimizing the binding domain of protein A, Biochem. Eng. J. 151 (2019) 107327.
[3] G. Vidarsson, G. Dekkers, T. Rispens, IgG subclasses and allotypes: from structure to effector functions, Front. Immunol. 5 (2014) 520.
[4] V. Irani, A.J. Guy, D. Andrew, J.G. Beeson, P.A. Ramsland, J.S. Richards, Molecular properties of human IgG subclasses and their implications for designing therapeutic monoclonal antibodies against infectious diseases, Mol. Immunol. 67 (2015) 171–182.
[5] W.L. DeLano, M.H. Ultsch, J.A. Wells, Convergent solutions to binding at a protein-protein interface, Science 287 (2000) 1279–1283.
[6] A. Forsgren, J. Sjöquist, "Protein A" from S. aureus I. Pseudo-immune reaction with human γ-globulin, J. Immunol. 97 (1966) 822–827.
[7] H. Hjelm, K. Hjelm, J. Sjöquist, Protein A from Staphylococcus aureus. Its isolation by affinity chromatography and its use as an immunosorbent for isolation of immunoglobulins, FEBS Lett. 28 (1972) 73–76.
[8] I. Johansson, IGG Separation Medium, Google Patents, 2002.
[9] H.Y. Aboul-Enein, G. Rigi, M. Farhadpour, A. Ghassempour, G. Ahmadian, Per Aqueous Liquid Chromatography (PALC) as a Simple Method for Native Separation of Protein A, Chromatographia, 2017, pp. 1–7.
[10] J. Sjödahl, Repetitive sequences in protein a from Staphyloccus aureus: three highly homologous Fc-binding regions, FEBS Lett. 67 (1976) 62–67.

[11] S. Aslıyüce, B. Mattiasson, A. Denizli, COMBINED PROTEIN A IMPRINTING AND CRYOGELATION FOR PRODUCTION OF SPERICAL AFFINITY MATERIAL, Biomedical Chromatography, 2019, e4605.
[12] S. Hober, K. Nord, M. Linhult, Protein A chromatography for antibody purification, J. Chromatogr. B 848 (2007) 40–47.
[13] M.N. Jaafar, J. Lowe, N. Ling, R. Jefferis, Immunogenic and antigenic epitopes of immunoglobulins—VII. The topographical distribution of Fcγ epitopes and the relationship of an iso-allotypic specificity to the presence of histidine 435, Mol. Immunol. 21 (1984) 137–143.
[14] D.R. Burton, Immunoglobulin G: functional sites, Mol. Immunol. 22 (1985) 161–206.
[15] J. Sjödahl, Repetitive sequences in protein A from Staphylococcus aureus: arrangement of five regions within the protein, four being highly homologous and fc-binding, Eur. J. Biochem. 73 (1977) 343–351.
[16] S. Khodaei, S. Ghaedmohammadi, M. Mohammadi, G. Rigi, P. Ghahremanifard, R. Zadmard, G. Ahmadian, Covalent immobilization of protein A on chitosan and aldehyde double-branched chitosan as biocompatible carriers for immunoglobulin G (igg) purification, J. Chromatogr. Sci. 56 (2018) 933–940.
[17] R. Lindmark, Estimation of the secondary structure of protein A from S. aureus by CD-spectroscopy, Mol. Immunol. 19 (1982) 957–959.
[18] A.L. Robertson, J. Horne, A.M. Ellisdon, B. Thomas, M.J. Scanlon, S.P. Bottomley, The structural impact of a polyglutamine tract is location-dependent, Biophys. J. 95 (2008) 5922–5930.
[19] M. Graille, E.A. Stura, A.L. Corper, B.J. Sutton, M.J. Taussig, J.-B. Charbonnier, G. J. Silverman, Crystal structure of a Staphylococcus aureus protein A domain complexed with the Fab fragment of a human IgM antibody: structural basis for recognition of B-cell receptors and superantigen activity, Proc. Natl. Acad. Sci. Unit. States Am. 97 (2000) 5399–5404.
[20] J. Deisenhofer, T.A. Jones, R. Huber, J. Sjödahl, J. Sjöquist, Crystallization, crystal structure analysis and atomic model of the complex formed by a human Fc fragment and fragment B of protein A from Staphylococcus aureus, Hoppe-Seyleŕ s Zeitschrift für physiologische Chemie 359 (1978) 975–986.
[21] J. Deisenhofer, Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9-and 2.8-, ANG. resolution, Biochemistry 20 (1981) 2361–2370.
[22] R. Li, V. Dowd, D.J. Stewart, S.J. Burton, C.R. Lowe, Design, synthesis, and application of a protein A mimetic, Nat. Biotechnol. 16 (1998) 190–195.
[23] A.E. Sauer-Eriksson, G.J. Kleywegt, M. Uhlén, T.A. Jones, Crystal structure of the C2 fragment of streptococcal protein G in complex with the Fc domain of human IgG, Structure 3 (1995) 265–278.
[24] H. Torigoe, I. Shimada, M. Waelchli, A. Saito, M. Sato, Y. Arata, 15N nuclear magnetic resonance studies of the B domain of staphylococcal protein A: sequence specific assignments of the imide 15N resonances of the proline residues and the interaction with human immunoglobulin G, FEBS Lett. 269 (1990) 174–176.
[25] H. Gouda, H. Torigoe, A. Saito, M. Sato, Y. Arata, I. Shimada, Three-dimensional solution structure of the B domain of staphylococcal protein A: comparisons of the solution and crystal structures, Biochemistry 31 (1992) 9665–9672.
[26] I.S. Shah, S. Lovell, N. Mehzabeen, K.P. Battaile, T.J. Tolbert, Structural characterization of the Man5 glycoform of human IgG3 Fc, Mol. Immunol. 92 (2017) 28–37.
[27] S. Yoshida, D. Murata, S. Taira, M. Takano, K. Iguchi, Y. Nakano, Protein capable of binding specifically to immunoglobulin, and immunoglobulin-binding affinity ligand, in: Google Patents, 2011.
[28] M. Kangwa, V. Yelemane, A. Ponnurangam, M. Fernandez-Lahore, An engineered Staphylococcal Protein A based ligand: production, characterization and potential application for the capture of Immunoglobulin and Fc-fusion proteins, Protein Expr. Purif. 155 (2019) 27–34.
[29] G. Hale, A. Drumm, P. Harrison, J. Phillips, Repeated cleaning of protein A affinity column with sodium hydroxide, J. Immunol. Methods 171 (1994) 15–21.
[30] J.A. Capp, A. Hagarman, D.C. Richardson, T.G. Oas, The statistical conformation of a highly flexible protein: small-angle X-ray scattering of S. aureus protein A, Structure 22 (2014) 1184–1195.
[31] E. Majima, A. Shima, Y. Hara, Immunoglobulin affinity ligand, in: Google Patents, 2014.
[32] B. Nilsson, T. Moks, B. Jansson, L. Abrahmsén, A. Elmblad, E. Holmgren, C. Henrichson, T.A. Jones, M. Uhlén, A synthetic IgG-binding domain based on staphylococcal protein A, Protein Engineering, Design and Selection 1 (1987) 107–113.
[33] L. Jendeberg, M. Tashiro, R. Tejero, B.A. Lyons, M. Uhlén, G.T. Montelione, B. Nilsson, The mechanism of binding staphylococcal protein A to immunoglobin G does not involve helix unwinding, Biochemistry 35 (1996) 22–31.
[34] M. Tashiro, R. Tejero, D.E. Zimmerman, B. Celda, B. Nilsson, G.T. Montelione, High-resolution solution NMR structure of the Z domain of staphylococcal protein A, J. Mol. Biol. 272 (1997) 573–590.
[35] M. Linhult, S. Gülich, T. Gräslund, A. Simon, M. Karlsson, A. Sjöberg, K. Nord, S. Hober, Improving the tolerance of a protein a analogue to repeated alkaline exposures using a bypass mutagenesis approach, Proteins: Structure, Function, and Bioinformatics 55 (2004) 407–416.
[36] K. Minakuchi, D. Murata, Y. Okubo, Y. Nakano, S. Yoshida, Remarkable alkaline stability of an engineered protein A as immunoglobulin affinity ligand: C domain having only one amino acid substitution, Protein Sci. 22 (2013) 1230–1238.
[37] R.-M. Bao, H.-M. Yang, C.-M. Yu, J.-B. Tang, Oriented covalent immobilization of engineered ZZ-cys onto maleimide-sepharose: an affinity platform for IgG purification, Chromatographia 79 (2016) 1271–1276.
[38] J. Nilsson, P. Nilsson, Y. Williams, L. Pettersson, M. Uhlen, P.Å. Nygren, Competitive elution of protein A fusion proteins allows specific recovery under mild conditions, FEBS J. 224 (1994) 103–108.
[39] R. Bywater, G.-B. Eriksson, T. Ottosson, Desorption of immunoglobulins from Protein A-Sepharose CL-4B under mild conditions, J. Immunol. Methods 64 (1983) 1–6.
[40] A.R. Mazzer, X. Perraud, J. Halley, J. O'Hara, D.G. Bracewell, Protein A chromatography increases monoclonal antibody aggregation rate during subsequent low pH virus inactivation hold, J. Chromatogr. A 1415 (2015) 83–90.
[41] S. Gülich, M. Uhlén, S. Hober, Protein engineering of an IgG-binding domain allows milder elution conditions during affinity chromatography, J. Biotechnol. 76 (2000) 233–243.
[42] T.M. Pabst, R. Palmgren, A. Forss, J. Vasic, M. Fonseca, C. Thompson, W.K. Wang, X. Wang, A.K. Hunter, Engineering of novel Staphylococcal protein A ligands to enable milder elution pH and high dynamic binding capacity, J. Chromatogr. A 1362 (2014) 180–185.
[43] A. Zwolak, C.N. Leettola, S.H. Tam, D.R. Goulet, M.G. Derebe, J.R. Pardinas, S. Zheng, R. Decker, E. Emmell, M.L. Chiu, Rapid purification of human bispecific antibodies via selective modulation of protein A binding, Sci. Rep. 7 (2017) 15521.
[44] S. Sato, Polypeptide, an affinity chromatography material, and a method for separating and/or purifying immunoglobulin, in: Google Patents, 2012.
[45] I. Koguma, S. Yamashita, S. Sato, K. Okuyama, Y. Katakura, Novel purification method of human immunoglobulin by using a thermo-responsive protein A, J. Chromatogr. A 1305 (2013) 149–153.
[46] W. Krepper, P. Satzer, B.M. Beyer, A. Jungbauer, Temperature dependence of antibody adsorption in protein A affinity chromatography, J. Chromatogr. A 1551 (2018) 59–68.
[47] J. González-Valdez, A. Yoshikawa, J. Weinberg, J. Benavides, M. Rito-Palomares, T.M. Przybycien, Toward improving selectivity in affinity chromatography with PEGylated affinity ligands: the performance of PEGylated protein A, Biotechnol. Prog. 30 (2014) 1364–1379.
[48] J. Dong, T. Kojima, H. Ohashi, H. Ueda, Optimal fusion of antibody binding domains resulted in higher affinity and wider specificity, J. Biosci. Bioeng. 120 (2015) 504–509.
[49] A.M. Scott, J.D. Wolchok, L.J. Old, Antibody therapy of cancer, Nat. Rev. Canc. 12 (2012) 278–287.
[50] J. Orange, Clinical Update in Immunoglobulin Therapy for Primary Immunodeficiency Diseases, Immune Deficiency Foundation, 2011.
[51] K.-P.S. Dancil, D.P. Greiner, M.J. Sailor, A porous silicon optical biosensor: detection of reversible binding of IgG to a protein A-modified surface, J. Am. Chem. Soc. 121 (1999) 7925–7930.
[52] T. Ikeda, Y. Hata, K.-i. Ninomiya, Y. Ikura, K. Takeguchi, S. Aoyagi, R. Hirota, A. Kuroda, Oriented immobilization of antibodies on a silicon wafer using Si-tagged protein A, Anal. Biochem. 385 (2009) 132–137.
[53] Y. Niu, A.I. Matos, L.M. Abrantes, A.S. Viana, G. Jin, Antibody oriented immobilization on gold using the reaction between carbon disulfide and amine groups and its application in immunosensing, Langmuir 28 (2012) 17718–17725.
[54] S.V. Sonti, A. Bose, Cell separation using protein-A-coated magnetic nanoclusters, J. Colloid Interface Sci. 170 (1995) 575–585.
[55] S. Mazzucchelli, M. Colombo, C. De Palma, A. Salvade, P. Verderio, M.D. Coghi, E. Clementi, P. Tortora, F. Corsi, D. Prosperi, Single-domain protein A-engineered magnetic nanoparticles: toward a universal strategy to site-specific labeling of antibodies for targeted detection of tumor cells, ACS Nano 4 (2010) 5693–5702.
[56] K.J. Widder, A.E. Senyei, H. Ovadia, P.Y. Paterson, Magnetic protein A microspheres: a rapid method for cell separation, Clin. Immunol. Immunopathol. 14 (1979) 395–400.
[57] T.Q. Huy, P. Van Chung, N.T. Thuy, C. Blanco-Andujar, N.T.K. Thanh, Protein A-conjugated iron oxide nanoparticles for separation of Vibrio cholerae from water samples, Faraday Discuss 175 (2015) 73–82.
[58] G. Matic, T. Bosch, W. Ramlow, Background and indications for protein A-based extracorporeal immunoadsorption, Ther. Apher. Dial. 5 (2001) 394–403.
[59] H.C. Lu, H.M. Chen, Y.S. Lin, J.W. Lin, A reusable and specific protein A-coated piezoelectric biosensor for flow injection immunoassay, Biotechnol. Prog. 16 (2000) 116–124.
[60] G. Shen, C. Cai, K. Wang, J. Lu, Improvement of antibody immobilization using hyperbranched polymer and protein A, Anal. Biochem. 409 (2011) 22–27.
[61] S.L. Baltierra-Uribe, J.J. Chanona-Pérez, J.V. Méndez-Méndez, M.d.J. Perea-Flores, A.C. Sánchez-Chávez, B.E. García-Pérez, M.C. Moreno-Lafont, R. López-Santiago, Detection of Brucella abortus by a platform functionalized with protein A and specific antibodies IgG, Microsc. Res. Tech. 82 (2019) 586–595.
[62] O.B. Gorbatiuk, A.O. Bakhmachuk, L.V. Dubey, M.O. Usenko, D.M. Irodov, O. V. Okunev, O.M. Kostenko, A.E. Rachkov, V.A. Kordium, Recombinant Staphylococcal protein A with cysteine residue for preparation of affinity chromatography stationary phase and immunosensor applications, Biopolym. Cell 31 (2015) 115–122.
[63] X. Zhang, Y. Duan, N. Han, Y. Wu, Increase in IgG-binding capacity of recombinant protein a immobilized on heterofunctional amino and epoxy agarose, in: IOP Conference Series: Materials Science and Engineering, IOP Publishing, 2018, 012042.
[64] K. Terpe, Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems, Appl. Microbiol. Biotechnol. 60 (2003) 523–533.
[65] Y. Cao, Q. Zhang, C. Wang, Y. Zhu, G. Bai, Preparation of novel immunomagnetic cellulose microspheres via cellulose binding domain-protein A linkage and its use for the isolation of interferon α-2b, J. Chromatogr. A 1149 (2007) 228–235.

[66] P. Füglistaller, Comparison of immunoglobulin binding capacities and ligand leakage using eight different protein A affinity chromatography matrices, J. Immunol. Methods 124 (1989) 171–177.
[67] R. Hahn, R. Schlegel, A. Jungbauer, Comparison of protein A affinity sorbents, J. Chromatogr. B 790 (2003) 35–51.
[68] G.R. Bolton, K.K. Mehta, The role of more than 40 years of improvement in protein A chromatography in the growth of the therapeutic antibody industry, Biotechnol. Prog. 32 (2016) 1193–1202.
[69] S. Margel, Agarose polyacrolein microsphere beads: new effective immumoabsorbents, FEBS (Fed. Eur. Biochem. Soc.) Lett. 145 (1982) 341–344.
[70] I. Nathan, M. Aharon, G. Reisenfeld, A. Dvilansky, A novel agarose acrobeads protein A column for selective immunoadsorbance of whole blood: performance, specificity and safety, Biomaterials, Artificial Cells and Immobilization Biotechnology 20 (1992) 23–30.
[71] J. Ren, K. Tian, L. Jia, X. Han, M. Zhao, Rapid covalent immobilization of proteins by phenol-based photochemical cross-linking, Bioconjugate Chem. 27 (2016) 2266–2270.
[72] X. Zhang, Y. Duan, X. Zeng, Improved performance of recombinant protein A immobilized on agarose beads by site-specific conjugation, ACS Omega 2 (2017) 1731–1737.
[73] L. Zhao, K. Zhu, Y. Huang, Q. Li, X. Li, R. Zhang, Z. Su, Q. Wang, G. Ma, Enhanced binding by dextran-grafting to Protein A affinity chromatographic media, J. Separ. Sci. 40 (2017) 1493–1499.
[74] A. Denizli, A.Y. Rad, E. Pişkin, Protein A immobilized polyhydroxyethylmethacrylate beads for affinity sorption of human immunoglobulin G, J. Chromatogr. B Biomed. Sci. Appl. 668 (1995) 13–19.
[75] H. Ayhan, V. Bulmus, E. Piskin, Protein A immobilized poly (methylmethacrylate-co-hydroxyethylmethacrylate) microbeads for IgG adsorption, J. Bioact. Compat Polym. 14 (1999) 490–503.
[76] A. Denizli, Y. Arica, Protein A-immobilized microporous polyhydroxyethylmethacrylate affinity membranes for selective sorption of human-immunoglobulin-G from human plasma, J. Biomater. Sci. Polym. Ed. 11 (2000) 367–382.
[77] P.L. Coleman, M.M. Walker, D.S. Milbrath, D.M. Stauffer, J.K. Rasmussen, L. R. Krepski, S.M. Heilmann, Immobilization of protein A at high density on azlactone-functional polymeric beads and their use in affinity chromatography, J. Chromatogr. A 512 (1990) 345–363.
[78] Z. Pan, H. Zou, W. Mo, X. Huang, R. Wu, Protein A immobilized monolithic capillary column for affinity chromatography, Anal. Chim. Acta 466 (2002) 141–150.
[79] S. Katoh, M. Imada, N. Takeda, T. Katsuda, H. Miyahara, M. Inoue, S. Nakamura, Optimization of silica-based media for antibody purification by protein A affinity chromatography, J. Chromatogr. A 1161 (2007) 36–40.
[80] F. Zhou, D. Muller, J. Jozenfonvicz, Double-coated silica supports for high-performance affinity chromatography of proteins, J. Chromatogr. A 510 (1990) 71–81.
[81] K. Maeno, A. Hirayama, K. Sakuma, K. Miyazawa, An activated medium with high durability and low nonspecific adsorption: application to protein A chromatography, Anal. Biochem. 409 (2011) 123–129.
[82] K. Nakanishi, M. Tomita, H. Nakamura, K. Kato, Specific binding of immunoglobulin G to protein A–mesoporous silica composites for affinity column chromatography, J. Mater. Chem. B 1 (2013) 6321–6328.
[83] X. Hou, C. Zhao, Y. Tian, S. Dou, X. Zhang, J. Zhao, Preparation of functionalized Fe3O4@ SiO2 magnetic nanoparticles for monoclonal antibody purification, Chem. Res. Chin. Univ. 32 (2016) 889–894.
[84] S. Kim, D. Sung, J.H. Chang, Highly efficient antibody purification with controlled orientation of protein A on magnetic nanoparticles, MedChemComm 9 (2018) 108–112.
[85] T. Iype, J. Thomas, S. Mohan, K.K. Johnson, L.E. George, L.A. Ambattu, A. Bhati, K. Ailsworth, B. Menon, S.M. Rayabandla, A novel method for immobilization of proteins via entrapment of magnetic nanoparticles through epoxy cross-linking, Anal. Biochem. 519 (2017) 42–50.
[86] K. Salimi, D.D. Usta, İ. Koçer, E. Çelik, A. Tuncel, Protein A and protein A/G coupled magnetic SiO2 microspheres for affinity purification of immunoglobulin G, Int. J. Biol. Macromol. 111 (2018) 178–185.
[87] Z. Wang, Y. Shen, Q.-H. Shi, Y. Sun, Insights into the molecular structure of immobilized protein A ligands on dextran-coated nanoparticles: comprehensive spectroscopic investigation, Biochem. Eng. J. 146 (2019) 20–30.