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Cogent Biology (2019), 5: 1665406
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Abstract: Recombinant proteins have wide applications in the development of pharmaceutical compounds, industrial applications of enzymes, and basic proteomics research. In this way, efficient production of recombinant proteins with high purity needs efficient purification methods. Various strategies have been devised to improve these proteins purification such as affinity purification and physicochemical purification methods which the affinity purification has some advantages over the others. Affinity strategies especially fusion strategies have been devised as indispensable tools for the massive parallel production, identification and purification of recombinant proteins from the host systems. These strategies facilitate commercial and industrial formulations of recombinant proteins, improve the study of protein interactions at the molecular level, and develop highly sensitive and specific bioassays. Recently, various surface-modified nanoparticles have been widely developed to enhance recovery and purification of recombinant proteins such as Hydrophobic polymer nanoparticles and Oleosin nanoparticles. In this review, we aim to discuss affinity purification technologies and address the principles, advantages, limitations and potential applications of them.

ABOUT THE AUTHOR

Sahar Mahmoodi has worked on the development of visual strip test for point of care detection of recombinant proteins.

Mohammad Pourhassan-Moghaddam is currently focusing on the preparation of bio and nanomaterials, including recombinant proteins, for application in the fields of cellular and molecular diagnostics.

David W. Wood is known for groundbreaking research in self-cleaving affinity tag technology for the purification of recombinant proteins. Current applications include new ways to purify recombinant proteins, bacterial biosensors that incorporate human drug targets, and new capabilities in drug discovery and drug delivery.

Hasan Majdi has worked on the development of SPR-based biosensors for detection of different proteins, including recombinant proteins.

Nosratollah Zarghami’s group is working on targeting cancer cells using various materials. Recently, his group is focused on the development of recombinant coagulases for targeting tumour vascularisation.

PUBLIC INTEREST STATEMENT

Purification is an essential part of developing protein-based products. The detailed knowledge of protein purification methods is required for researchers of molecular biology and biochemistry field to well tune the most suitable strategy for purification of their protein of interest from protein factories. Therefore, in this scientific article, we have reviewed the most popular methods for purification of recombinant proteins.
1. Introduction

The heterologous expression of proteins is a necessary step for the study of biological functions of genes, development of pharmaceutical compounds, industrial applications of enzymes, and basic proteomics research. The main advantage of using recombinant proteins is that a wealth of information is often already available about the product and major impurities, thereby simplifying detection assays, sample preparation methods, and purification strategies. This information allows the rapid development of strategies for both industrial and research-scale production of specific recombinant protein targets. Advances in protein expression systems have allowed for the production of nearly any desirable protein and peptide, often with high yields. Nevertheless, these products must be purified to permit their study and application (Banki, Gerngross, & Wood, 2005; Healthcare, 2007).

The variety of different purification techniques has increased due to the different qualities and quantities of recombinant proteins required for research and industrial purposes. On the other hand, yields of recombinant proteins are also affected by the expression conditions used. Taken together, the economical production of recombinant proteins requires implementation of efficient purification methods as well as high-yielding expression hosts (Healthcare, 2007). To address this need, various strategies have been devised to improve the recovery rates of the desired recombinant proteins, including physicochemical purification methods and affinity purification methods (Kimple, Brill, & Pasker, 2013; Kosobokova, Skrypnik, & Kosorukov, 2016; Wingfield, 2015).

In an ideal purification strategy, there should be a balance among a number of parameters that affect the final result. These include the speed of purification, the recovery rate, the capacity and the resolution. Resolution refers to the ability of the technique to produce fully separated (baseline resolved) peaks. Nevertheless, impurities with similar properties to the target protein hamper the achievement of the desirable resolution in each step of the purification process. The quantity of target protein that can be loaded on a single unit during the purification process is termed capacity and can have a major impact on the overall economics of the process. Recovery reflects the fraction of the expressed recombinant protein that is ultimately purified from the contaminants of the host organism. In general, the physicochemical properties of the sample and the required purification level are the most influential factors that determine the final purification strategy (Healthcare, 2007).

For these reasons, the development of simple, reliable, scalable and rapid methods for recombinant protein purification remains a critical goal for new bioseparation technologies. In particular, platform methods that can be easily applied to new protein targets with minimal optimization are attractive. These general methods can accelerate research and development of new products and shorten the time required for commercial delivery.

In recent years, various fusion tags have been developed to improve recombinant protein detection or purification, solubility enhancement (Loughran & Walls, 2017) and tag removal (Arnau, Lauritzen, Petersen, & Pedersen, 2006; Young, Britton, & Robinson, 2012). It is important to mention that purification process is facilitated by fusing proteins to various types of tags; these tags are used to increase expression and purification yield. Also, they are applied to enhance stability and solubility of protein of interest, and also to decrease the toxicity of recombinant protein on host cell (Arnau et al., 2006; Young et al., 2012).

With regards to the importance of these tags in the recombinant protein production, we aimed to address recent advances in this field and discuss the basics and characteristics of different recently developed affinity strategies.
2. **Affinity purification**

An important platform method, which can be applied reliably to different families of proteins, relies on natural biological affinities between the target proteins and their known ligands. This has allowed for the development of different affinity matrices that selectively and effectively bind to a specific site exposed only on the target protein. Examples include a designed concanavalin A matrix, which can reversibly bind to the carbohydrate moiety of glycoproteins; or immobilized antibodies that identify specific areas of a target protein; or the use of immobilized enzyme substrate or cofactor for binding to target enzymatic proteins or nickel-nitrilotriacetic acid matrices (Ni\(^{2+}\)-NTA) and cobalt-carboxymethylaspartate matrices (Co\(^{2+}\)-CMA) in which the metal ions interact with histidine residues in the affinity tag (Asliyuce, Mattiasson, & Mamo, 2016; Khoury et al., 2015). In many cases, target proteins may have no known affinity partners or the construction of a suitable affinity matrix may be too expensive for the required application. In these cases, recombinant DNA technology can be used to introduce specific modifications to the target proteins to facilitate their recovery, purification and detection. When using this technology, a specific tag sequence or peptide can be joined to the protein at the DNA level, leading to the expression of a tagged fusion protein (Figure 1). The fusion tag is designed to enable a simple, economic and effective purification. There are several affinity tags or proteins such as histidine tag (His-tag), Arg-tag, glutathione S-transferase (GST) tag, maltose-binding protein (MBP) tag, Strep-tag II, FLAG tag, calmodulin-binding peptide, the cellulose-binding domain and Small ubiquitin-related modifier (SUMO) (Guerrero, Ciragan, & Iwai, 2015; Yadav, Yadav, Yadav, Haque, & Tuteja, 2016). Among all the tags, the histidine tags are the most commonly used, because they are small, less disruptive than other tags, non-immunogenic and the polyHis-tagged proteins can be purified up to 95% with a recovery rate of 90% in a single purification step under both native and denaturing conditions (Manjasetty, Turnbull, Panjikar, Büsow, & Chance, 2008). Some tags such as GST and MBP or N-utilization substance A (NusA) have dual functions and can be used for both affinity purification and also increased solubility of the recombinant proteins (Walls & Loughran, 2011). It is important that the fused tag does not affect the folding or biological function of the tagged protein, and in many cases, it should be removable after the purification.

The Small ubiquitin-like modifier (SUMO) family proteins with covalent binding to lysine side chains of the target proteins have some roles in posttranslational modifications in eukaryotic cells, which is important for nuclear transporation, signal transduction, and protein stabilization. Actually, they with such covalent binding help to correct folding and solubility of target proteins in the cells. So, this aspect of SUMO proteins made them to be attractive candidates for the induction of solubility and correct folding of recombinant proteins through fusion at N-terminal of target proteins at the DNA level. In addition, SUMO conformation more particularly the conserved Gly-Gly motifs are been recognized by a SUMO protease, S. cerevisiae Ulp1, which is an important feature for specific purification of recombinant proteins. Although this purification method is more effective tool in prokaryotic hosts, eukaryote hosts have a disadvantage with this method that they naturally have SUMO protease for cleavage of SUMO-tag (Guerrero et al., 2015; Kimple et al., 2013; Malakhov et al., 2004; Marblestone et al., 2006;
Panavas, Sanders, & Butt, 2009). Since the purification method is based on the tag, rather than the target, the same fusion peptide can be used to purify various types of recombinant proteins based on the same purification methodology (Young et al., 2012). Currently, fusion tags can be removed via chemical methods (such as cyanogen bromide or hydroxylamine) or enzymatic methods. Chemical methods are somewhat unspecific and may cause protein denaturation and side-chain modification of amino acids in desirable proteins. However, enzymatic methods are more specific and usually cleave under mild conditions. Several endoproteases have been used for the tag removal such as enterokinase, factor Xa, SUMO protease, tobacco etch virus (TEV) protease, thrombin, 3C, Granzyme B and Caspase-6. Among them, enterokinase, thrombin and factor Xa are the most commonly applied enzymes for the tag removal purpose without requiring a specific amino acid or sequence at the cleavage site. Other enzymatic strategies are the using of exopeptidases. Exopeptidases consist of aminopeptidases (like aminopeptidase M, Aeromonas aminopeptidase) and carboxypeptidases (like carboxypeptidase A and B) (Arnaud et al., 2006; Yadav et al., 2016).

2.1. Affinity chromatography
Affinity chromatography (AC) is one of the most efficient systems available for the one-step purification of recombinant proteins with high selectivity and usually high capacity (Arnaud et al., 2006; Hage et al., 2017; Khoury et al., 2015; Young et al., 2012). Affinity tags are exogenous amino acid (aa) residues that selectivity bind to a biological or chemical ligand placed on a solid support (e.g., his-tags connected to immobilized Ni) or an immobilized protein partner (e.g., the use of protein A affinity chromatography for purification of monoclonal antibodies) (Arnaud et al., 2006; Healthcare, 2007). Separation by the AC method occurs by the reversible binding of the protein with a ligand specific for the protein (Figure 2). Regarding the nature of purification, the chromatography based on metal-affinity tag interactions is called immobilized metal ion affinity chromatography (IMAC) (Healthcare, 2007). For instance, histidine-tagged proteins can selectively be purified by means of the interaction between polyhistidine tag with several cations such as Ni²⁺, Cu²⁺, Zn²⁺ and Co²⁺ (Kimple et al., 2013). This method was introduced by Porath et al. in 1975 (Porath, Carlsson, Olsson, & Belfrage, 1975). IMAC is considered as a critical tool for the purification of fusion proteins with synthetic or natural tags in a single-step procedure (CDaga, Bochkariov, Jokhadze, Hopp, & Nelson, 1995; Porath et al., 1975). An example purification involves a natural poly-histidine affinity tag of 19 amino acids (from lactate dehydrogenase) fused to the N-terminal of green fluorescent protein-UV-enhanced variant, dihydrofolate reductase and chloramphenicol acetyl transferase. The results showed a very efficient purification of all three proteins using cobalt (II) ions immobilized on carboxymethyl aspartate cross-linked agarose under near-physiological conditions. The purification was carried out in a single chromatographic step in less than 1 h, and the recovery rate for each separated protein was higher than 77% (CDaga et al., 1999; Lin, Zhao, Xing, Zhou, & Wang, 2015; Yang, Pistolozzi, & Lin, 2018).

In another study of a different amino acid tag, namely polyarginine, the tag was fused to the C-terminal of human urogastrone to help purification of the target. This peptide generates a positively charged “tail”, leading to powerful binding to the sulfated cation exchanger SP Sephadex (ion-exchange matrix containing negative charge) at pH 5.5. Below pH 12, arginine has a positive charge and therefore links to any ion exchanger containing negative charge. On the other hand, most native bacterial host proteins have a negative charge above pH 5, and thus the bacterial proteins will not bind to the negatively charged ion exchanger. This charge difference allows for the selective purification of the polyarginine-tagged protein (Brewer & Sassenfeld, 1985; Pina, Batalha, & Roque, 2014). Further, the tag can be removed by carboxypeptidase B, an exopeptidase which specifically and sequentially digests the amino acids arginine and lysine at the C-terminal. In this study, after polyarginine removal by carboxypeptidase B, an additional level of purification was achieved by means of repeating the SP-Sephadex column chromatography. Urogastrone purified by this strategy was very pure (>95%). This technique is also scalable due to the simplicity and low cost of ion-exchange chromatography (Akparov, Sokolenko, Timofeev, & Kuranova, 2015; Fang, Lin, & Yao, 2018; Porodko et al., 2018). In additional studies, beta-galactosidase (Ullmann, 1984), chloramphenicol acetyltransferase (Ullmann, 1984) and protein A (Lofdahl, Uhlen, Lindberg, & Sjoquist, 1992; Nilsson et al., 1985) have been also...
Figure 2. Scheme of steps involved in the purification of recombinant proteins by affinity chromatography. At first, a specific tag is fused to target proteins at DNA level in order to express tagged fusion protein, thus protein mixture is collected and added to column containing specific ligands for protein of interest. Unwanted molecules are washed and finally a protease is added to separate the target protein from the affinity tag.
purified using N-terminal protein fusions through affinity chromatography (Sassenfeld & Brewer, 1984). The purification columns and affinity media are commercially available for the purification of recombinant proteins. Two types of chromatography media are available for the purification of tagged proteins such as pre-charged media by different ions and uncharged media. In order to adjust the selectivity, different metal ions can be used to charge the uncharged media. The main parameter in the selection of right column and affinity media is the physicochemical properties of the sample. In order to show the potential of IMAC in recombinant protein purification, a study has been conducted to compare the purification of recombinant untagged and N-terminal Histagged Bacillus amyloliquefaciens pyroglutamyl aminopeptidase (pGAP) (Arnau et al., 2006). The purification procedure of untagged pGAP purification consisted of precipitation by ammonium sulfate, two sequential separation steps using phenyl-Sepharose and a final step using Q Sepharose HP, resulting in the contamination of the final protein with host proteins despite several attempts to eliminate these contaminants. Also, this process exhibited only 40% recovery rate with important protein loss at steps one and three, respectively. Meanwhile, the amount of Histagged-pGAP was increased in the cell extract, maybe due to the higher expression level of this protein and remarkably, a very pure protein with an un-optimized yield of 96% was achieved with a single IMAC step. Furthermore, affinity tags, especially his-tags, for high output procedures may represent a general platform for the purification of proteins where biochemical properties are not available (Arnau et al., 2006; Dando, Fortunato, Strand, Smith, & Barrett, 2003). In standard chromatographic methods, numerous subsequent chromatographic phases are performed to obtain a relatively pure protein. Therefore, these methods are time-consuming approaches with a low yield of recovery, whereas affinity chromatography leads to high yields (over 90%), reduction in the number of steps, costs and impurities (Arnau et al., 2006; Jain, 2006; Korf et al., 2005). Additionally, this approach is chemically resistant to the long-time cleaning processes commonly utilized in pharmaceutical production (Healthcare, 2007; Young et al., 2012).

2.2. Tandem affinity purification (TAP)

The use of two different affinity tags (dual affinity tags) for purification and separation is termed tandem affinity purification and results in high and effective purification of protein (Figure 3) (Cass, Pham, Kamen, & Durocher, 2005; Gingras, Aebersold, & Rauhut, 2005; Gong-Hong, De-Pei, & Liang, 2004; Gräslund et al., 2002; Prinz et al., 2004; Rubio et al., 2005; Waugh, 2005). In one study, the combination of protein A and calmodulin-binding peptide was used for purification using this strategy. The TAP-tagged proteins were fixed on IgG Sepharose by the protein A and then they are incubated with TEV protease in order to separate the protein complex. After that, the TAP-tagged protein was immobilized on calmodulin-Sepharose through its calmodulin-binding peptide in the presence of calcium. Then, calcium chelation results in the release of the TAP-tagged protein complex (Gingras et al., 2005; Gong-Hong et al., 2004; Rubio et al., 2005). Therefore, the target protein was purified by two affinity chromatography. The combination of dual tags with fluorescent proteins can be used to monitor the expression procedure. The use of each of tags for one-step purification has drawbacks in terms of yield, purity and recovery rate. In another study, His8 and Strep-tag-II were located at the C-terminal of Discosoma sp. red fluorescent protein, vascular endothelial growth factor and secreted alkaline phosphatase. These proteins were then purified through successive IMAC and StrepTactin affinity that resulted in rapid and efficient purification, up to >99% homogeneity, with the yields ranging from 29% to 81%. Primary purification through IMAC eliminates most biotinylated proteins and biotin while pre-concentrating the recombinant protein that leads to more effective binding to the StrepTactin column. In addition, resizable IMAC columns allow for the maximal recovery rate of recombinant proteins. As another contributing factor, StrepTactin chromatography is performed in a mild buffer and leads to the purification of the recombinant protein with high homogeneity as well as eliminates buffer exchange process, thus simplifying the purification process (Cass et al., 2005).

2.3. Affinity precipitation

Affinity precipitation combines the selectivity of an affinity ligand with the advantages of conventional precipitation such as the relatively simple equipment requirements and the potential for scale-up. The affinity precipitation has been used as a potential technology for the separation and purification of
proteins from large volume of diluted solution materials via reversible soluble-insoluble polymers (such as temperature-, pH- and light-response polymers) linked to an affinity ligand (Hilbrig & Freitag, 2003). For example, Zhou et al. synthesized a thermo-response polymer (with hydrophobic butyl groups as the ligand) for purification of lipase (Zhou, Wan, & Cao, 2010), and also Chen and Hoffman designed a copolymer of N-isopropyl acrylamide and N-acryloxysuccinimide to immobilize Protein A on this copolymer to purify IgG via affinity-precipitation strategy (Chen & Huffman, 1990). In addition, Ling and Zhu applied a thermo-sensitive copolymer comprising N-vinyl-2-caprolactam (NVCL) and methacrylic acid, with copper as the ligand for purification of BSA (Ling, Nie, Brandford-White, Williams, & Zhu, 2012). In another group, N-methylol acrylamide (N-MAM), N-isopropyl acrylamide (NIPA) and butyl acrylate (BA) was applied as monomers for designing of a thermo-sensitive polymer to immobilize L-thyroxin (an affinity ligand) on the polymer for affinity precipitation of human serum albumin (HSA) which their results indicate that the affinity precipitation was a practical and feasible method for the purification of HSA with high purity in a single step (Ding & Cao, 2013). Elastin-Like polypeptides (ELPs), the synthetic thermally responsive biopolymers, contain repetitive sequences of Valine-Proline-Glycine-Xaa-Glycine (VPGXG) where X can be any amino acid except for Proline.
Elastin-Like polypeptides (ELPs), the synthetic thermally responsive biopolymers, contain repetitive sequences of Valine-Proline-Glycine-Other-Amino Acid-Glycine (VPGXG) where X can be any amino acid except for Proline and below their critical temperature, they are completely soluble in aqueous solutions, but, they gradually aggregate above this temperature, leading to the formation of insoluble proteins in aqueous solutions (Banki, Feng, & Wood, 2005; Ge et al., 2005; Meyer, Trabbic-Carlson, & Chilkoti, 2001; Trabbic-Carlson, Liu, Kim, & Chilkoti, 2004). Inverse transition cycling (ITC) technique exploits the thermally responsive property of ELP for the purification of recombinant proteins (Meyer & Chilkoti, 1999) (Figure 4). Generally, the environmental sensitivity and reversible solubility occurs for recombinant proteins when an ELP is fused to them and thus in ITC, a recombinant ELP fusion protein can be separated from other proteins to a high purity during repetitive steps of aggregation, centrifugation, and re-solubilization of the fusion protein (Coolbaugh, Tang, & Wood, 2017; Meyer et al., 2001). This technique is simple, rapid, scalable, and requires no specialized tools or substances. It is suitable for high-throughput protein expression and purification and significantly improves solubility and preserves functional activity of the protein. Although it is an inexpensive non-chromatographic method for protein purification, this technology is not applicable to all types of proteins (Conley, Mohib, Jevnikar, & Brandle, 2009; Floss et al., 2009, 2008; Meyer & Chilkoti, 2004; Scheller, Leps, & Conrad, 2006; Shamji et al., 2007; Shimazu, Mulchandani, & Chen, 2003; Trabbic-Carlson et al., 2004, 2004). Anyhow the robustness of ELP tags needed to be addressed for large-scale purifications (Meyer & Chilkoti, 2004; Trabbic-Carlson et al., 2004). A particular study demonstrated that ELP tags containing 30 pentapeptide repeats have a positive impact on the recovery and the purity of recombinant proteins. Interestingly, the C-terminal ELP fusion tags yield higher recovery rates of fusion proteins, compared with N-terminal ELP fusions. Thus, the size and orientation of the ELP tag affect the final yield of recombinant proteins and their purification quality (Conley, Joensuu, Jevnikar, Menassa, & Brandle, 2009). Recently, an Elastin-Like polypeptide (ELP) fusion protein with a small synthetic antibody-binding Z-domain (Z-ELP) originated from the B domain in Protein A for several affinity precipitation applications has been introduced for several affinity precipitation purposes. They eradicated the limitations of traditional ELP precipitation via designing a new ELP scaffold that could efficiently capture antibodies at high temperature (19-25°C) while minimizing the salt required for aggregation (Swartz, 2018).

**Figure 4. Schematic of recombinant proteins purification by elastin-like polypeptide (ELP)**

Aggregation of the fusion protein is triggered by increasing the solution temperature and/ or ion strength. After centrifugation (or filtration), the supernatant (or filtrate) comprising the contaminating soluble molecules is discarded finally, the target protein is separated from the ELP via cleavage by site-specific protease at a recognition site between the target protein and ELP.
2.4. Affinity partitioning

Affinity partitioning strategy depends on the tendency some solute molecules to dissolve in a certain liquid phase of an immiscible liquid–liquid system such as an aqueous two-phase system (ATPS). The affinity partitioning in ATPS is extensively used for purification of biomolecules. The aqueous two-phase systems affinity extraction/partitioning (ATPAP) consists of high specificity of an affinity technique with the strong conditions of traditional liquid–liquid extraction systems (ATPS) for recovery and purification of various biological molecules. The ATPAP strategy provides high recovery yield and is also easily to scale up and set up. This strategy is also a selective, economic and environmental-friendly method. For optimization of ATPAP, the effect of several factors must be characterized such as the ionic state of active groups in affinity ligands (according to pH level), ionic strengths of the system (depending on salt concentrations), affinity ligand’s nature and concentrations, modified polymer concentrations, the general processing time in the activation technique, the chemical nature of the active groups in the polymers and the post-activation ligand coupling to the polymer. In addition, the ATP systems also can be designed via two polymers (such as polyethylene glycol and/or dextran), a polymer and a salt, an ionic liquid and a salt, or a low molecular weight alcohol and a salt mixed over a limit concentrations thus forming two immiscible phases, micellar and/or reverse micelle aqueous two-phase systems and ionic and/or non-ionic surfactants (Ruiz-Ruiz, Benavides, Aguilar, & Rito-Palomares, 2012; Ruiz-Ruiz, Benavides, & Rito-Palomares, 2017). Numerous molecules can be linked to ATPS polymer components as affinity ligands. Thaumatin and trypsin were purified via ATPAP systems which designed by means of PEG 8000 and DEX T500. Their results proved that the recovery and purification of the target proteins could be dramatically increased via modified ATPS with biospecific ligands linked to PEG molecules without major changes in the system structure (Andrews et al., 1990). It should be noted that polymers have to be chemically active by chemical reactions to reach high ligand coupling. Actually, several activation strategies have been established to achieve this aim, for example, in the case of PEG and DEX, the strategies rely on the active hydroxyl groups of the polymers. The Affinity ligand can be either free in solution phase or chemically linked to phase forming components. Recent studies have highlighted the importance of using ligand free in solution to avoid chemically activation of the polymers. The main advantages of the applying ligands free in solution are no polymer activation and ligand coupling, and decreasing resource consumption and time (Barbosa, Hine, Brocchini, Slater, & Marcos, 2008; Maestro et al., 2008). Maestro et al. purified recombinant proteins tagged with a choline-binding module (C-LytA) via ATPAP with free choline (a natural ligand) which this method is a simple, quick, effective, scalable strategy (Maestro et al., 2008). Another example of this strategy is the partition of chitinases in systems made through PEG 6000, dipotassium hydrogen orthophosphate (K2HPO4) and free
chitosan as affinity ligand (Teotia, Lata, & Gupta, 2004). Nowadays, there is a vibrant attention towards the using of natural polymers and affinity tags like Reppal PES starch, Guar Gum Galactomannans and carbohydrate-binding modules (CBMs). Antov et al. used polysaccharide guar gum galactomannan and starch in a new ATPAP system in order to purify mannanase from Cellulomonasfimi. This enzyme has a carbohydrate-binding module (CBM) as a natural tag with high specificity towards mannans. Their results demonstrated that different CBM could be applied for development of new and unique purification strategies if each target molecule has been connected with a specific binding module (Antov, Anderson, Andersson, Tjerneld, & Stålbrand, 2006).

2.4.1. Hydrophobin fusion technology
Certain fungi secrete small surface-active proteins called hydrophobins to control interfacial forces. Hydrophobin has a distinct structural property as one portion of its surface harbors hydrophobic aliphatic side chains that create a "hydrophobic patch" on the surface of the protein. This amphipathic feature is closely similar to the behavior of surfactants with one hydrophobic and one hydrophilic part. Due to this feature, hydrophobins can alter the hydrophobicity of their fusion partner and therefore can be used for effective purification by a surfactant-based aqueous two-phase system (ATPS). In this system, a surfactant separates fusion proteins towards the surfactant phase while the majority of the sample remains in the aqueous phase. Nondenaturing organic solvents, such as isobutanol, can be then easily separate fusion protein from the surfactant phase. ATPS is a simple, rapid, high capacity, inexpensive and scalable method. Also, using this method, proteins are not subjected to denaturation (Conley, Joensuu, Richman, & Menassa, 2011; Linder et al., 2004; Selber et al., 2004). Recently, the combination of hydrophobin I (HFBI) from Trichoderma reesei with GFP was used to simplify the purification process of GFP from plant leaf extracts using ATPS method. This study proved the potential of this method to selectively recover up to 91% of the GFP-HFBI while the HFBI fusion did not inhibit the fluorescence behavior of GFP (Joensuu et al., 2010). The hydrophobin fusion technology has certain advantages such as being appropriate for hard-to-express and toxic proteins and the hydrophobin part not inhibiting the function of the target protein. Also, hydrophobins have a robust and specific interaction with polymeric surfactants which can be used to improve protein purification. Taken together, these reports suggest hydrophobins as promising tags in advancing the clinical, industrial and research goals related to recombinant protein purification (Conley et al., 2011; Linder et al., 2004). Table 1 summarizes the various types of technologies used for the purification of recombinant proteins.

2.5. Self-cleaving affinity purification
The proteolytic removal of the affinity tags is an impediment in expanding the affinity purification method due to the possible non-specificity of the protease used, which may lead to cleavage at off-target places within the fusion protein. Moreover, most of the cleavage reactions occur at high temperatures that may denature the produced protein. Besides, the cleavage site may not be available for all fusion proteins. Recently self-cleaving affinity tags have been developed based on the self-splicing protein elements known as inteins that effectively eliminate the step of protease treatment of the purified fusion protein (Shah & Muir, 2014; Wood et al., 2000; Xu & Evans, 2001).

The cleavage activity of inteins is evidently related to the genetic mobility of the intein coding sequences. Inteins start with Serine or Cysteine and end in Asparagine. These residues often function as nucleophiles. Inteins as intron-like elements can catalyze protein splicing process through organization of a splicing precursor with a homing endonuclease domain (Wood & Camarero, 2014). Protein splicing is a post-translational process which involves four nucleophilic displacements directed by the intein plus the first C-extein residue (Serine, Threonine or Cysteine). This process can be used as a protein engineering tool. The desirable protein is fused to the N-terminus or C-terminus of the intein. Thiols such as dithiothreitol (DTT), β-mercaptoethanol and free cysteine trigger peptide bond cleavage at the intein N-terminus or C-terminus, thus the target protein is separated from the rest of the fusion protein immobilized on the column (Shah & Muir, 2014; Volkman & Mootz, 2013). The self-cleavage occurs under mild reaction conditions.
which protects the purified proteins from denaturation and loss of their activity (Wood et al., 2000; Xu & Evans, 2001). In a study, the target protein is linked to the C-terminus of a modified intein; on the other hand, a small affinity tag is fused in a loop region of the endonuclease domain of the intein for affinity purification (Xu & Evans, 2001). This group found specific mutations at the C-terminal splice junction of the intein result in manageable C-terminal peptide bond cleavage, as well as, the C-terminal peptide bond cleavage activity of intein is mostly affected by pH, temperature and the first residue of the target protein. The development of pH and temperature-sensitive inteins has further contributed to the simplicity and cost-effectiveness of this method (Southworth, Amaya, Evans, Xu, & Perler, 1999; Wood, Wu, Belfort, Derbyshire, & Belfort, 1999; Xu & Evans, 2001) (Figure 5).

2.6. Nanoparticles in affinity separation

Surface modified nanoparticles can be used in affinity separation of recombinant proteins, particularly his-tagged protein. Immobilized metal ion affinity chromatography applies different solid supports for capturing affinity ligand by means of immobilized metal ions. Polymeric beads with covalently immobilized nickel ions (Ni²⁺) are commonly used in order to purify 6xHis-tag recombinant proteins. However, this approach has some drawbacks such as the need for pretreatment to eliminate colloidal pollutants existent in the cell, high usage of solvents, and is time-intensive. To overcome these problems, separation through Magnetic-beads has been suggested as a suitable alternative. Among the different kinds of Magnetic-beads, hydrophilically modified iron oxide nanoparticles have higher binding rate because of their high surface-to-volume ratio and good dispersibility in an aqueous medium. Recently, nitrotriacetic acid-modified Fe-Pt nanoparticles were used for the purification of 6xHis-Tagged protein by IMAC (Xu et al., 2004). But, this strategy is not suitable for large-scale purification due to its high cost and difficult synthetic procedure. In another study, iminodiacetate functionalized superparamagnetic Fe₃O₄@silica core-shell nanoparticles chelated by Ni²⁺, have been used to purify 6xHis-Tagged proteins (Mohapatra, Pal, Ghosh, & Pramanik, 2007a). As hydrophilicity increases the purification rate, the use of silica for encapsulation of magnetic nanoparticles has been adopted as a promising strategy, because it offers an inert surface for the nanoparticulate systems and thus increases their hydrophilicity and dispersibility. This study demonstrated Ni²⁺ charged superparamagnetic silica nanoparticles have specific affinity towards 6xHistidine-Tagged recombinant protein, as well as being a highly effective, economic, bio-compatible, and flexible approach (Mohapatra et al., 2007a; Mohapatra, Pal, Ghosh, & Pramanik, 2007b).

2.6.1. Hydrophobic polymer nanoparticles

Polyhydroxyalkanoates (PHA) are a microbial family of hydrophobic biopolymesters (Wang et al., 2008). The surface of PHA granules is decorated with several proteins including PHA synthase (PhaC), PHA depolymerase (PhaZ), repressor protein (PhaR) and phasing proteins. In addition, several studies demonstrate these proteins as potential affinity tags for the purification of recombinant proteins (Malhotra, 2009; Wang et al., 2008). For example, polyhydroxybutyrate (PHB) is the simplest form of PHA that in combination with a PHB-specific binding protein causes the creation of a self-contained protein expression and purification method. In this method, the target protein effectively binds to the granules via the PHB-binding tag, and then, this compound can be easily recovered through simple mechanical methods and tools. This method has been successfully applied in laboratory scale for the purification of proteins at a reasonable yield with high activity level and it is also a simple, affordable and reliable strategy for the purification of native target proteins under mild conditions. This technique is applicable in many other expression systems and can be easily scaled-up for the large-scale production of recombinant proteins (Banki et al., 2005). A study reports a new one-step purification strategy containing PHA nanoparticles, PHA granule-linked phasin (used as affinity tag) and a pH-inducible self-cleaving intein (Figure 5). In this method, the nanoparticles linked fusion proteins were concentrated by means of centrifugation, followed by releasing the protein into the solution through self-cleavage of intein, and finally the released proteins were collected via pelleting the nanoparticles by another centrifugation step. The authors purified three proteins comprising of enhanced
green fluorescent protein (EGFP), maltose-binding protein (MBP) and β-galactosidase using this strategy, without compromising their activity and with considerable yields (Trabbic-Carlson et al., 2004). This strategy is an effective, simple and reliable system for separating recombinant proteins and the preparation of nanoparticles is very easy and inexpensive. Because this strategy does not require the use of affinity matrices and proteases, it has considerable economic benefits. This method only consists of simple washing and centrifugation steps and also decreases the possibility of degradation and denaturation of target proteins by recruiting the self-cleaving peptides. In order to expand the application range of this method to various other proteins, this group separated the phases of protein production in vivo and purification in vitro via exploiting synthetic hydrophobic polymer nanoparticles. Anyways, PHAs are costly and often not available to numerous laboratories. To address these problems, the authors proved that the phasin has a nonspecific interaction to three kinds of nanoparticles including PHBHHx, PLA and PCL (Wang et al., 2008), providing inexpensive alternatives for PHAs. Similarly, it is obvious that numerous other common and inexpensive hydrophobic polymers can be exploited for the preparation of nanoparticles usable in protein purification purposes. Unwanted premature cleavage of intein, i.e. the cleavage before its time, could occur during the protein expression process and cell lysate incubation with nanoparticles. For example, the use of greatly controllable thiol-inducible inteins can solve this problem (Perler, 2002). Another way to control this unwanted cleavage, inteins are engineered structurally where deletion of some residues can solve this problem; For instance, a mutant SspDnaBintein with a 11-amino acid stretch at its N terminal leads to removing premature cleaving during expression (Wood & Camarero, 2014). Another technical issue is the aggregation of nanoparticles during the centrifugation procedure leading to the reduction of total yield. Thus, well-dispersed nanoparticles should be provided through innovative and progressive methods to avoid the aggregation problem. One particular solution to avoid aggregation is the application of magnetic beads coated with hydrophobic polymers. These bare magnetic nanoparticles are more affordable and easy to synthesize compared with the existent commercial protein-modified magnetic beads used for purification procedure (Gu, Xu, Xu, & Xu, 2006).

2.6.2. Oleosin fusion technology
Seed oil bodies are plant organelles that store lipids as a fuel for the germination process. They comprise of a triacylglycerol (TAG) matrix enclosed by a monolayer of phospholipid and proteins. They are highly stable both in the host cells and in isolated preparations due to the steric limitation and electronegative repulsion created by the specific structural proteins known as oleosins (Frandsen, Mundy, & Tzen, 2001; Lin, Tai, Peng, & Tzen, 2002). Seeds containing oleosin-fused proteins, extracted from transgenic plants, have been proven to be suitable carriers to produce and purify recombinant proteins (Figure 6(a)) (Van Rooijen & Moloney, 1995). In one study, a novel method was developed to efficiently produce target recombinant proteins in E. coli and to easily isolate the target recombinant proteins by artificial oil bodies (AOB), similar to the method of protein purification by recombinant oil bodies from transgenic plants. In this study, GFP was effectively produced and purified in a high quantity and quality (Peng, Chen, Shyu, Chen, & Tzen, 2004). AOB is technically created by TAG, PL, and oleosin (Figure 4(b)) (Beisson, Ferté, Voultoury, & Arondel, 2001; Peng, Lin, Lin, & Tzen, 2003; Sorgan, Miles, Chi-Chung, & Jason, 2002), and in comparison with ligand affinity purification methods, this strategy is relatively inexpensive due to avoiding the use of expensive ligand-coupled columns (Terpe, 2003). Compared with the seed oil-body system (Van Rooijen & Moloney, 1995), the use of the AOB method leads to considerable boosting of the yield with a simple and more rapid process. In the AOB expression/purification system, soluble and self-folding polypeptides are selected as target proteins to prevent aggregation of recombinant proteins and save their functionality. In this study, firstly, oleosin–Xa–GFP was expressed in Escherichia coli (Xa is a linker sequence for proteolytic cleavage) and then artificial oil bodies were created by the mixture of triacylglycerol, phospholipid, and oleosin–Xa–GFP. After centrifugation, the oleosin–Xa–GFP was located on the surface of AOB and after cleavage of factor Xa the soluble GFP was
released from oleosin and the GFP was collected in a high yield and purity via concentrating the final supernatant (Peng et al., 2004).

3. Conclusion
The purification and separation of functional recombinant proteins are one of the most challenging tasks in biotechnology. In recent years, this problem has been partially addressed via the use of different strategies such as physicochemical purification methods, affinity purification methods and fusion technology. As evident from this article, fusion technology provides a potent strategy for high-throughput purification. We believe that the combination of the fusion strategy with nanotechnology is a promising approach in this specialized research field, and different innovative strategies can be devised by using various nanomaterials combined with self-cleaving peptides for one-step, high-yield, economical and large-scale purification of recombinant proteins.

Acknowledgements
This article has been extracted from the MSc thesis number "95/2-2/1". The authors would like to thank the Research Deputy of Tabriz University of Medical Sciences for their financial support.

Funding
This work was supported by the Research Deputy of Tabriz University of Medical Sciences [95/2-2/1].

Competing Interests
The authors declare no competing interests.

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Conflict of Interest
The authors declare no conflict of interest.

Citation information
Cite this article as: Current affinity approaches for purification of recombinant proteins, Sahar Mahmoodi, Mohammad Pourhassan-Moghaddam, David W. Wood, Hasan Majdi & Nosratollah Zarghami, Cogent Biology (2019), 5: 1665406.

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https://doi.org/10.1080/23312025.2019.1665406

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