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Regeneration and Recycling of Supports for Biological Macromolecules Purification

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1. Introduction

Great evolution and improvement in biological molecules purification have been achieved in the last 20 years, giving advantages in both product quality and yield, and speed of purification methods. Purity levels, once poorly suitable for large scale production, as well as requiring long and very expensive procedures, are today achievable using simple procedures. For example, till few decades ago, the only way to obtain ultrapure nucleic acids was the ultracentrifugation on cesium chloride gradient; today, the same -if not higher- purity is easily achieved using solid-phase anion-exchange separations. The purification of recombinant proteins has also been dramatically improved by using more precise affinity techniques.

However, despite their routine use, these procedures are often quite expensive, making it very convenient the possibility of using the same purification devices several times, instead of wasting them after one use only.

The possibility of recycling purification matrices has to be considered desiderable and convenient not only in the laboratory research field, where most purifications are anyway performed on small-medium scale, but also in large scale production. Several attempts to reuse purification systems have been made in the last years, showing how many critical points have to be considered.

In fact, most of the previous procedures tested, especially on DNA purification columns, failed to fully decontaminate them (Chang *et al.*,1999; Fogel and McNally, 2000; Kim *et al.*, 2000), resulting in a substantial carry-over contamination, because of the remaining of substantial amounts of material into the matrix after elution (Esser *et al.*, 2005), so that the main challenge in every regeneration procedure is the complete removal of any detectable trace of the previously purified molecules, to avoid the presence of contaminating molecules in downstream applications. Moreover, the use of very sensitive analysis systems (like PCR) reduced dramatically the threshold of acceptable contamination levels. Only in recent years reliable decontamination methods have been proposed, as it will be discussed in the chapter.

Regeneration procedures of columns used in nucleic acids purification are based on nucleic acids hydrolysis, and take advantage of DNA and RNA chemical properties, so that different protocols may be needed for DNA and RNA efficient removal, depending on the decontamination procedure used. To this aim, the proper knowledge of the basis of binding

and elution (including the incomplete release of sample molecules), chemical properties and tolerance of both binding matrices and biomolecules to different reagents is needed, to ensure the proper management and improvement of decontamination procedures without impairing the matrix performances.

1.1 DNA columns

DNA purification strategies using columns take advantage of the chemical nature of the molecule, an highly negatively charged polyanion. Silica, glassfiber and silica-based anion exchange supports are among the most used DNA purification systems, ensuring rapid procedures with good yields and quality without any organic extraction. Such columns are commercially available for the purification of either small molecules (PCR fragments, plasmids, etc.) and genomic DNA, suitable for most applications. Anion-exchange matrices are usually based on polysaccharidic or mineral (silica) derivatized supports, where the active chemical group on resin surface is usually the DEAE (DiEthyl-AminoEthyl). The density of DEAE groups, much higher in silica based matrices (as found in Qiagen resins, Fig. 1A), seems to strongly affect not only the column capacity, but also the binding properties and the selective release of different molecules, each in specific conditions.
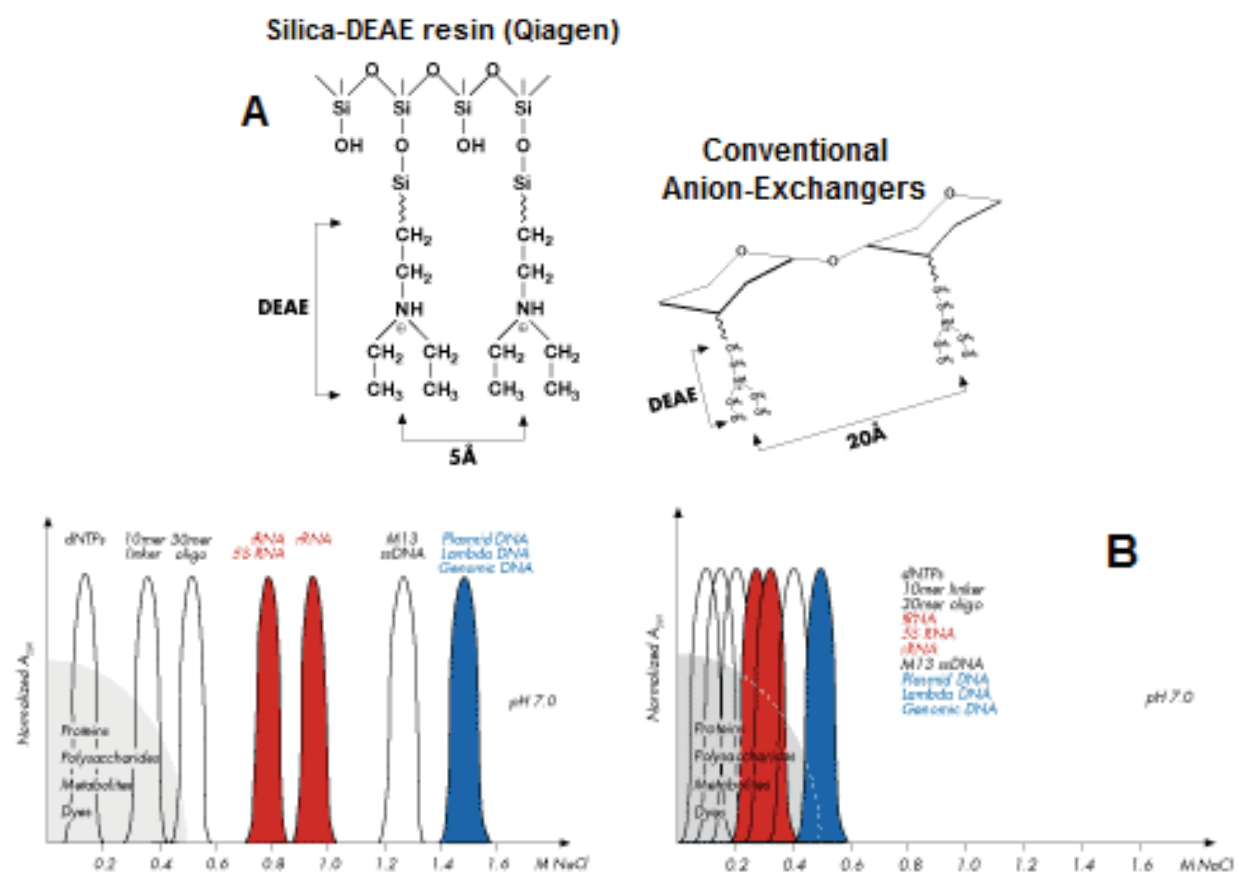

Fig. 1. Structure of anion-exchange DEAE resins (A) and elution profiles (B) (from Qiagen, Purification Technologies)

Such devices have a very broad range of separation, and allow the highly selective binding of nucleic acids, due to the negatively charged phosphate backbone, to the positively charged DEAE groups. Different column suppliers propose various purification conditions, using

either changes of both pH and ionic strength or constant pH (usually at neutral values) and different saline concentration to achieve binding (sometimes in the presence of some amount of ethanol or isopropanol) and elution. These parameters are also tuned to sequentially purify DNA and RNA, or nucleic acid molecules of different sizes (Fig. 1B)

The DNA purification by means of silica gel, proposed first in batch and later in column, has been the most largely used method to recover DNA from both dissolved gel bands and cell lysates, giving high quality DNA without time consuming procedures.

Silica and glassfiber matrices bind DNA because of its negative charge. However, the interactions between silica and nucleic acids occur in different ways depending on chemical conditions.

Binding properties of silica surface depend on its hydratation status (silica-gel) and on the pH value.

In particular, acidic or neutral pH combined with high ionic strength (Fig. 2) allows the silica surface to be positively charged and the DNA to tightly bind silica particles. Binding occurs in the presence of high concentrations of chaotropic salts (usually guanidinium hydrochloride or thiocyanate, sometimes sodium perchlorate). These chemicals alter the hydratation status of macromolecules and facilitate silica-nucleic acid interactions. Under these conditions, DNA remains selectively bound to the matrix, while other molecules (RNA, proteins, polysaccharides and other biological molecules) flow through. Such interaction is still strong under low ionic strength in the presence of high alcohol concentrations, so that this condition is used to perform one or more washes, allowing any trace of salts or soluble contaminants to flush out. Finally, DNA is recovered by elution in pure water or very low salt buffer (frequently Tris-HCl 10 mM, pH 7.5-8.5). In most columns, the maximum DNA release occurs above pH 8.
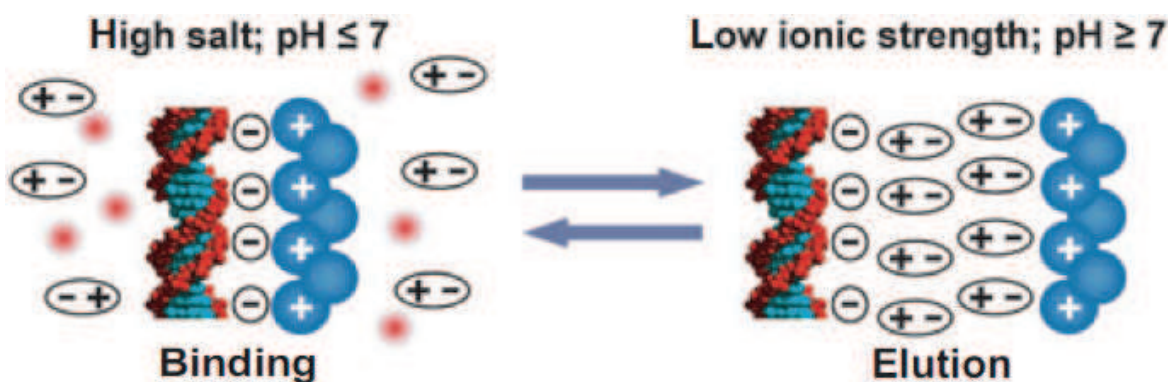

Fig. 2. Interactions between nucleic acids and silica in conditions employed for binding and elution (from Esser *et al.*, 2006)

1.2 RNA columns

Devices for fast total RNA purification are based on the same principles described above. In these columns, a guanidinium salt is always used to protect RNA from degradation, besides promoting its binding to the resin.

Columns can be employed which allow the purification of DNA or RNA, or both (silica-DEAE resins in particular), simply using different buffers. Some silica-based columns, like silica gel itself, show an enhanced RNA binding capacity when the chaotropic, high salt

binding buffer is supplemented with ethanol and/or 2-propanol; in these conditions, DNA binds the silica with low efficiency. Due to the different supports and/or binding and elution conditions, molecules shorter than 200 nucleotides usually fail to bind or are lost during washes, although recently columns allowing the recovery of the whole RNA population, including molecules few tens of nucleotides long, became commercially available.

Affinity columns are largely used to purify the poly-A$^+$ fraction of eukaryotic mRNA. Such purifications can be performed either in batch or in columns, although the mechanism is the same. The resin employed consists of polymeric, hydrophilic beads (often agarose) whose surface is coated of covalently linked oligo-dT. The interaction with target RNAs occur because of the complementarity between the mRNA poly-A tail and the resin-linked oligo-dT, leaving other molecules unbound. The following elution allows the recovery of the poly-A$^+$ fraction (Fig. 3).
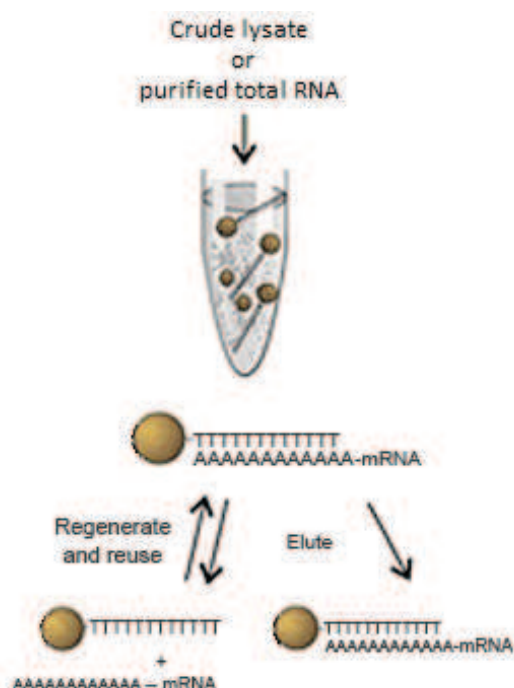

Fig. 3. Purification of the eukaryotic poly-A$^+$ mRNA fraction. (modified from Invitrogen)

All the systems described above are largely used in most molecular biology laboratories and in many biotechnological companies for both research applications and large scale productions, as they offer the possibility to get large quantities of high quality products using short and simple procedures.

2. Limits

The major disadvantage of the purification systems and devices described above is the cost, as they can only be used once because of the substantial amount of DNA which remains into the matrix after elution.

In fact, the nucleic acids recovery has been estimated to be not more than 90-95% of the input. The remaining part is lost during purification (because of binding failure or leakage

during washes) or remains inside the matrix. The incomplete elution may have various explanations:

1. some molecules are not released from the resin;
2. a small volume of eluent is always retained by the resin, leaving free molecules inside;
3. some molecules might be included into unsolubilized protein particles;
4. some molecules might be associated with cellular fragments, especially of bacterial origin.

Whatever the cause, after the elution (even if sequential rounds are performed) the resin contains relatively large amounts of sample (Fig. 4), making it impossible to reuse the same matrix for further purifications, especially of different samples, because of the high risk of cross-contamination and reduction in the column binding capacity due to trapped particles (especially when a crude lysate had passed trough the column).

All these conditions require particular attention for any attempt of recycling purification columns, so that only methods whose reliability has been proven should be employed.
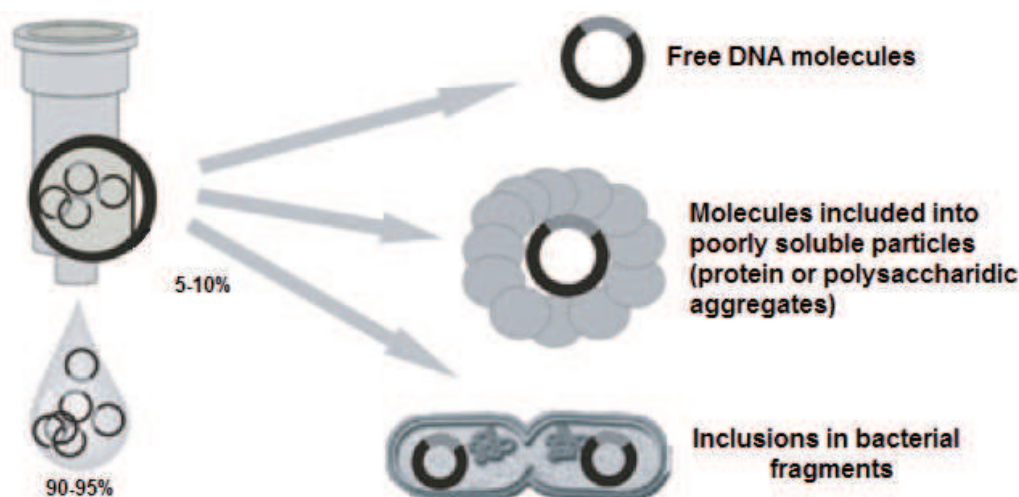

Fig. 4. Residual DNA into the column after elution (modified from Esser *et al.*, 2006).

3. Regeneration

Whereas working with a large number of DNA samples (or large volumes) could represent a problem because of the columns or, in general, purification matrices cost, the possibility of recycling them becomes attractive. The main challenge in every regeneration procedure is the complete elimination of any detectable DNA trace.

In the last years several attempts have been made to set fast and safe procedures ensuring a true complete decontamination without impairing the resin binding properties.

Thus, chemical procedures are needed that could ensure the complete hydrolysis or functional/chemical inactivation of nucleic acids. Moreover, the treatment should be able to remove, at least partially, particles (mainly composed of proteins) trapped into the matrices.

The methods proposed earlier did not avoid carry-over contamination (Chang *et al.*,1999; Fogel and McNally, 2000; Kim *et al.*, 2000).

The first reliable method has been proposed (Esser *et al.*, 2005) and became commercially available as a kit (patented in USA in 2009).

It is based on the hydrolysis (single and double strand breaks) of nucleic acids in the presence of ferric salts in a reducing, buffered acidic environment. Other biomolecules, including lipids and proteins are damaged by the treatment, too. This procedure is claimed to be effective within minutes or hours, ensuring the complete decontamination (assessed by PCR assay) of columns used to purify plasmids or PCR products. Moreover, the composition of the two active solutions included in the kit is claimed to be safe and not hazardous. In fact, the first solution presumably consist of ferric chloride, citric acid, ascorbic acid (as reducing agent), detergents and phosphate buffer at mild acidic pH.

The ability of iron salts (and other transition elements salts, like those of copper, zinc, cobalt, etc.) to damage DNA and RNA is well known since a long time, so that Fe^{2+}-mediated DNA hydrolysis has been used in earlier studies on chromatin structure (Hertzberg and Dervan, 1982) and even today for the footprinting of DNA-protein complexes (Swapan and Tullius, 2008).

Several reactions have been hypothesized to occur, and the so called Fenton's reaction is the most widely recognized and used up today in several fields where fast and efficient oxydative degradation of organic compounds is required.

Fenton's reaction (see below) consists in the iron(II) salt-dependent decomposition of hydrogen peroxide (hypothesized to occur *via* an oxoiron(IV) intermediate), which generates the highly reactive hydroxyl radical. When a reducing agent is added, it leads to a cycle which greatly enhance the damage to biomolecules.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^\cdot + OH^-$$

This reaction is believed to occur together with the Haber-Weiss reaction, which triggers the following cycle:

$$H_2O_2 + OH^\cdot \rightarrow H_2O + O_2^- + H^+$$

$$H_2O_2 + O_2^- \rightarrow O_2 + OH^- + OH^\cdot$$
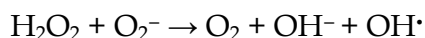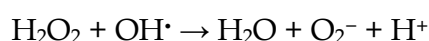
Although the exact sequence of these reactions and the identity of the reactive species involved in the various conditions (i.e. presence or absence of chelating agents and/or reducing agents) is still controversal (for a discussion, see Barbusiński, 2009), it's widely accepted that the formation of the hydroxyl radical is determinant for the subsequent DNA damage.

Besides the reactions reported above, the presence of chelated Fe(III) instead of Fe(II), together with reducing agents, can lead to strong DNA damage resulting in multiple strand breaks. For example, it has been reported that Fe(III)-nitrilotriacetate (NTA) in the presence of either H_2O_2 (able to act both as reductant and oxidizing agent (Buettner and Jurkiewica, 1996), ascorbate (which in certain conditions can act as pro-oxidizing) or cysteine, produced DNA single and double strand breaks as a function of reductant concentration, *via* a mechanism involving the reduction of Fe(III) to Fe(II) and the formation of H_2O_2. The latter, in turn, enters in the Fenton/Haber-Weiss reactions, where the presence of a reducing agent supports the "iron redox cycle". In all these cases, H_2O_2 seems to be a common intermediate (in fact, catalase activity is able to block these events, leading to the reduction of DNA damage), while the OH$^\cdot$ hydroxyl radical is the reactive species which attacks DNA (Toyokuni and Sagripanti, 1992). Moreover, the auto-oxidation of ascorbate in the presence of Fe(II) ions, chelating agents and

phosphate buffer, with the concurrent formation of hydroxyl radicals (OH˙) has been reported (Prabhu and Krishnamurthy, 1993). Figure 5 shows the chemical reaction resulting in DNA (or RNA) multiple breaks, although other reactions may occur simultaneously, which result in direct bases damage caused by the OH˙ radical.
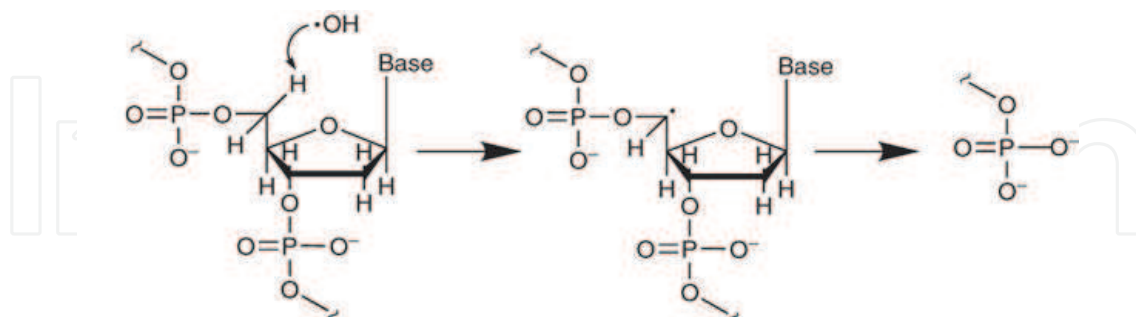

Fig. 5. DNA break by exposure to hydroxyl radicals (modified from Swapan and Tullius, 2008)

Thus, this method makes use of relatively non-toxic and environmental friendly chemicals, allowing the virtually complete removal of small nucleic acid molecules from purification columns. A disadvantage is that the solutions are guaranteed for 18 months only, because reagents undergo chemical modifications making them ineffective. The supplier of the recycling kit claims that resins can undergo about 20 regeneration cycles. Unfortunately, no data are available on the possibility of efficiently decontaminate devices used for genomic DNA purification, whose features are expected to make it more resistant to chemical treatmets, thus leading to the possibility of DNA carry-over.

The first report of a simple and efficient home-made decontamination method has been published in 2008. It achieves the nucleic acids removal by acidic hydrolysis, but its major limits were the need of very long incubation times (Siddappa *et al.*, 2007), and the secure efficacy on low molecular weight nucleic acids only.

The principle used in that method was the DNA and RNA degradation by a treatment with strong acids. In particular, nucleic acids are known to be susceptible to acid catalyzed hydrolysis, which involves the cleavage of the *N*-glycosidic bond of purine nucleosides. As shown in Fig. 6, the reaction results in the formation of an AP-site (apurinic or abasic sites), which causes DNA or RNA break. In fact, the hydrolysis of the *N*-glycosidic bond unmasks the latent aldehyde functionality at the C1' position, rendering the 3'-phosphate group susceptible to β-elimination (1), which results in strand break. Moreovever, such products are highly sensitive to further alkaline hydrolysis (Fig. 6), so that depurinate molecules can easily undergo fragmentation following exposure to bases (2). These are, for example, reactions used for the controlled, partial DNA hydrolysis in molecular biology protocols, where the short exposure to relatively low concentrations of HCl lead to DNA fragmentation.

However, when the acid concentration is high and/or the exposure time is extended, the depurination extent is so high that, after alkaline treatment, very short DNA fragments or even nucleotides are obtained.

The column regeneration method proposed by Siddappa *et al.* (2007) consisted in a 24 hours incubation of used columns in a HCl solution, followed by several washes. Data reported showed that no detectable nucleic acids were still present in the column, whose binding capacity were claimed to be mantained.
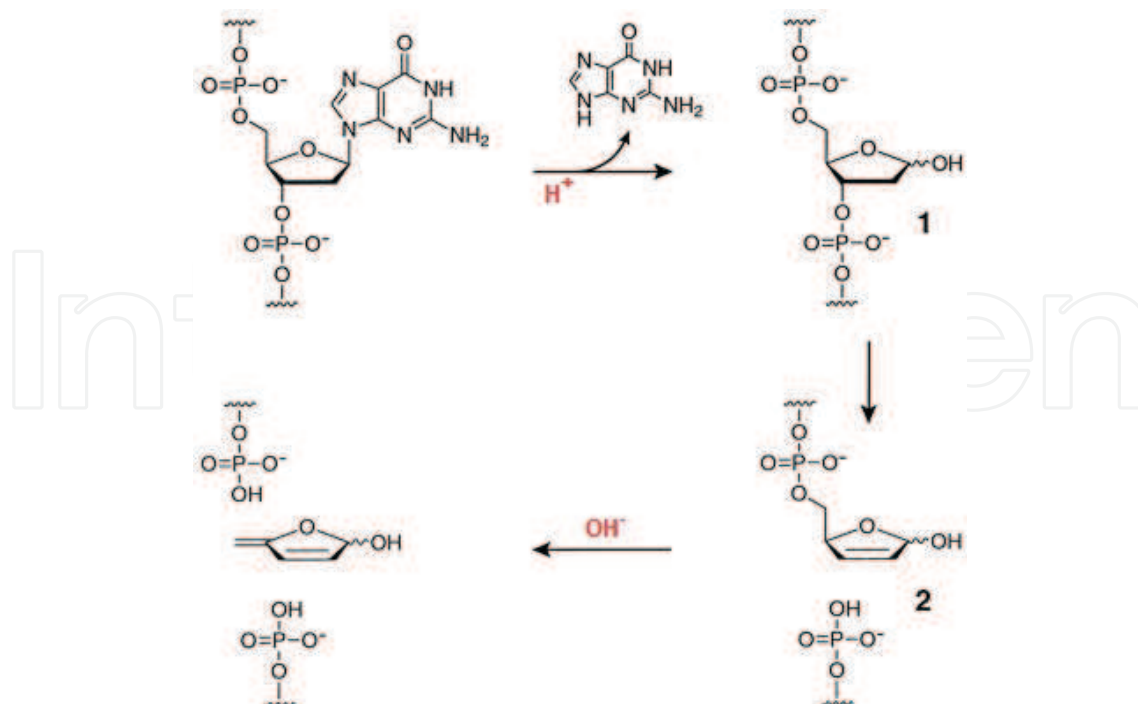
Fig. 6. DNA strand break resulting from H⁺ catalyzed depurination and subsequent β-elimination at the AP site. Hydrolysis results in release of the purinic nucleotide and formation of an AP site (1). The α- and β-hemiacetals are in equilibrium with the open chain aldehyde, which is susceptible to β-elimination that results in cleavage of the adjacent 3′ phosphoester (2). This product in turn undergoes cleavage of the 5′ phosphoester under alkaline conditions. (modified from Sheppard *et al.,* 2000).

The lack of evidences about the efficacy of the method even on columns contaminated by genomic DNA and the time-expensiveness of the procedure prompted further tests to improve the procedure.

In fact, if silica columns are used to purify small molecules, contaminating DNA can be virtually completely eliminated by commercial kits (Esser *et al.*, 2005) or using the procedure reported in Siddappa *et al.* (2007), as they make any trace of the previous sample undetectable. However, the efficacy of both methods in eliminating genomic DNA remains uncertain.

The fastest and most effective home-made procedure available up today for the decontamination of silica-based columns consist in an improvement of that described above, as it's also based on DNA depurination and hydrolysis, and addresses the main limits of previously proposed protocols.

Silica-bound DNA could be expected to be efficiently depurinated and removed by treatments with strong acids even after short exposures. However, after such a regeneration procedure small amounts of amplifiable DNA are actually still detectable.

Such failure might be hypothesized to be due to an incomplete permeation of the acidic solution into the silica matrix, where the nucleic acid might be still bound to silica or trapped because of its high molecular weight (Esser *et al.*, 2005). Moreover, any molecules included into aggregates might be somewhat resistant to chemical treatments. All these conditions might allow variable amounts of DNA to escape the depurinating agent, resulting in residual amplifiable traces, making it necessary a very long incubation in HCl solutions.

These limitations have been overcome by the procedure described by Tagliavia *et al.* (2009) and reported below. It can be completed in about 45' (instead of more than 24 hours), and allows not only to regenerate silica columns contaminated by DNA of any size, but also to save time.

The method consists in sequential alkaline and acidic treatments which denature and depurinate, respectively, any DNA still present into the column (depurination rate in denaturated DNA is higher than in native DNA (Lindahl and Nyberg, 1972). A further alkaline treatment hydrolyzes long depurinated DNA molecules reducing them into very small fragments (Siddappa *et al.*, 2007). These chemical treatments are performed in the presence of a non-ionic detergent at low concentration, which seems to enhance their action. In fact, given the structure of the column resins, the detergent is supposed to allow a more even permeation of the solutions employed in the treatment, as it modifies their surface tension. Moreover the tensioactive (which is important to be non-ionic to reduce any dependence of its action on pH and ionic strength), along with the initial alkaline treatment, helps dissolving aggregates, making trapped molecules more exposed to NaOH and HCl.

The efficacy of the method has been demonstrated both by assays using radiolabeled DNA and by PCR, using columns contaminated by large amounts of either genomic DNA or short PCR products.

The protocol steps are briefly reported in box 1.

Box 1. DNA silica column regeneration protocol

1. Load the silica columns with a 1 N NaOH/0.1% Triton X-100^{TM} solution
2. Incubate 5 minutes at room temperature
3. Spin for 30 seconds
4. Load the silica columns with a 1 N HCl/0.1% Triton X-100^{TM} solution
5. Let a little amount of the solution flow through by gravity
6. If dropping tends to empty the column, put it into a tube containing the same solution
7. Incubate at room temperature for 30 minutes
8. Spin for 30 seconds
9. Load the silica columns with a 1 N NaOH/0.1% Triton X-100^{TM} solution
10. Incubate at room temperature for 5 minutes
11. Spin for 30 seconds
12. Load the column with sterile ddH$_2$O
13. Spin 30 seconds

Note: it's essential to treat not only the resin, but any surface even potentially contaminated by DNA.

The use of the regeneration systems described above is safer on silica-based columns, but not on those supports consisting of polysaccharidic compounds, as they might be hydrolysed or their structure impaired by chemicals employed. Alternative methods, some of which based on radical-driven nucleic acids degradation different from that described above, are under investigation.

Regeneration methods, besides their first application in reusing purification supports, might become of wider use, even for the pre-treatment of new columns before first use. There are many commercially available kits that rely on DNA binding columns to extract and purify DNA from tissues or cultured cells, and a recent paper (Erlwein et al., 2011) reported that, in independent tests, some DNA purification columns from different kits were contaminated with DNA of diverse provenance, including human and murine DNA. Although further investigations are needed, the need of a preliminar columns decontamination step should be considered, at least for particular experiments or analyses have to be carried out.

3.1 RNA columns

Total RNA purification is carried out using columns working exactly like those employed in DNA purification. As discussed earlier, different conditions used for binding and/or elution allow the selective recovery of RNA only.

The same problems described for DNA columns occur in RNA columns, too. However, RNA is well known to be very sensitive to a variety of conditions and chemicals, but treatments are needed that ensure not only the complete degradation of any residual RNA, but also the maintainance of the columns RNase-free state.

The commercial system based on the earlier discussed iron-mediated degradation is effective, but a home-made, simple and inexpensive method is available (Nicosia *et al.*, 2010).

In fact, the methods described in two previous reports (Siddappa *et al.*, 2008; Tagliavia *et al.*, 2009) are time expensive or include steps not required for RNA hydrolysis, so that a faster and more efficient protocol has been set up. It is based on the RNA high sensitivity to alkali, omitting acidic treatments. Indeed, the exposure of RNA to high pH is able to completely hydrolyse RNA, since it is directly cleavable by the OH^- due to the presence of the 2'-OH group in the molecule (Fig. 7).
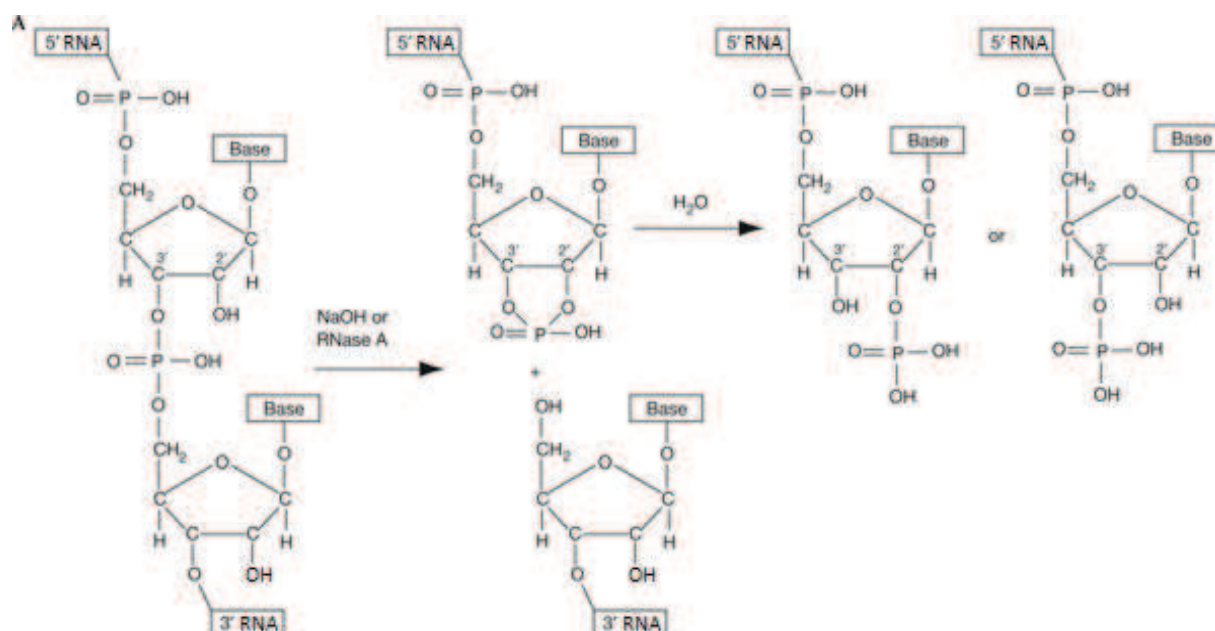

Fig. 7. Alkali catalyzed RNA hydrolysis. The 2'-OH group, present in RNA only, makes it OH^- sensitive. Besides a 5'-OH end, a cyclic 2',3'-P intermediate is released, which in turn produces a 3'-P or 2'-P end (modified from Vengrova and Dalgaard, 2005).

Thus, a strong base like NaOH is employed in the presence of low concentrations of a non-ionic surfactant, whose role has been earlier discussed. Treatments are performed using prewarmed solutions, so as to allow the reduction of both alkali concentration and exposure time. Indeed, it should be remembered that silica does not tolerate high alkali concentrations, as it forms silicates, resulting in matrix destruction and loss of binding properties. This is the reason why time of exposure to NaOH, its concentration and the temperature, as described in Nicosia *et al.* (2010), are crucial for the successful decontamination without impairing the columns integrity and efficieny, making it possible to reuse them several times.

The regeneration protocol is briefly reported below.

Box 2. RNA silica column regeneration protocol

1. Fill the silica column with a prewarmed (75°C) solution containing 0.2 N NaOH and 0.1% (v/v) Triton X-100™

2. Incubate 5 minutes at room temperature

3. Spin for 1 minute

4. Repeat step 1, incubate for 10'

5. Spin for 1 minute

6. Add a volume of a 50 mM sodium acetate/acetic acid buffer (pH 4) solution

7. Incubate at room temperature for 1 minute

8. Spin for 1 minute

9. Load the column with sterile ddH_2O

10. Spin for 1 minute

A different strategy is used to purify the poly-A$^+$ fraction of eukaryotic mRNAs, aiming to exclude the most abundant RNA classes like rRNAs, where oligo-dT covalently linked on the surface of polysaccharidic beads or similar solid supports are employed, as described earlier.

Many suppliers indicate, in instruction of such kits, that oligo-dT supports may be reused, and provide regeneration protocols always based on RNA hydrolysis by NaOH treatments, which will destroy any RNA traces, leaving unmodified the DNA component (oligo-dT).

4. Protein purification resins

4.1 IMAC

The use of immobilized-metal affinity chromatography (IMAC) for protein purification was firstly described and showed by Porath *et al.* (1975). Initially developed for purification of native proteins with an intrinsic affinity to metal ions, IMAC shows numerous application fields spanning from chromatographic purification of metallo and phosphorylated proteins, antibodies and recombinant His-tagged proteins. IMAC is also used in proteomics approaches where fractions of the cellular protein pool are enriched and analyzed differentially (phosphoproteome and metalloproteome).

IMAC is a chromatography method that can simply be scaled up linearly from milliliter to liter volumes (Block *et al.*, 2008; Hochuli *et al.*, 1988; Kaslow and Shiloach, 1994; Schäfer *et al.*, 2000) and Ni-NTA Superflow columns are in use for biopharmaceutical production processes.

It is based on the known affinity of transition metal ions such as Zn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+} to certain amino acid in aqueous solutions (Hearon, 1948). Amino acids as histidine, cysteine, tryptophan, tyrosine, or phenylalanine, working as electron donors on the surface of proteins, are able to reversibly bind transition metal ions that have been immobilized by a chelating group covalently bound to a solid support. Histidine represents the preferential choice in protein purification using IMAC since it binds selectively immobilized metal ions even in presence of free metal ions excess (Hutchens and Yip, 1990b); additionally, copper and nickel ions have the greatest affinity for histidine.

Great improvement in development of IMAC chromatographic procedures was achieved by the introduction of DNA engineering techniques allowing the construction of fusion proteins in which specific affinity tags as 6xHis tag are added to the N-terminal or C-terminal protein sequence; the use of these strategies simplifies purification of the recombinant fusion proteins (Hochuli *et al.*, 1988). Moreover the identification or invention of chelating agent able to be both covalently bound to a support and interact with transitional metal ions contributed to the definition of IMAC for high-quality protein purification.

The chelating group that has been first used for IMAC proteins purification is iminodiacetic acid (IDA) (Porath *et al.*, 1975). IDA was charged with metal ions such as Zn^{2+}, Cu^{2+}, or Ni^{2+}, and then used to purify a variety of different proteins and peptides (Sulkowski, 1985)

The tridentate IDA group binds to three sites within the coordination sphere of divalent metal ions such as copper, nickel, zinc, and cobalt (Fig. 8). When copper ions (coordination number of 4) are bound to IDA, only one site remains available for interaction with proteins (Hochuli *et al.*, 1987). For nickel ions (coordination number of 6) bound to IDA, three valencies are available for imidazole ring interaction while it is unclear whether the third is sterically able to participate in the interaction binding to proteins. Thus Cu^{2+}-IDA complexes are stable on the column but have lower capacity for protein binding. Conversely, Ni^{2+}-IDA complexes bind proteins more avidly, but Ni^{2+}-protein complexes are more likely to dissociate from the solid support.
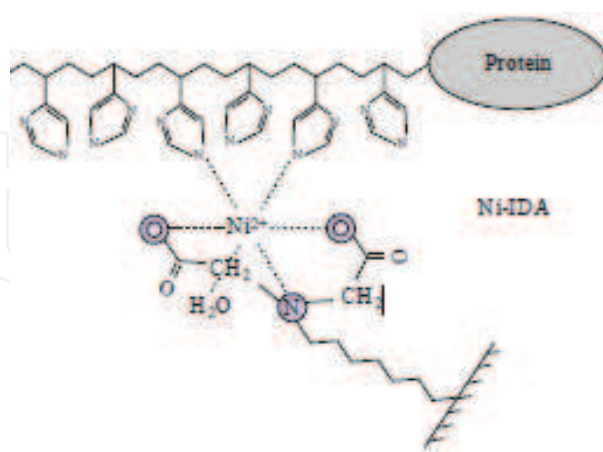

Fig. 8. Model of the interaction between residues in the His tag and the metal ion in tridentate (IDA) IMAC ligand.

The development of a new metal-chelating adsorbent, nitrilotriacetic acid (NTA), has provided a convenient and inexpensive tool for purification of proteins containing histidine residues (Hochuli *et al.*, 1987). The NTA chelating agent coordinates Ni^{2+} with four valencies

(tetradentate, coordination number 4) leaving two valencies available for binding to electron donor groups (i.e., histidine) on the surface of proteins (Fig. 9).
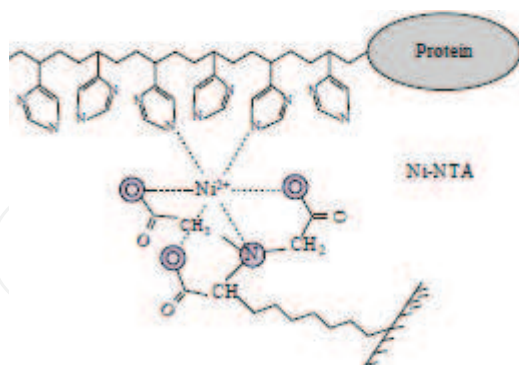

Fig. 9. Model of the interaction between residues in the His tag and the metal ion in tetradentate (NTA) IMAC ligand.

The coordination number plays an important role regarding to the quality of the purified protein fraction but not in protein yield. IDA has only 3 metal-chelating sites and cannot tightly bind metal ions, a relative weak binding leads to ion leaching after loading with strongly chelating proteins or during washing steps. This results in impure products, and metal-ion contamination of isolated proteins; meanwhile protein recovery is usually similar between the two chelating agent. Thus the advantage of NTA over IDA is that the divalent ion is bound by four rather than three of its coordination sites. This minimizes leaching of the metal from the solid support and allows for more stringent purification conditions (Hochuli, 1989).

The NTA also binds Cu^{2+} ions with high affinity, but this occupies all of the coordination sites, rendering the resulting complex ineffective for IMAC. Another tetradentate ligand is a chelating agent commercially known as Talon resin, consisting in carboxymethyl aspartate (CM-Asp), available as cobalt-charged (Chaga *et al.*, 1999).

The lowest metal leaching is obtained using *N*,*N*,*N'*-tris(carboxymethyl)ethylenediamine (TED), a pentadentate ligand (Fig.10). Because TED coordinates ions extremely tightly, such chelators represent a valid alternative expecially if low metal ion contamination is needed; nevertheless only one coordination site is avalaible for His tag binding and protein recovery is substantially lower than IDA or NTA
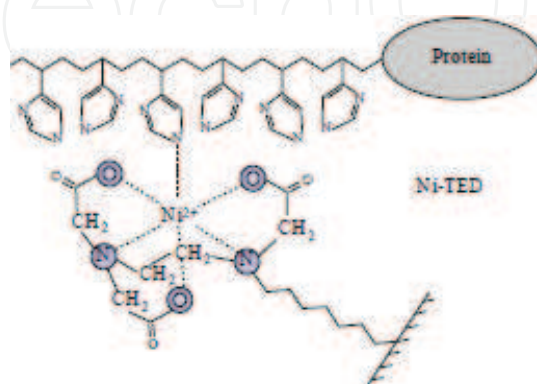

Fig. 10. Model of the interaction between residues in the His tag and the metal ion in pentadentate (TED) IMAC ligand.

The choice of the metal ion immobilized on the IMAC ligand depends on the application.

Whereas trivalent cations such as Al^{3+}, Ga^{3+}, and Fe^{3+}(Andersson and Porath, 1986; Muszynska et al., 1986; Posewitz and Tempst, 1999) or tetravalent Zr^{4+} usually immobilized to IDA (Zhou et al., 2006) are preferred for phosphoproteins and phosphopeptides capturing, divalent Cu^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+} ions are preferentially used for purification of His-tagged proteins. Combinations of a tetradentate ligand that ensure strong immobilization, and a metal ion that leaves two coordination sites available free for imidazole interaction (Ni^{2+} and Co^{2+}) allow similar recovery yield and eluted proteins quality. Immobilized copper or nickel ions bind native proteins with a K_d of $1x10^{-5}$ M and $1.7x10^{-4}$ M, respectively (Hutchens and Yip, 1990a). The K_d value is reduced for protein produced, using recombinant DNA technology, as chimeric constructs with an epitope containing six or more histidine residues. Addition of six histidines to the protein results only in 0.84 kDa protein mass excess whereas other fusion protein systems utilize much larger affinity groups that must be often removed to allow normal protein function (e.g., glutathione-S-transferase, protein A, Maltose Binding Protein). Furthermore the lack of His-tag immunogenic activity allows injection into animals for antibody production without tag removal. Addition of a His-tag results in an enhanced affinity for Ni^{2+}-NTA complex binding due to K_d value of 10^{-13} M at pH 8.0 even in the presence of detergent, ethanol, 2 M KCl (Hoffmann and Roeder, 1991), 6 M guanidine hydrochloride (Hochuli et al., 1988), or 8 M urea (Stüber et al., 1990) allowing protein purification under both native and denaturing conditions, as well as both oxidizing and reducing conditions providing a stringent environment avoiding host strain proteins co-purification (Jungbauer et al., 2004). Nevertheless proteins intrinsically expressing chelating amino acids, such as histidine on their surface, are able to interact with an IMAC support and, although usually with lower affinity than a His-tagged protein, co-purify. In E. coli, proteins observed to copurify with His-tagged target proteins, especially in native conditions, can be classified into four groups (Bolanos-Garcia and Davies, 2006):

1. proteins with natural metal-binding motifs,
2. proteins displaying histidine clusters or stretches on their surfaces,
3. proteins interacting directly or not with heterologously expressed His-tagged proteins,
4. proteins showing affinity to IMAC support such agarose or sepharose based supports.

Furthermore, some copurifying proteins seem to have a binding preference for Co^{2+} over Ni^{2+} (or other ions) and others vice versa. Several options have been developed in order to reduce the contaminating amount of copurified quote or avoiding their adsorption to the matrix, including additional purification steps, adjusting the His-tagged protein to resin ratio, to using an engineered host strain that does not express certain proteins, using an alternative support, tag cleavage followed by reverse chromatography and reduction of non specific binding by including imidazole in the lysis and washing buffer.

Since there is an higher potential of binding background contaminants under native conditions than under denaturing conditions, low concentrations of imidazole in lysis and wash buffers (10–20 mM) could be used. The imidazole ring is part of histidine structure and it's responsible for Ni-NTA interaction (Fig. 11).

At low imidazole concentrations, non specific binding is prevented, while 6xHis-tagged proteins, because of the K_d value derived, still bind strongly to the Ni-NTA matrix allowing greater purity in fewer steps.
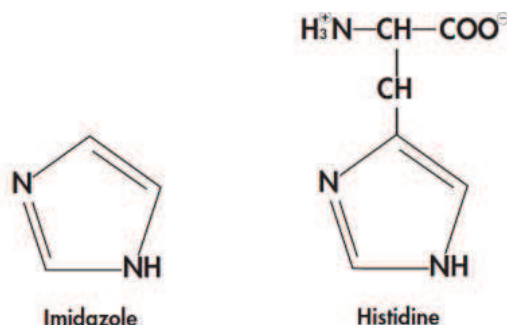
Fig. 11. Chemical structures of histidine and imidazole.

Binding of tagged proteins to Ni-NTA resin is not conformation-dependent and is relatively not affected, within a certain concentration range, by most detergents and denaturants, so Triton X-100 and Tween 20 (up to 2%), or high salt concentrations (up to 2 M NaCl) can be used, resulting in nonspecific binding reduction without affecting specific interaction.

As previously described, purification of tagged proteins under native conditions is often associated with copurification of coupled proteins such as enzyme subunits and binding proteins present in the expressing host (Le Grice and Grueninger-Leitch, 1990; Flachmann and Kühlbrandt 1996). Purification in denaturing condition is performed in presence of strong chaotropic agents such as 6 M GuHCl or 8 M Urea. Under these conditions the 6xHis tag on the protein surface is fully exposed so that binding to the Ni-NTA matrix will improve, and the efficiency of the purification procedure will be maximized by reducing the potential of non specific binding. The histidine tail binds to the Ni^{2+}-NTA resin via the imidazole ring of the histidine residues. At pH $\geq$7.0, the imidazole side chain is deprotonated, leading to a net negative charge interacting with Ni^{2+}-NTA; at pH 5.97 (corresponding to imidazole pK_a), 50% of the histidines are protonated; finally, within pH values $\leq$4.5, almost all of the histidines are protonated and unable to interact with Ni^{2+}-NTA. Thus, there are, generally, three different methods for His-tagged proteins recovery after washing steps based on chemical and cinetical counterpart features that can be used for both native or denaturing purifications.

A "competition derived approach" based on Ni^{2+}-NTA affinity for imidazole, working as competitor, increasing imidazole concentrations results in protein displacement from the support at constant pH. Under these conditions the 6xHis-tagged protein can no longer bind to the nickel ions and will dissociate from the Ni-NTA resin.

An alternative procedure uses buffers of decreasing pH to elute the histidine tail ensuring efficient recovery from Ni^{2+}-NTA (Hochuli *et al.*, 1988). Disadvantages are that the pH must be maintained accurately at all temperatures and that some proteins may not be able to withstand the extreme pH change required for protein elution.

An optional method is based on the stripping ability of certain reagents such as EDTA or EGTA in chelation of nickel ions and their removal from the NTA groups. This results in the 6xHis-tagged protein elution as a protein–metal complex. NTA resins, so stripped, appear white in color because they have lost their nickel ions and must be recharged if additional purification steps have to been performed.

Whereas all elution methods (imidazole, pH, and EDTA) are equally effective, imidazole is recommended under native conditions, when the protein would be damaged by a pH reduction or when the presence of metal ions in the eluate needs to be avoided

4.2 Cleaning and regeneration of Ni-NTA resins

The suitability of IMAC for industrial production purposes has been largely demonstrated and it can be expected that IMAC-based procedures will acquire increasing application because of its robustness and relatively low requirements for individual optimization. In contrast to these facilities it's noteworthy the production of a large amount of discarted materials consisting in metal-chelating groups, IMAC supports such as agarose and sepharose ones and, above all, considerable metal transition amounts to be disposed.

In order to reduce the environmental impact of such wastes, several IMAC commercially manufacturers have introduced and developed protocols allowing to reuse the same resin after regeneration and equilibration step cycles. Regeneration methods, enabling the flush out of any contaminating materials from previously purified samples, can be divided into 2 different classes:

1. CIP (cleaning-in-place) protocols;
2. Stripping and recharging.

A simple and effective cleaning procedure for Ni-NTA resins used to purify proteins from different samples is represented by the incubation of such resins with a non-flammable, bacteriostat 0.5M NaOH solution for 30 min in 15 column volumes (Schäfer *et al.*, 2000) allowing denaturation and desorption of unspecifically resin-attached proteins.

<div style="border:1px solid #ccc;padding:1em;">

Box 3. Ni-NTA agarose regeneration protocol

1. Wash the column with 2 volumes of Regeneration Buffer (6 M GuHCl, 0.2 M acetic acid).

2. Wash the column with 5 volumes of H_2O.

3. Wash the column with 3 volumes of 2% SDS.

4. Wash the column with 1 volume of 25% EtOH.

5. Wash the column with 1 volume of 50% EtOH.

6. Wash the column with 1 volume of 75% EtOH.

7. Wash the column with 5 volumes of 100% EtOH.

8. Wash the column with 1 volume of 75% EtOH.

9. Wash the column with 1 volume of 50% EtOH.

10. Wash the column with 1 volume of 25% EtOH.

11. Wash the column with 1 volume of H_2O.

12. Wash the column with 5 volumes of 100 mM EDTA, pH 8.0.

13. Wash the column with H_2O.

14. Recharge the column with 2 volumes of 100 mM $NiSO_4$.

15. Wash the column with 2 volumes of H_2O.

16. Wash the column with 2 volumes of Regeneration Buffer.

17. Equilibrate with 2 volumes of a suitable buffer

</div>

Resins stored for long terms in up to 1 M NaOH do not show any significant effect on metal-leaching rates corresponding to 1 ppm under any conditions without compromising its performance.

For repeated reuse of a Ni-NTA column, the CIP procedures had to be followed by a reequilibration step. Furthermore for long-term storage, resin may be kept in 30% (v/v) ethanol to inhibit microbial growth. No significant changes of metal-ion leaching were observed during five CIP runs, moreover the binding capacities for 6xHis-tagged protein of Ni-NTA resins remained unchanged from run 1 to run 5 (Schäfer *et al.*, 2000).

Due to the high chelating strength and the resulting low metal-leaching rate of all Ni-NTA IMAC resins, stripping is not required even after repeated reuse or long-term storage. However, reduction in binding capacity or resin damages for example, by repeated purification of samples containing chelating agents, could happens. In this cases Ni-NTA may be stripped and recharged with nickel or a different metal ion using combination of chelating steps (EDTA treatments) ensuring a Ni^{2+} free medium, followed by nickel salts incubation. Metal chloride and sulfate salts, (e.g. 0.1 M $NiSO_4$) are commonly used. Here we report (box 3)a stripping and recharging protocol based on Qiagen instruction for relative Ni-NTA agarose resins

4.3 IMAC for industrial-scale protein production and Ni^{2+} environmental impact

IMAC for production of proteins in industrial scale, has not been used until quite recently due to worries regarding allergenic effects of nickel leaching from an IMAC matrix. During protein purification 1ml or resins is usually used for each 30-40 mg recombinant proteins. Several data describing nickel leaching from resins show that nickel concentrations in the peak elution fractions is below 1 ppm under all conditions, including denaturant or native conditions. More specifically even after several purification steps followed by CIP, the level of nickel contamination in the peak elution fractions is comprised between 0.3 and 0.6 ppm for native and denaturing conditions, respectively (Schäfer *et al.*, 2005). The discarded cations are released as liquid or dry waste into the environment where it's just present under many forms.

Nickel, occurs naturally in the earth's crust, in various forms such as nickel sulphides and oxides, its sources arise from earth's molten core where it is trapped and unusable to volcanic eruptions, soils, ocean floors, and ocean water (Stimola, 2007).

Such divalent cation is used not only in metallurgic industries to make stainless steel but also in other application fields such as in coinage in various forms of 'costume' or 'fashion' jewellery. The different forms of nickel include elemental nickel (Ni), nickel oxide (NiO), nickel chloride ($NiCl_2$), nickel sulphate ($NiSO_4$), nickel carbonate ($NiCO_3$), nickel monosulfide (NiS), and nickel subsulfide (Ni_3S_2) (ATSDR, 2005).

Human exposure to nickel is associated with drinking water, food, or smoking tobacco containing nickel or direct contact with nickel-containing products, such as jewelry, stainless steel and coins. The average concentration of nickel in different categories of soil span from 4 to 80 ppm, but this number has increased significantly (up to 9,000 ppm) around nickel producing industries (ATSDR, 2005). Skin contact is the usual source of contamination from the ground unless for children who are more likely to ingest soil particles. Foods such as tea, coffee, chocolate, cabbage, spinach and potatoes contain high levels of nickel, making these foods a major source of exposure. The average amount of nickel introduced is 70 micrograms of nickel per day.

This rapid analysis suggests nickel concentrations typically observed in protein preparations obtained from tetradentate IMAC resins are low and content in expected daily doses of protein used such as biopharmaceutical will be far below the typical daily intake of nickel.

4.4 Amylose affinity chromatography

The expression and purification of recombinant proteins compared to native ones represent an efficient system to product any protein. As previously described for IMAC tag, recombinant DNA techniques allow the construction of fusion proteins in which specific affinity tags are added to the protein sequence of interest, facilitating the recombinant fusion proteins purification by the use of affinity chromatography methods.

Maltose-binding protein (MBP) is one of the older and more popular fusion partners used for recombinant proteins production in bacterial cells; it's coded by the *malE* gene of *Escherichia coli* as part of maltose/maltodextrin system (Nikaido, 1994). MBP, despite the molecular weight (42.5 kDa) is considered one of the best choises to solve problems related to heterologous protein expression since it acts as protein production and solubilisation enhancer by mechanisms far to be completely understood (Randall *et al.*, 1998; Nomine *et al.*, 2001; Sachdev and Chirgwin, 1998). Several commercial plasmid DNA vectors have been constructed allowing expression of a cloned protein or peptide by fusing it to MBP (Guan *et al.*, 1988; Bedouelle and Duplay, 1988; Maina *et al.*, 1988). The isolation and purification of recombinant proteins MBP fused can be performed using an easy affinity column procedure amylose based resins dependeding on MBP affinity for maltose packaged in the amylose resins (K_d value of MBP for maltose is 3.5 µM) (Kellerman and Ferenci, 1982). A crude cell extract, in absence of detergent or chaotropic agents, is prepared and passed over a column containing an agarose resin derivatized with amylose, a polysaccharide consisting of maltose subunits.
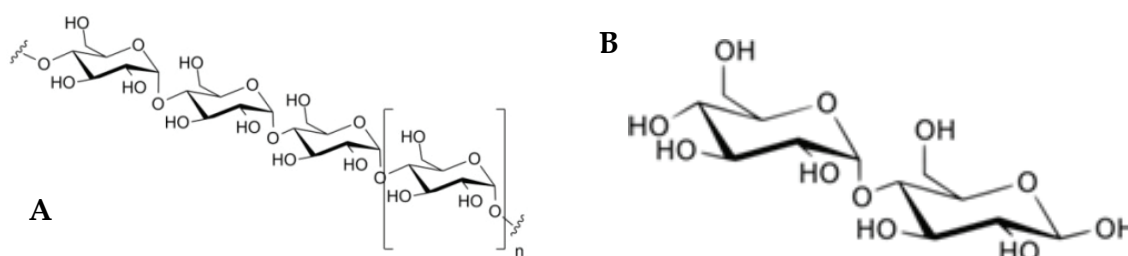

Fig. 12. Chemical structures of amylose (A) and maltose (B). Glucose monomers (2 units in maltose, several hundreds in amylose) are joined with an α(1→4) bond.

Such resin can be purchased from commercial suppliers in it's original form (amylose based) or in an maltoheptaose version similar to amylose one, but with lower molecular weight glucose polymers resulting in a theorical larger number of potential binding sites. Three amylose affinity chromatography matrices are manufactured by New England BioLabs (Cattoli and Sarti, 2002):

1. Amylose magnetic beads;
2. Amylose agarose resin;
3. High flow support matrix.

Amylose magnetic beads have a binding capacity up to 10 µg/mg (supplied as a 10 mg/ml suspension). Amylose agarose has a binding capacity of 3 mg/mL for MBP and 6 mg/ml for an MBP-β-galactosidase protein. The typical flow velocity of the amylose resin is 1 ml/min in a 2.5 cm x 10 cm column, and the matrix can withstand small manifold vacuums (universally known as "piglet"). The amylose matrix can suffer from flow restrictions. So that total protein loading should be ≤2.5 mg/ml. Amylose high flow has a binding capacity of approximately 7 mg/ml for an MBP-paramyosin protein. The exact chemical nature of the

matrix is not described but has a pressure limit of 0.5 MPa (75 psi), a maximum flow velocity of 300 cm/h, and recommended velocities are below 60 cm/h being 10–25 ml/min (for Ø1.6-cm and Ø2.5-cm columns respectively).

Alternatively, home-made amylose-agarose resin can be prepared following procedures described by Lee *et al.* (1990). Pratically, sepharose beads are washed with water and then incubated with 1M sodium carbonate pH 11 allowing to react in presence of vinyl sulfonic acid. Activated resin is derivatized by mixing, in 1 M sodium carbonate pH 11 environment, with an amylose solution. The resulting matrix can be freshly used or in 20% ethanol stored.

In contrast with an IMAC conformation-independent binding of tagged proteins to Ni-NTA resin, MBP's affinity to amylose and maltose depends on hydrogen bonds patterns derived from the three-dimensional structure of the protein; agents interfering with hydrogen bonds or the protein structure interfere with binding as well. For these reasons protein purification of tagged proteins can be performed under native conditions only, (Tris-HCl, MOPS, HEPES, and phosphate, buffers at pH values between 6.5 and 8.5) in presence or absence of optional additives as 1 mM sodium azide, 10 mM β-mercaptoethanol or 1 mM DTT. Such reducing agents can be added to mantein reduced cysteins avoiding non specific disulphide bridges formation resulting in tedious aggregations. Moreover higher ionic strength does not adversely affect MBP binding to amylose, so that 1M NaCl can be used to reduce non specific protein binding to resin.

Despite MBP's affinity of some fusions to amylose is dramatically reduced in presence of nonionic detergents (0.2% Triton X–100 or 0.25% Tween 20) resulting in<5% binding, other fusions are unaffected. Binding is efficient in the presence of 5% ethanol or acetonitrile, as well as in 10% glycerol. 0.1% SDS completely eliminates binding.

Furthermore low levels of residual detergents, especially from regeneration solutions, (see below) can still remain; removal of detergent and mixed micelles can be achieved using dilute methanol-containing solutions

After several washing steps, protein elution and recovery is performed in a "competition derived approach" based on MBP affinity for maltose. Maltose working as competitor at 10 mM concentration, results in protein displacement from amylose at constant pH value.

Because the presence of substantial amounts of amylases in the crude extracts interferes with binding, by cutting the fusion off the column or by releasing maltose that elutes the fusion from the column, the amylose resin "half-life" depends on incubation time with trace amounts of contaminant. Manufacturers instructions and recommendations explain (e.g. NEB): "Under normal conditions defined as 15 ml of amylose agarose matrix processing, 1 liter of LB media supplemented with 0.2% glucose (producing 40 mg MBP fusion protein); a matrix binding capacity reduction of 1–3% after each purification step is reported. It is stated that such a column may be used up to 5 times before a decrease in yield is detectable (5–15% lost binding capacity), and up to 10 times to achieve an evident reduction (10–30% lost binding capacity)".

Column reuse and regeneration can be performed according to New England BioLabs following sequence of washes in water, saline buffer (20 mM Tris-HCl, 200 mM NaCl and 1 mM EDTA), and 0.1% SDS, or by a very short treatment using 0.1 N NaOH followed by a neutralization step.

Alternatively Pattenden *et al.* (2008) proposed a regeneration procedure based on sequential amylose resin treatments with two different regeneration solutions:

1. Regeneration 1: 50 mM HEPES, 4 M Urea, 0.5% w/v SDS pH 7.4.
2. Regeneration 2: 50 mM HEPES, 150 mM $(NH_4)_2SO_4$, 2 mM EDTA, 2 mM EGTA pH 7.4.

Regenerated resin can be stored in 20% ethanol at 4 °C

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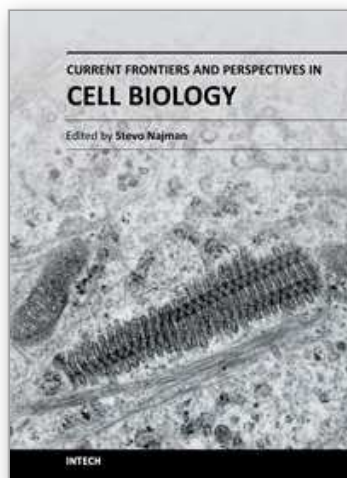
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