Review

Affinity chromatography approaches to overcome the challenges of purifying plasmid DNA

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The diversity of biomolecules present in plasmid DNA (pDNA)-containing extracts and the structural and chemical similarities between pDNA and impurities are some of the main challenges of improving or establishing novel purification procedures. In view of the unequalled specificity of affinity purification, this technique has recently begun to be applied in downstream processing of plasmids. This paper discusses the progress and importance of affinity chromatography (AC) for the purification of pDNA-based therapeutic products. Several affinity approaches have already been successfully developed for a variety of applications, and we will focus here on highlighting their possible contributions to the pDNA purification challenge. Diverse affinity applications and their advantages and disadvantages are discussed, as well as the most significant results and improvements in the challenging task of purifying plasmids.

Introduction

Research concerning biotechnological applications of DNA has gained some relevance during recent decades. With the aid of advanced genetic tools, it is now possible to manipulate the genetic information of many organisms to introduce desired characteristics, such as in the promising and potentially revolutionary DNA-based therapies of DNA vaccination, gene therapy (Box 1) [1–10] or recombinant biopharmaceuticals.

The successful implementation of clinical approaches using these therapeutics, especially plasmid DNA (pDNA)-based strategies, will require the continuous improvement of production and purification procedures.

Characterization of pDNA

In therapeutic applications, pDNA is employed to deliver the desired genetic information into the cells and to induce the production of relevant proteins.

Due to space restrictions, the genomic DNA structure inside a cell is highly compact, and the right-handed DNA double helix is further twisted in the opposite sense, resulting in a negatively supercoiled (sc) DNA. Biologically, negative supercoiling is advantageous because it promotes the necessary unwinding and strand separation during replication and transcription [11]. Likewise, plasmids mainly exist in an active sc structure also known as covalently closed circular DNA (cccDNA) [12], although other forms can be generated (Box 2). The occurrence of different pDNA structures depends on the characteristics of their DNA sequence, as well as on supercoiling stress or unfavourable environment conditions, such as extreme pH or high temperature [11,13]. Temperature has an effect on the helical repeat of the DNA; temperature increase will result in extended thermal motion, which promotes a gradual unwinding of the DNA helix, as demonstrated by recent circular dichroism analysis [14], with the result that the extent of supercoiling of pDNA will be reduced. Because other forms of pDNA result from the damage to the sc form (Box 2), the only naturally intact and undamaged form of pDNA is its sc form, leading to an increased interest in developing efficient methods for recovering this pDNA form with high purity.

pDNA chromatography

In the development of plasmid purification methods for therapeutic applications, it is essential to consider the starting material, which is usually an *Escherichia coli* (*E. coli*) lysate with a high concentration of cell impurities that need to be removed [17] to conform with criteria demanded by regulatory agencies such as the US Food and Drug Administration (FDA). One of the limitations to the recovery of desired pDNA is that it is only present in very low amounts in the *E. coli* extract, representing less than 3% (w/w) of content. Moreover, most of the critical impurities share common characteristics of negative charge [RNA, genomic DNA (gDNA) and endotoxins] molecular mass (gDNA, endotoxins) and hydrophobicity (endotoxins) [17] with pDNA. These contaminant characteristics significantly constrain the separation and purification of pDNA, independent of the actual technique being considered. Thus, extensive efforts have been undertaken to attempt to minimize the amounts of these impurities in steps before lysate purification. The gDNA content can be reduced with a denaturation step during the alkaline lysis procedure [18], and RNA can be reduced by addition of RNA-digesting enzymes, such as RNase. However, this approach cannot be used for the purification of therapeutics because the use of animal-derived materials, such as commercially available RNases, is not permitted

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Box 1. Advantages of pDNA therapeutics and ongoing pDNA-based clinical trials for gene therapy and DNA vaccination

Both plasmid DNA (pDNA) vaccines and recombinant viral vectors have been and are currently being tested in clinical trials [1]. The advantages of pDNA vaccines are that they are easy to manufacture and, as indicated by a large body of evidence, are also safer [2]. Their poor immunogenicity is often stated as a main disadvantage [3]; however, several strategies for improving the immunogenicity of pDNA are under evaluation [1]. In 2007, around 30% of gene therapy clinical tests used plasmids as vectors.

Examples of pDNA-based clinical trials currently in progress

- Malaria [4]: induction of antigen-specific cytotoxic T lymphocytes – Humans – Phase I
- HIV [5]: stimulation of HIV-specific T-cell responses – Humans – Phase I
- Ebola virus [6]: induction of Ebola virus-specific antibody and T-cell responses – Humans – Phase I
- H5N1 Influenza virus [7,8]: induction of specific antibody (IgG or IgA) – Mice
- Cancer [9]: development of immune responses to a melanoma antigen – Humans – Phase I
- Critical limb ischaemia [10]: *HGF* gene for critical limb ischaemia – Humans – Phase III

Box 2. Structural and chemical characterization of pDNA

- Circular, double-stranded DNA molecule
- Length: 2–20 Kb
- Molecular weight: 10^6–10^7 Daltons
- Hydrophilic backbone (sugar and phosphate group)
- Hydrophobic interior of double helix (planar bases stacked on each other)
- Typical isoforms: both oc and linear forms can be randomly damaged at different gene locations, which make these forms less efficient in inducing gene expression [15], especially if promoter or gene coding regions have been destroyed [12,16].

[17,19]. An alternative strategy is to reduce the presence of RNA and other impurities, such as proteins, throughout the primary isolation process by using clarification and concentration steps [20], as schematized in Figure 1. The removal of endotoxins is particularly important because these lipopolysaccharide components of the *E. coli* cell wall can produce symptoms of toxic shock syndrome if present in sufficient quantities *in vivo* [21].

The purification of pDNA for research or clinical applications requires efficient technologies to obtain a reproducible and appropriate plasmid quality, that is, with an sc pDNA content higher than 97% [17]. Liquid chromatography, a high-resolution analysis method that is well characterized and well established in the biopharmaceutical industry, is often used to purify sc pDNA. Chromatographic processes can advantageously explore pDNA properties such as size, charge, hydrophobicity, accessibility of the nucleotide bases, the topological constraints imposed by supercoiling and/or affinity. However, the purification of pDNA by chromatography also faces some limitations related to both the characteristics of the molecules involved (i.e. pDNA and impurities) and the available stationary phases, which have a low capacity for binding large biomolecules such as pDNA. In general, poor selectivity and considerable co-elution are problems associated with the similarities between pDNA and impurities, as previously discussed [18].

The high molecular weight of pDNA is responsible for the high viscosity of DNA extracts and their lower diffusion coefficients (10^{-8} cm^2/s) compared with those of proteins, which are generally one order of magnitude larger [22]. This is a significant problem when using conventional chromatographic media, which consist of small pores that are not engineered to handle large molecules [18]. A new generation of supports have been developed to overcome the diffusion limitation, as well as to improve the binding capacity of the support for the target molecules, which is also considered to be a limiting factor in pDNA chromatography. Such superporous supports [23,24], together with monoliths [25–28] and adsorptive membranes [29,30], are advanced approaches that have shown to be able to partially solve the problems associated with low capacity.

Several chromatographic methodologies (Table 1), including size-exclusion, ion-exchange, hydrophobic interaction, reversed-phase, thiophilic adsorption and affinity chromatography, have been utilized for pDNA chromatography, either as an isolated step or integrated in an overall purification process.

Size-exclusion chromatography (SEC) distinguishes the components of a clarified lysate based on their differing sizes. Because the lysate is a complex mixture of different molecules with a great variety of molecular mass, the resolution capability of SEC is limited [31], and the isolation of sc plasmids in one single step is also limited [19]. However, SEC can be used to separate less complex samples because large molecules, such as the different forms of pDNA and gDNA that are unable to access the inside of agarose beads, can be separated from RNA and other small impurities [19].

In anion-exchange chromatography (AEX), strong ligands, such as quaternary amines, are typically coupled to polymeric matrices [32]. Nucleic acids as polyanionic molecules interact with the positively charged functional groups [33]. After binding, a salt gradient can be used to displace the different nucleic acids that in principle should elute in the order of an increasing overall net charge, which in turn is a function of chain length [32]. However, not all pDNA purification protocols include a salt gradient for plasmid elution. On a laboratory scale, the AEX column can be washed with a salt/alcohol mixture, followed by plasmid elution with water. AEX has become the most extensively described technique for pDNA purification processes (Table 1). The possibility of performing rapid separations and analysis, the lack of requirement for organic solvents, possible sanitisation with sodium hydroxide and the large commercial availability of stationary phases are some of the factors that contributed to the popularity of using AEX to purify pDNA [33], both at preparative and analytical scale [17]. AEX has been shown to be able to separate the sc isoform of pDNA, which is more compact and has a higher charge density than the open circular form [32]. However, some inversions in retention time were already described and attributed to the high adenine and thymine (AT) content [34,35]. In addition, due to the contaminants' similarity to pDNA in terms

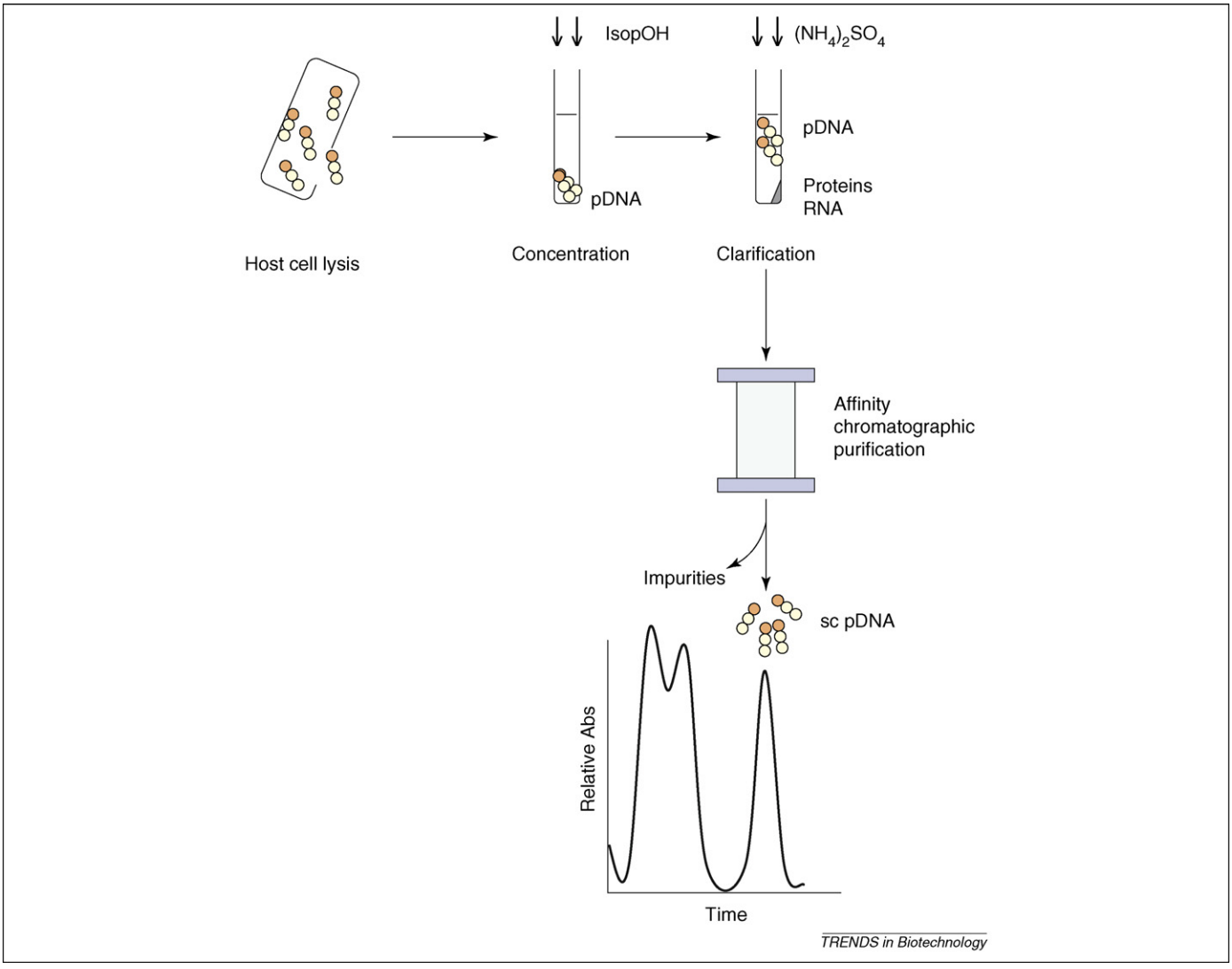

Figure 1. Affinity chromatography (AC) as part of the downstream processing of sc pDNA preparation. After pDNA production in the host organisms, cells are lysed, followed by an initial isolation (including concentration and clarification steps) and the final step of AC to purify and isolate pDNA. Abbreviations: IsopOH, isopropanol; $(NH_4)_2SO_4$, ammonium sulphate; sc pDNA, supercoiled plasmid DNA.

of chemical composition and structure (gDNA, RNA) or charge (endotoxins), their separation can in some cases be insufficient [2,33].

Hydrophobic interaction chromatography (HIC) provides purification based on hydrophobicity and has minimal solvent requirements [36]. In this method, solute retention occurs at high salt concentrations, driven mainly by a displacement of ordered water molecules around biomolecules and ligands. The requirement for high salt concentration is often viewed as a disadvantage, especially with regard to the industrial application of this method, because the use of salt is associated with higher costs and environmental impact. Elution is achieved by decreasing the salt concentration of the mobile phase, which weakens

Table 1. Chromatography methods used for pDNA purification

Chromatography type	Advantages	Drawbacks	Refs
Size-exclusion	Isolation of pDNA and gDNA from RNA and small molecules; Powerful as a final 'polishing' step	Low resolution; High dilution; Co-elution of pDNA isoforms and gDNA	[19] [31]
Ion-exchange	Application in preparative and analytical scales; Separation of sc from oc pDNA;	RNA co-elutes with pDNA; Relatively low resolution between pDNA and endotoxins	[20] [32,33,35,43,44]
Hydrophobic interaction	Application at preparative, analytical and industrial scales; Efficient separation of pDNA from endotoxins and single-stranded nucleic acids;	Non-binding of pDNA; Elution at high salt concentration; Difficult separation of pDNA isoforms	[38–42,45]
Reversed-phase	Possibility of isolating sc pDNA; Separation of pDNA from crude lysates	Requirement of organic solvents	[35]
Thiophilic adsorption	Isolation of pDNA from impurities; Separation of pDNA isoforms	Elution at high salt concentration	[46]

the hydrophobic interactions [36,37]. In the particular case of nucleic acids, the separation occurs by exploiting the differences in hydrophobicity of pDNA, single-stranded nucleic acid species and endotoxins [38,39]. Hence, the retention of nucleic acids with HIC is mainly affected by their size, base composition and structure [19]. Successful examples of HIC application include the purification of a cystic fibrosis gene therapy vector [40] and of a DNA vaccine against rabies [41], as well as the implementation of analytical methods [38,42].

Affinity chromatography of pDNA

The pursue of highly selective, reliable and economical processes to conform with the strict quality assurance in production of therapeutic biomolecules has been accompanied with an improvement of downstream strategies that are based on affinity chromatography (AC) [47]. This method is unique in that it uses a specific binding agent to analyse or purify biomolecules on the basis of their biological function or individual chemical structure [47–49]. In fact, affinity methods have the advantages of eliminating additional steps, increasing yields and improving process economics; however, they also do present some limitations, particularly in regard to the biological origin of the ligands [47]. Because these ligands tend to be fragile and associated with low binding capacities, a new approach is aimed at designing synthetic ligands that would combine the selectivity of natural ligands with the high capacity and durability of synthetic systems [47,50]. Although important advances in ligand design have already been achieved for specific proteins, its development for nucleic acids has not been extensively described.

Although the design of selective ligands for the purification of biomolecules is complex, time consuming and expensive, their implementation into AC processes would result in important economical advantages [47], such as the reduction of downstream steps and the improvement of the product quality, therefore justifying the initial investments. Thus, the development of selective ligands must be advanced before manufacturers will become interested and the method can become commercially viable. The concept underlying affinity interactions is not only associated with chromatography, and other interesting approaches, such as biomimetics [51] and bio-nanoengineering [52], have been developed by using affinity interactions as molecular tools.

AC separates biomolecules on the basis of a reversible interaction between the target biomolecule and its specific ligand, which is coupled to a chromatography matrix. The choice of matrix and conditions to be used will depend on the molecular properties of biomolecules and the physicochemical and thermodynamic nature of their molecular interactions [27,53]. Elution steps can be performed either specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity depending on the matrix used and the chemical characteristics of the biomolecules.

The specific interactions occurring between ligand and target molecule can be the result of electrostatic interactions, hydrophobic interactions, van der Waals forces and/or hydrogen bonding. The specific nature of the underlying interactions is a major advantage of AC because it results in a high selectivity and high resolution [54]. Overall, in a single step, affinity purification can offer immense

Table 2. Affinity chromatography methods for purification of nucleic acids

Affinity type	Principle	Specific binding	Advantages	Limitations	Refs
Immobilized metal-ion	Chelating ligands charged with divalent metal ions specifically interact with aromatic nitrogen atoms through π-d orbital overlap	Single-stranded nucleic acids (particularly purine bases)	Efficient resolution of RNA from gDNA and pDNA; High endotoxin removal; Separation of denatured pDNA	pDNA in the flowthrough; Incomplete RNA capture in complex mixtures; Co-elution of all DNA forms	[55–59]
Triple-helix	Specific sequences present on DNA are recognized by an immobilized oligonucleotide, forming a triple-helix	Double-stranded DNA	Discrimination of different plasmids based on their sequence; sc pDNA isolation in one chromatographic step; Reduction of RNA, gDNA and endotoxin contamination levels; Possibility for scale-up	Loss of pDNA during wash step; Low yields; Slow kinetics of triple-helix formation; Long chromatographic run times	[60,61]
Polymyxin B	Immobilized polymyxin B specifically recognizes the lipid structure of endotoxins	Endotoxins	Elimination of endotoxin contamination from pDNA preparations	Non-specific interaction of ligands with pDNA; Poor yields; Toxicity of polymyxin B	[75]
Protein-DNA	A protein or protein complex immobilized on the matrix specifically recognizes a DNA motif	pDNA	Discrimination of different plasmids based on their sequence; pDNA isolation from clarified lysates; Elimination of proteins and RNA from preparation	Relatively low yields; Contamination with gDNA	[62–67]
Amino acid-DNA	Multiple interactions occur between immobilized amino acids and nucleic acids	sc pDNA	sc pDNA purification in a single chromatographic step; Efficient elimination of RNA, gDNA, proteins and endotoxins	Elution with high salt concentration and relatively low yields (for histidine)	[71–73]

advantages over other less-selective and time-consuming multi-step procedures (Figure 1).

As previously discussed, the separation of pDNA can be particularly challenging, so in view of its supremacy and unmatched selectivity, AC technology might find a widespread application in nucleic acid technology (Table 2). Below we will discuss the different types of AC that have been utilized for the purification of pDNA.

Immobilized metal ion-affinity chromatography (IMAC)

Immobilized metal ion-affinity chromatography (IMAC) was first established as a technique to fractionate proteins on solid supports based on their differential affinity towards immobilized metal ions [55]. IMAC matrices are charged with chelating agents, such as nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA), that coordinate transition metal ions, more commonly Ni(II) and Cu(II) [56,57]. The reversible interactions in IMAC can be disrupted under mild conditions, usually by adding a competing agent like imidazole [56,58].

Several research groups have recently attempted to isolate nucleic acid molecules and to purify pDNA by exploiting IMAC technology. It has been reported that IMAC matrices were able to selectively adsorb single-stranded nucleic acids through metal ion interactions with aromatic base nitrogens [56], especially those present on purine bases (adenine and guanine) [58], whereas oligonucleotide duplexes, pDNA and gDNA, showed low IMAC binding affinity [56]. The feasibility of using IMAC for the purification of pDNA directly from an alkaline cell lysate was also reported by other authors [59]. The inaccessibility of aromatic nitrogen bases in pDNA to the immobilized metal ions hindered their affinity interaction with any IMAC matrix. On the contrary, the flexibility of endotoxin molecules induced the highest affinity interaction for all transition metals tested [59]. The preference of affinity interaction with immobilized ion metals followed the order: endotoxins>RNA>pDNA [59]. The main disadvantage of IMAC was that a separation of pDNA from gDNA was only possible if the gDNA structure was destabilized, thus exposing the bases and enhancing interactions [58]. The high affinity of chelated metals for nucleic acid bases makes IMAC suitable for the purification of pDNA and RNA, for the removal of contaminants and primers from PCR reaction products and for the detection of mismatches in DNA heteroduplexes [56].

Triple-helix affinity chromatography (THAC)

Triple-helix affinity chromatography (THAC) is based on the sequence-specific interaction of a triple-helix-forming oligonucleotide with pDNA [60,61]. A pyrimidine oligonucleotide is covalently linked to a chromatographic matrix and will bind to duplex DNA via the major groove and through the formation of Hoogsteen hydrogen bonds (Figure 2a). The triple-helix interaction is only possible if a suitable target homopurine sequence has been previously inserted into the pDNA [60,61]. However, due to the requirement for cytosine protonation, the triple-helices are only stable at mild acidic pH, where hydrogen bond formation is the major driving force [61]. The dissociation of triple-helices occurs in alkaline conditions.

Wils and collaborators studied the particular case of purification of the plasmid pXL2563, which contains the polypurine $(GAA)_{17}$ sequence and can bind to a $(CTT)_7$ immobilized oligonucleotide under an acidic pH [60]. This study provided evidence that it is possible to purify sc pDNA with THAC and, furthermore, to significantly reduce the level of contaminating RNA, endotoxins and gDNA in a single step [60] (Table 2). However, the relatively low recovery (32%) was one of the major drawbacks associated with this study [60]. In addition, the slow kinetics of triple-helix formation requires a column-binding step of at least one hour, which renders the process time-consuming.

To overcome the observed low yields, a large pore affinity support was developed for the purification of pDNA via triplex affinity interaction [61]. Despite the improved yield, the enrichment of undesired denatured pDNA limited the success of this approach [61].

Protein-DNA affinity chromatography

A first example for the use of protein-DNA chromatography was the isolation of pDNA directly from a crude lysate with the use of a bifunctional protein-based affinity linker consisting of a zinc finger (ZF) DNA-binding protein that was fused to glutathione S-transferase (GST-ZF) [62]. Here, the ZF domain of the protein bound to a 5′-GGGGCGGCT-3′sequence, while the GST domain bound to a glutathione Sepharose™ affinity matrix. The protein-pDNA complexes formed on the column can usually be recovered by competitive elution with reduced glutathione [62]. Using this technique, it was possible to distinguish plasmids that contained the specific 5′-GGGGCGGCT-3′ sequence from other plasmids [62], as well as to recover pDNA from a clarified lysate, albeit with a relatively low yield [63].

A different strategy was developed that exploited the natural interaction between the lac operon sequence contained in the pDNA and its repressor, the lacI protein [64,65]. The binding of the DNA to the lac repressor occurs via complex hydrophobic interactions and hydrogen bonding [65]. The DNA–protein complexes are typically eluted with the addition of a NaCl/Isopropyl-β-D-1-thiogalactopyranoside (IPTG) solution, which induces an allosteric change in lacI, allowing the release of the plasmid [66]. In this method, no interaction between RNA and the repressor molecules was detected, but gDNA and pDNA showed a specific interaction with the repressor [64]. Thus, this technique allowed the separation of single-stranded from double-stranded molecules, but its applicability for selectively isolating pDNA is limited (Table 2) because gDNA contamination might remain [66]. Compared with linear or open circular pDNA isoforms, sc pDNA displayed a higher selectivity for these protein-affinity matrices [65,67]. One possible explanation for the preferential binding of sc pDNA to repressor molecules could be that in the cell this interaction mostly occurs with physiologically active sc pDNA [65,67].

Amino acid-DNA affinity chromatography

The use of less selective, but at the same time more robust, small molecular ligands (also known as pseudobio affinity ligands) was first introduced by Vijayalakshmi and

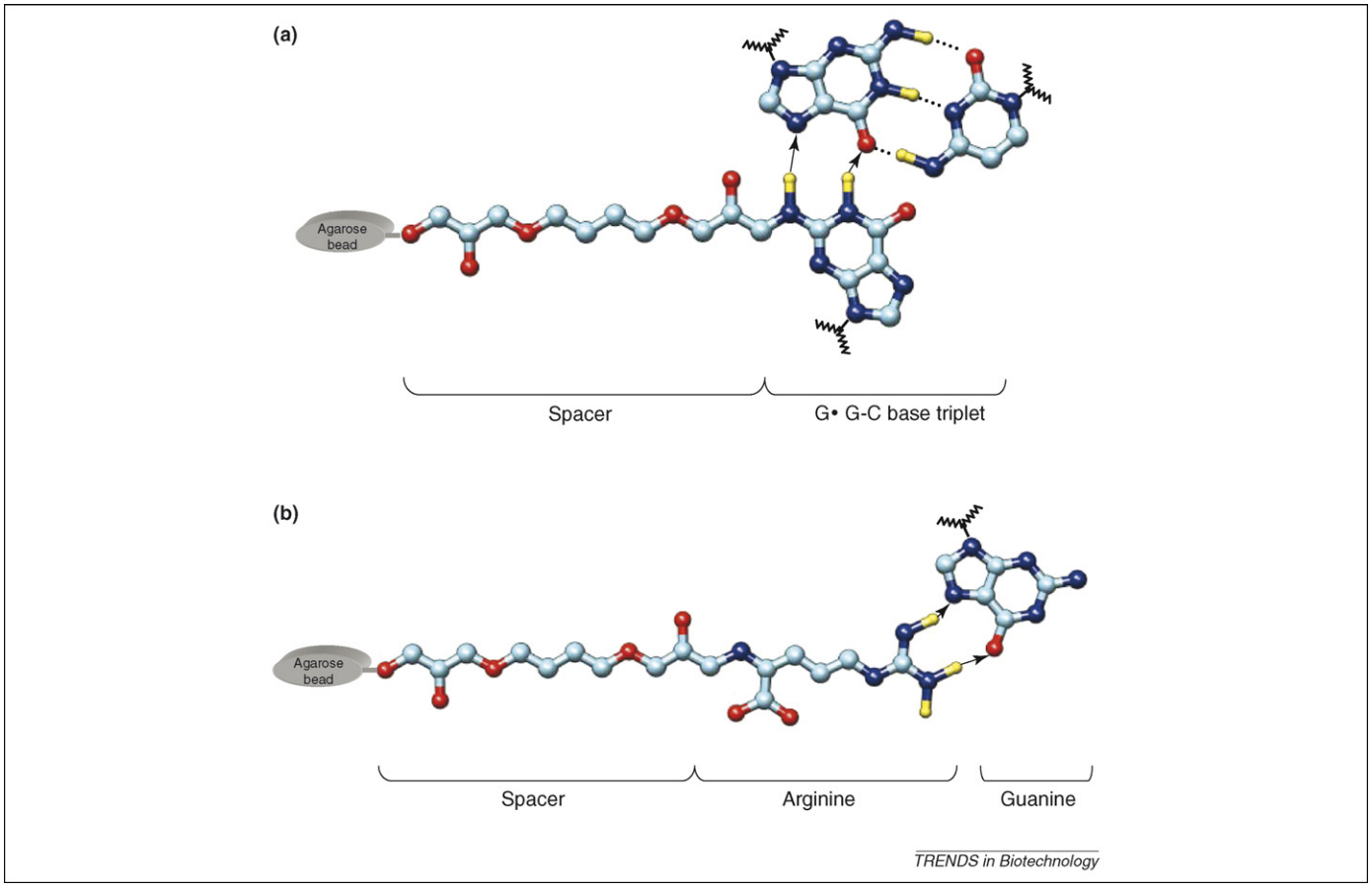

Figure 2. Schematic representation of affinity interactions. Oxygen atoms are represented in red, carbon in light blue, nitrogen in dark blue and hydrogen in yellow. The nucleotide structure is represented by the zigzag line. **(a)** Triple-helix affinity chromatography (THAC). This is a achematic illustration of a G•G-C base triplet formed between a G on the immobilized oligonucleotide and a G-C plasmid base pair. The dotted lines represent the Watson-Crick hydrogen bonds in the double-stranded pDNA, and the arrows represent Hoogsteen interactions. **(b)** Amino acid-DNA affinity chromatography. This is a schematic diagram of one type of 'bidentate' interaction, involving two hydrogen bonds, between arginine immobilized ligand and guanine. Arrows indicate the hydrogen bonding between the atoms involved and point from the donor to the acceptor atoms.

coworkers for the purification of immunoglobulins [48] and a wide variety of proteins [68], which has confirmed its exceptional potential. The concept of using less-selective ligands has also been applied to pDNA purification, mainly by using amino acids as immobilized ligands, which, as predicted from atomic studies, might preferentially interact with specific nucleic acid bases [69,70].

Histidine [71] and arginine [72] have been used as amino acid ligands, and their ability to isolate sc pDNA proved the presence of specific interactions of pDNA with both amino-acid-based matrices. The potential of histidine AC to efficiently separate sc pDNA from host impurities was further demonstrated [71,73] in a study that showed that only sc pDNA interacted with the histidine ligand, whereas open circular (oc) pDNA and gDNA did not. Furthermore, RNA was strongly retained in the column because of the higher base exposure, and its elution occurred with a decreasing ammonium sulphate gradient. The interaction of histidine with the DNA bases might include hydrogen bonding, ring stacking/hydrophobic interactions and water-mediated hydrogen bonds [69,70]. As a consequence of the sc structure, the bases of this isoform became more exposed than the bases of the oc isoform [71], facilitating the specific interaction with histidine ligands. Moreover, the interaction between histidine and pDNA was found to be dependent on the base composition; for instance, the presence of adenine- or guanine-rich sequences enhanced this interaction [14]. The pDNA obtained with this histidine affinity approach was of high quality: contamination with gDNA and endotoxins was within acceptable levels and neither RNA nor proteins were detected [73]. Transfection experiments using this purified pDNA also confirmed that it could efficiently drive gene expression in eukaryotic cells [73]. The binding capacity for pDNA binding was reasonable and comparable with those of agarose-based supports [74]. The relatively low yield and the high salt concentrations required for elution are the main drawbacks of this technique.

This technique might be improved by using arginine as the immobilized ligand. In fact, arginine-base interactions have been recognized as the most prevalent interactions in numerous protein-DNA structures [69,70]. Preliminary studies that employed arginine matrices to purify pDNA revealed the presence of specific interactions with plasmid molecules and, importantly, a significant recognition of the sc isoform [72]. In this example, the elution could be performed either by using an increased sodium chloride gradient or by addition of an arginine-supplemented buffer [72]. Apart from increased stability due to the interaction with pDNA backbone, the ability of the arginine-agarose to

distinguish and differentially interact with both pDNA isoforms further suggests a specific recognition of the sc isoform based on hydrogen-bond interactions between the ligand and the bases of the sc plasmid (Figure 2b). The increased proximity between nucleic acid chains arising from the superhelicity of sc pDNA would also favour multiple-contact, complex interactions with arginine. Atomic studies performed on protein-DNA complex structures have shown that arginine has a strongly favoured interaction with guanine [69,70].

Taken together, the use of this matrix resulted in strong interactions of pDNA, while at the same time elution and complete plasmid recovery was achieved using only low concentrations of salt or arginine [72]. Moreover, the ability of the arginine matrix to purify sc pDNA directly from a clarified lysate presents a significant advantage over other chromatographic techniques.

Conclusions and future trends

To conform to the strict quality assurance required for the production of therapeutic pDNA, more selective, reliable and efficient purification processes are needed that will allow the obtainment of plasmid product with the necessary purity. pDNA-containing extracts isolated from host cells are complex, and the impurities present have structural and chemical similarities with pDNA, resulting in extensive challenges for the selective purification of pDNA. Here, we emphasized the potential of AC in pDNA purification. As discussed above, THAC and protein-DNA AC utilize highly specific interactions to purify pDNA, allowing an efficient separation of single-stranded from double-stranded DNA. However, the resulting specificity is not always sufficient to separate gDNA from pDNA or to isolate the different pDNA isoforms. In our view, amino acid-based AC represents a particularly promising approach because it combines the selectivity of a naturally occurring biological interaction with the simplicity of a single small molecule. However, the low capacity of available supports for pDNA is a problem that remains to be solved, as well as the low diffusivity of pDNA samples due to their molecular weight. These problems will require further efforts to design more suitable ligands and supports, in a similar way to the developments already seen for protein purification matrices intended for preparative and analytical purposes.

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