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Review

Ribonucleic acid purification

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ABSTRACT

Research on RNA has led to many important biological discoveries and improvement of therapeutic technologies. From basic to applied research, many procedures employ pure and intact RNA molecules; however their isolation and purification are critical steps because of the easy degradability of RNA, which can impair chemical stability and biological functionality. The current techniques to isolate and purify RNA molecules still have several limitations and the requirement for new methods able to improve RNA quality to meet regulatory demands is growing. In fact, as basic research improves the understanding of biological roles of RNAs, the biopharmaceutical industry starts to focus on them as a biotherapeutic tools. Chromatographic bioseparation is a high selective unit operation and is the major option in the purification of biological compounds, requiring high purity degree. In addition, its application in biopharmaceutical manufacturing is well established. This paper discusses the importance and the progress of RNA isolation and purification, considering RNA applicability both in research and clinical fields. In particular and in view of the high specificity, affinity chromatography has been recently applied to RNA purification processes. Accordingly, recent chromatographic investigations based on biorecognition phenomena occurring between RNA and amino acids are focused. Histidine and arginine have been used as amino acid ligands, and their ability to isolate different RNA species demonstrated a multipurpose applicability in molecular biology analysis and RNA therapeutics preparation, highlighting the potential contribution of these methods to overcome the challenges of RNA purification.

Contents

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1. Introduction

Until recently, RNA was overlooked compared to DNA or proteins, consigned to a simple intermediate role in the flow of information from genes to functioning molecules in living cells. RNA is now known to play many more functional roles and to be responsible for a multitude of essential biological processes [1]. In the last 20 years RNA was the subject of four Nobel prizes winning discoveries – 1989 for catalytic RNA, 1993 for splicing, 2006 for RNA interference (RNAi), and 2009 for the ribosomal structure [2] and new roles for RNA in biology continue to emerge at a glance. All of these discoveries have revealed so far that RNA is truly a remarkable and multi-talented cellular component with fundamental implication on biotic evolution and heredity. Furthermore, the widespread involvement of RNA in the regulation of numerous genes has highlighted its vast therapeutic potential [3]. These and similar breakthroughs have led to the emergence of numerous types of RNA-based therapeutics either using RNA as a therapeutic agent or a therapeutic target. Table 1 shows potential therapeutic approaches for RNA, specifying the involved RNA or RNA-based molecules and their mechanism of activity.

The successful results of these novel therapeutic approaches are reinforcing the focus on RNA investigation and are rendering RNA molecules into new targets for pharmaceutical and biotechnological industries [4]. Due to the increasing number of structural, biophysical and biomedical studies that require large quantities of homogeneous good-quality RNA, a widespread need to improve production scale and RNA isolation and purification schemes has been recognized.

As RNA emerges into the new class of biotherapeutic products, pharmaceutical-grade RNA, produced under the current good manufacturing practice (cGMP) is crucial. Thus, it will be essential that the bioproduct fulfil the requirements of regulatory authorities such as Food and Drug Administration (FDA), European Medicine Agency (EMA) and World Health Organization (WHO). However, non-consensus still exists for regulation of mRNA vaccination and RNA oligonucleotides-based therapies. In the European Union, mRNA-based therapies are based on the regulation for advanced therapy medicinal products – EC No 1394/2007 – [5] which refers to directive 2001/83/EC [6]. Here, a 'Gene therapy medicinal product is an active substance which contains or consists of a recombinant nucleic acid used in or administered to human beings'. In the United States of America, in contrast, mRNA vaccines are not categorized as gene therapy [7]. On the other hand, RNA oligonucleotides products, such as siRNAs or aptamers, are regulated as drugs under FDA's Centre for Drug Evaluation and Research and are not considered as an advanced therapy in the European Union (not classified as gene therapy) [8]. Currently, no regulatory authority has formal guidelines available for RNA oligonucleotide products or mRNA molecules. The guidelines established for human cell-based medicinal products [5] and DNA vaccines [9] are providing the guidance in the regulatory framework for RNA-based therapies [8,10]. Nevertheless, those guidelines do not focus on particular features of RNA, such that as shown in clinical trials, RNA-based therapies do not confer the risks of integration into the genome. The scientific community, in the form of volunteer members from the industry and regulatory agencies worldwide, are actively engaged in addressing topics such as quality specifications and impurities in RNA bioproducts [11].

Consequently, economically feasible processes for RNA isolation and purification, as well as the implementation of methodologies able to control RNA quality suitable for industrial manufacturing, will be increasingly necessary, especially when the RNA products are finally released to the market.

However, the isolation and purification of RNA are critical steps because of the easy degradability of RNA, consequence of the peculiar structural chemistry, which can impair chemical stability and biological functionality, and can limit the success of subsequent RNA investigations.

Therefore, this paper focuses the challenging task of isolating and purifying RNA molecules. The current state of the isolation and purification methodologies used for RNA preparation will be discussed regarding the growing demands in RNA applicability. Moreover, promising affinity approaches based on chromatographic purification exploiting the biorecognition between amino acids ligands and RNA molecules will be introduced. These new strategies bring new insights into the way RNA can be purified, contributing to the future development of new and more robust bioseparation methods.

2. RNA chemistry

RNA has a number of unique chemical characteristics that have profound structural consequences with remarkable implications in cell biology and are a real challenge in research activities.

RNA is a polymer organized in a long chain of ribonucleotide monophosphates, but it resembles DNA in many ways (Fig. 1). RNA shares the same chemical units as DNA, however there are two fundamental differences that distinguish DNA from RNA. RNA has the nucleotide uracil (U) instead of thymine (T) and the ribose 2′-OH group on each RNA nucleotide is absent in DNA. Consequently, the deoxyribose sugar in DNA is less reactive because of C-H bonds. This leads to a greater resistance of DNA to alkaline hydrolysis. Accordingly, RNA is less stable than DNA because its vicinal 2′-OH group makes the 3′-phosphodiester bond susceptible to nucleophilic cleavage, and so it is readily hydrolyzed by hydroxide ions [12]. In addition, RNAs adopt dissimilar shapes when they are base-paired into a double helix. RNA takes on the geometry structure referred as an A-form helix while DNA takes on the B-form. DNA is commonly found in a double-stranded structure while RNA often comes as single-strand and is quite flexible [13]. However, single-stranded DNA can also occur as an intermediate in some biological processes. During these processes, single-stranded DNA assume specific folded structures to perform essential biological functions during DNA replication, recombination, repair, and transcription [14]. In its turn, RNA can twist itself into a variety of complex structures. Its propensity to form secondary structures facilitates RNA interactions with other molecules by covering some sequences and exposing others for recognition. Besides, RNA can assume tertiary structures that present surfaces for interactions and contain internal environments that create binding sites for metal ions, so that they can promote catalytic reactions [15].

The high chemical reactivity of RNA provides more instability to the molecule, increasing the susceptibility to degradation. This instability is very important for cells, as they can change their patterns of protein synthesis very quickly in response to biological needs [16].

Table 1
RNA molecules with therapeutic involvement.

RNA type	Cell function	Therapeutic concept	References
Protein synthesis			
Messenger RNA (mRNA)	Codes for protein	Vaccination	Kreiter et al. [7]
Ribossomal RNA (rRNA)	Translation	Antibiotic target	Tenson and Mankin [118]
Transfer RNA (tRNA)	Translation Apoptosis regulation	Understand many human diseases	van Raam and Salvesen [119], Belostotsky et al. [120]
Post-transcriptional modification or DNA replication			
Small nuclear RNA (snRNA)	Splicing and other functions	Understand many human diseases	Matera et al. [121]
Small nucleolar RNA (snoRNA)	Nucleotide modification of RNAs	Understand many human diseases	Kiss [122]
Regulation			
Antisense RNA	Transcriptional attenuation mRNA stabilization or degradation Translation block	Inhibitor of mRNA translation	Dias and Stein [123], Brantl [124]
MicroRNA (miRNA)	mRNA cleavage and Translation repression	Gene silencing	Kusenda et al. [125], Lin et al. [126]
Small interfering RNA (siRNA)	mRNA cleavage and Translation repression	Gene silencing	Doench et al. [127], Ghildiyal and Zamore [128]
Ribozymes	RNA enzyme. Catalyze RNA cleavage and ligation reactions	mRNA reprogramming and repair	Phylactou, et al. [129]
Riboswitch	Regulate gene expression by binding to small metabolites	Regulate gene expression Antibacterial drug target	Tucker and Breaker [130], Wittmann and Suess [25], Blount and Breaker [131]
Aptamers	Oligoribonucleotide part of a riboswitch that binds to a specific target molecule with high affinity	Decoy mechanism that inhibits various target proteins	Mayer [132], Ni et al. [133]

These properties consign RNA versatility in cellular processes, namely in gene regulation, which open the possibility of exploring new therapeutic opportunities [17]. On the other hand, the peculiar three-dimensional compaction and structural instability of RNA are huge challenges in laboratory, as the biological activity and integrity can be easily compromised during extraction and purification procedures. Thus, improved methodologies for recovering RNA of high quality is a constant concern [18–20].

3. RNA isolation and purification

RNA methods differ from those used for DNA and proteins [2]. The extraction, isolation, and analysis of RNA are routinely more difficult in comparison to that required for DNA. As raised before, RNA chemistry adds complexity to sample preparation because the ubiquitous presence of RNA-degrading enzymes (RNases) both in biological samples and in the laboratory environment easily degrade RNA, compromising the integrity and biological activity of RNA molecules [18]. Therefore, minimizing RNA degradation by protecting it against RNases requires that all glassware, plastic ware, instrument tubing and reagents be RNase free. Additionally, among the many challenges is the need for maximizing recovery yield, while removing unwanted components, minimizing sample transfers, and avoiding non-specific binding to containers [21]. Therefore, the quality and quantity of RNA preparations are the main concerns of isolation procedures, since the lack of integrity, the presence of contaminants or the low RNA quantity may strongly constrain the success of several RNA based-procedures in basic and clinical research [22]. Additional challenges also emerge with the advance of clinical trials using RNA intended to be administered in humans.

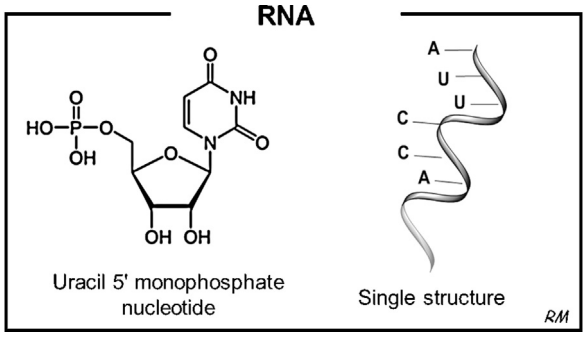

Fig. 1. Structural characteristic of RNA. RNA differs from DNA in the nucleotide uracil, as a thymine exists in DNA, as well as the constituent sugar molecule that is a ribose in RNA and a desoxyribose in DNA. DNA molecules take a double helix structure, while RNA molecules are originally synthesized as single-strands.

Presently, RNA molecules can be obtained by extraction from a biological matrix, such as cells or tissues, or they can be produced by chemical or enzymatic (*in vitro* transcription) synthesis. Chemical synthesis is normally used for the generation of short oligoribonucleotides (<50 nucleotides) while *in vitro* transcription can produce longer RNAs. Synthesized RNAs are being greatly employed in structural, biochemical and biophysical studies [23,24] as well as in the development of new therapeutic approaches by RNA interfering technology, RNA aptamers, ribozymes or mRNA vaccination [25–27]. In these cases, the final RNA product needs to be purified from impurities derived from the synthesis process. These impurities are, besides enzymes, nucleotides, aberrant oligonucleotides, salts or buffer. Longer oligoribonucleotides are more contaminated with aberrant species than short ones. The failure products are prematurely halted as shorter oligonucleotides. Some are mismatch failure sequences where there are missing nucleotides in the middle of the sequence, rather than at the end. Other by-products of synthesis may have greater molecular weight than the target oligoribonucleotide (heterogeneous RNAs in length). This is a result of incomplete post-synthesis deprotection, or due to the branching of an oligonucleotide backbone during the synthesis [28].

On the other hand, biological RNAs are preferably used in basic research for the study of cellular mechanisms, as they reflect intrinsic cell features [29], in clinical investigations for pharmacokinetic and pharmacodynamics analysis [30] and in some strategies of mRNA vaccination using bulk tumour mRNA [31]. In RNA extraction from a biological matrix, the main principle is the disruption of cells and subsequent elimination of host contaminants, such as genomic DNA (gDNA) and proteins, in order to obtain intact and pure RNA molecules [32].

Table 2
Isolation techniques used in RNA preparation (nt, nucleotides; PAGE, polyacrylamide gel electrophoresis; cGMP, current good manufacture practice).

Method	Principle	Advantages	Disadvantages	References
Acid guanidinium thiocyanate–phenol–chloroform	Chaotrope helps cells lysis and inactivates RNases. DNA and Proteins are denatured and removed in the organic phase during acid phenol/chloroform extraction. RNA is precipitated with alcohol and salt.	Inexpensive Enhanced protection against RNases Good yields and purity	Requirement of toxic chemicals Time-consuming Highly operator dependent Inhibit enzyme activity	Chomczynski and Sacchi [32]
Silica purification	Chaotrope helps cells lysis and inactivates RNases. Polyanionic RNA and DNA bind to silica particles by hydrogen interaction in the presence of chaotrope. DNA is digested with DNase. Contaminants are washed away and RNA is eluted with low ionic strength.	Fast Does not require use of organic solvents or alcohol precipitation Amenable to automation	Low binding capacities Purification based on adsorption/desorption mechanisms on solid surfaces. Does not discriminate between RNA or DNA Requires DNase treatment	Wen et al. [38]
Preparative denaturing PAGE	Separates molecules based on their electrical charge and hydrodynamic properties, which are a function of chain length. The desired RNA is eluted from the gel matrix, concentrated, equilibrated in buffer and refolded.	Highly purified product	Time-consuming Introduces contaminants Uses denaturing conditions Low yields	Doudna [24], Hagen and Young [51]
Lithium chloride precipitation	Selective separation of long or short RNA sequences from impurities. Elevated concentration of LiCl is added to impure RNA preparations follow by incubation at −20 °C for several hours or overnight. Precipitates or supernatants are recovered according to required RNA type.	Separation of small RNAs from long RNAs Recovery of long RNAs from impurities of *in vitro* transcription synthesis.	Inefficient precipitations Introduces lithium metal into preparations Employ phenol/chloroform extraction to improve isolation Time consuming	Pascolo [55], Baker, et al. [56], Nilsen [57], Romanovskaya, et al. [60]

Many methods have been developed in an attempt to circumvent the several challenges of purifying RNA molecules and to achieve the goal of good-quality RNA [33–36]. Therefore, considering the recent developments in RNA understanding and the growing demand on its purification, the next discussion intends to briefly introduce the isolation and purification methods used in RNA preparation. This description will draw particular attention to the main problems that can limit the success of RNA research or that can make the procedures not suitable to obtain RNA to be further applied in clinical investigation, also focusing on the cost-effectiveness for preparative-scale or large-scale industrial applications (Tables 2 and 3).

3.1. RNA isolation methods

3.1.1. Chemical and solid-phase extractions

Traditionally two types of isolations are used in RNA preparations, (1) chemical extraction using denaturing agents and organic solvent precipitation and (2) solid-phase extraction by immobilizing RNA on a glass support. These methods generally include a chaotropic agent, denaturant, or other chemical in the lysis step to inactivate RNases.

Although the RNA extraction methods are similar to DNA procedures, the main difference is the working pH. RNA is extracted under acidic conditions, while for DNA mild alkaline extractions are preferred [37]. This difference is related to the chemical stability of each molecule, as previously discussed (Section 2). The acidic pH is the critical factor to ensure the separation of RNA from DNA and proteins [32,33]. Therefore, chemical extraction that involves acid phenol/chloroform extraction is currently the most employed method either performed with home-made solutions or commercial ready-to-use reagents, because it leads to high recovery yields and purity of total RNA [32]. Briefly, this technique allows RNA separation from DNA and proteins after extraction with an acidic solution consisting of guanidinium thiocyanate, sodium acetate, phenol, and chloroform that allow the formation of two phases. RNA remains in the upper aqueous phase of the whole mixture, while proteins and DNA remain in the interphase or lower organic phase. Recovery of total RNA is then achieved by precipitation with isopropanol [33,37]. However, these extractions are extremely toxic and hazardous and highly operator dependent. Sometimes they can involve up to six or more steps and three sample transfers leading to time consuming and laborious RNA preparations. Although this method is almost always included in RNA purification schemes, the organic solvents such as alcohols and phenol/chloroform may interfere in the majority of routine molecular biology techniques because they can inhibit enzymes activity. Moreover, as those compounds convey health risks, this procedure is not tolerable for the welfare of the researcher and should not be an integral part of the process for a therapeutic formulation. However, RNAs that are being employed in clinical trials are often extracted using phenol/chloroform [31]. This should certainly be considered and advised by regulatory authorities.

The other methods for RNA isolation are based in solid-phase extraction using silica membranes, as prefilled columns or as magnetic beads, in combination with phenol/guanidine-based lysis of samples. This technology was developed to offer safer and simpler operations, as they are amenable to automation using liquid handling robotics [38]. In fact, these techniques significantly reduce sample preparation time, but can still involve multiple sample transfers and time-consuming evaporation steps. Low RNA yields are often obtained due to the low binding capacity of the cartridges, which can be easily overloaded [38,39]. Moreover, these processes lack specificity as they are non-discriminatory for DNA or RNA. Silica matrices have no specific characteristics to discriminate between nucleic acids. Silica surface is composed of

Table 3
Purification methods used in RNA preparation (PAGE, polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; dsRNA, double-stranded RNA; SPE, solid phase extraction; DEAE, diethylaminoethyl).

Chromatographic method	Matrix functionalization	Advantages	Disadvantages	References
Reversed-phase	Modified silica with hydrocarbon chains (normally C8 or C18)	Based on differences in hydrophobicity High resolution Efficient purification	Limited to short RNA sequences (<50nt) Requirement of toxic chemicals Uses denaturing conditions	McGinnis, et al. [21]
Ion-pairing reversed-phase	Polystyrene-divinyl benzene beads	In combination with SEC improves the purification of biological non-coding RNAs Synthetic mRNA purification at preparative scale Non-denaturing conditions Use of simpler solvents	Difficult to scale up Still use toxic solvents	Dickman [66], Chionh et al. [68], Kariko et al. [69]
Size exclusion	Cross-linked dextran Cross-linked agarose gel	Powerful as final polishing step in oligoribonucleotides purified by HPLC Effective alternative to preparative PAGE purification of *in vitro* transcribed RNA Non-denaturing conditions	Time-consuming Highly operator dependent	Lukavsky and Puglisi [52], Kim et al. [53], McKenna et al. [64]
Anion-exchange	Polystyrene/divinyl benzene poly-(methyl-methacrylate) poly(glycidyl methacrylate-co-ethylene dimethacrylate) functionalized with strong quaternary amine	Purification of synthetic dsRNA, rather than separated single-strands Effective alternative to preparative PAGE purification of *in vitro* transcribed RNA Non-denaturing conditions Large scale preparation of dsRNA using monolithic columns	RNA folding can be compromised by chromatographic conditions	Noll, et al. [75], Koubek, et al. [79], Romanovskaya, et al. [60]
	DEAE Sepharose	Effective alternative to preparative PAGE purification of *in vitro* transcribed RNA Natively folded RNA Non-denaturing conditions Scaled up methods	Three columns in series to achieve purification Does not purify RNAs with homogeneous 3′ends	Easton, et al. [20]
	Latex coated monolith anion-exchange (Quaternary ammonium ion, diethyl methyl amine)	Separates short synthetic RNAs from isomers impurities Evaluation and characterization of therapeutic synthetic RNAs High efficiencies and capacities Lab-scale	Difficult to scale up	Thayer et al. [78]

silanols and their presence is responsible for the adsorption properties of silica gel [40]. Interaction with silica surface has been intensively studied to illustrate adsorption/desorption processes [41–44]. However, the mechanistic details are not fully understood. In the majority of reported protocols, nucleic acids extraction from biological samples is performed by inducing the binding onto a silica surface in the presence of high salts concentration (*e.g.* 4 M or 6 M guanidinium salts) [45,46]. The chaotropic agent guanidinium salt favours nucleic acids binding to silica because it destroys the hydration shell that is surrounding the nucleic acids. This allows positively charged ions to form a salt bridge between the negatively charged silica and the negatively charged DNA and RNA under high salt conditions. By using this high salt environment, the nucleic acids can be washed, in order to remove all other contaminants [43]. It is also known that pH has a strong influence on the adsorption of nucleic acids on silica surfaces [41]. Several companies have tried to develop refined silica membranes in order to improve RNA binding over DNA. RNeasy® (Qiagen), PureLink™ RNA (Invitrogen), NucleoSpin® RNA (MACHEREY-NAGEL) are some examples of new silica membranes-based approaches with successful results. These technologies combine the selective binding properties of silica-based membranes with the speed of micro spin technology. In general, samples are lysed and then homogenized in the presence of large amounts of a highly denaturing guanidine salt-containing buffer, which protects RNA from endogenous RNases ensuring the intact purification of the molecules, and creates appropriate binding conditions for the favoured adsorption of RNA to the silica membranes. After homogenization, ethanol is added to the samples to improve the binding. Impurities are removed by subsequent washing buffers. High-quality RNA is then eluted in RNase-free water or under low ionic strength conditions. Nevertheless, in most cases the isolation of pure RNA is achieved by a secondary enrichment, either through enzymatic removal of DNA (DNase treatment) or by a second step using specific columns [39]. These limitations can greatly diminish the success of several molecular biology investigations, in particular gene expression analysis. Sample contamination with DNA will provide false results in gene amplification by real time-PCR [47]. In addition, the treatment with enzymes, such as commercial DNases that are often animal-derived, is not prudent in therapeutic applications [9].

Recent developments in high throughput technologies led to the design of large and complex instrumentation using silica technology to simplify the isolation of nucleic acids. Automation for nucleic acids extraction offers high-throughput sample processing, high quality and effective purification since the human impact is restricted to a minimum [39]. Therefore, less contaminants or inequalities concerning time, volumes of chemicals or improper handling are avoided. Automation therefore increases the efficiency, accuracy and velocity of RNA purification [48]. Nevertheless, cross contaminations can occur by the sample collector arm. In these systems, the principles of purification are based in solid-phase extraction, which presents some limitations, as previously mentioned. While the high cost disadvantage of these systems could be overcome by the high-throughput of automation, the lack of efficacy in the isolation method makes the investment not worth it. Actually, automated extraction systems may even be advantageous to some laboratories, since consistent and reproducible purified samples in sufficient quality and purity are achieved for subsequent application [49].

3.1.2. *Polyacrylamide gel electrophoresis (PAGE)*

The use of preparative denaturing polyacrylamide gel electrophoresis (PAGE) has also been extensively described to

complement the schemes described above in the preparation of RNA, especially synthetic RNA [24,28,50,51]. Preparative denaturing PAGE has been successful and continues to be the most popular method to resolve RNA samples, but it presents several disadvantages. The protocol is very lengthy as after denaturing PAGE, the desired RNA must be eluted from a gel matrix, concentrated, equilibrated in the desired buffer, and refolded [24]. Moreover, this introduces acrylamide contaminants, which are difficult to remove from RNA products and can interfere with RNA analysis, namely by reducing the information content on nuclear magnetic resonance (NMR). Although highly purified, the RNAs achieved by PAGE purification show low yields since RNA can irreversibly aggregate due to many precipitation steps, resulting in incomplete denaturation during electrophoresis and low accumulation in the desired gel band [52,53]. Moreover, denaturation of RNA molecules is another issue for RNA applicability. For many RNAs, significant time is spent optimizing refolding conditions to minimize unproductive conformations. Some well-known RNAs, such as *Escherichia coli* (*E. coli*) $tRNA^{Phe}$, cannot be refolded into a conformational homogeneous and active population [54]. Therefore, native purification techniques should be favoured. From the therapeutic viewpoint, PAGE purification is rather impracticable for many reasons. First, the prominent possibility of acrylamide contaminants presence in RNA preparations is not adequate or safe for human therapy. Furthermore, structural modifications due to denaturation, which can cause the loss of secondary structure, can greatly compromise the effectiveness of RNA therapeutic action.

3.1.3. Lithium chloride (LiCl) precipitation

Precipitation with high concentration of lithium chloride (LiCl) is another methodology often used in synthetic RNA isolation procedures [55–57]. Although precipitation of RNA molecules with alcohol and a monovalent cation such as sodium or ammonium ion is much more widely used, LiCl precipitation offers major advantages over other RNA precipitation methods because it does not efficiently precipitate DNA, proteins or carbohydrates [58]. Moreover, LiCl is very soluble in ethanolic solutions and is typically not co-precipitated with the nucleic acid. On the contrary, when using ammonium acetate or sodium acetate, a substantial weight fraction of the pelleted material is formed by the salts precipitation and it is often required the use of subsequent de-salting techniques to eliminate these ions in the recovered material [59]. LiCl precipitation can be used for the separation of small RNAs (<100 nucleotides long) (*i.e.*, siRNA and miRNAs) from other long RNAs or RNA-based impurities. Small RNAs are enriched in the supernatant phase, while remaining impurities or all other long RNAs tend to precipitate [56,57]. However, the efficient fractionation typically requires repetition of the LiCl precipitation procedure several times and the quality of resulting preparation is in general dependent on the secondary or tertiary conformation of RNA molecules [60]. Other applications describe the use of LiCl precipitation in *in vitro* transcripts reaction to eliminate most nucleotides, cap, deoxynucleotides, oligodeoxynucleotides and proteins by selectively precipitating RNA of more than approximately 100 bases, as mRNA transcripts [55]. Nonetheless, most of the time, this precipitation does not completely remove trace of contaminating plasmid DNA (pDNA) or proteins and worse it introduces lithium metal into preparations. Thus, to ensure long RNA purity, phenol/chloroform extraction often accompanies LiCl precipitation. Although this method can be suitable for preparation of mRNA molecules for research, it is not recommended to be used in clinical applications, as in vaccination approaches, since the safeness of those preparations are not guaranteed when applied to humans [55].

3.2. RNA purification methods

In recent years, several strategies employing chromatographic techniques have been explored to overcome the increasing challenges in RNA purification. Advances in synthetic RNA chemistry brought more efficient and affordable methods for large scale production of RNA. Accordingly, synthetic techniques opened the possibility to induce chemical modifications on RNA molecules, which can reduce RNA instability and degradation [61]. The improved RNA stability also represent an advantage for the application of crystallography, spectroscopy, calorimetry or NMR techniques used in structural, biochemical and biophysical analysis of regulatory siRNA, miRNA, rybozymes and aptamers. Therefore, synthetic RNA production can greatly improve and accelerate many RNA therapeutic investigations.

However, not so many efforts have been made for the improvement of biological RNAs purification.

Reversed-phase (RP) and ion-pairing (IP) RP [61–63], as well as anion-exchange chromatography (AEC) have been extensively described for RNA purification (reviewed in [21]). In addition, size exclusion chromatography (SEC) has also accompanied the development of schemes for RNA preparation [64].

3.2.1. Reverse phase (RP) and ion pairing (IP) RP chromatography

RP chromatography relies on hydrophobicity as a mechanism of separation while IP chromatography describes a RP-based improved technique in which an ion-pairing reagent is used allowing enhanced resolution [65]. In fact, a number of studies for the purification and analysis of RNA are already available, demonstrating the versatility of IP RP HPLC in different applications with synthetic oligoribonucleotides or biological RNA (reviewed in [66]). Although these analytical studies include reliable information on RNA purification, very few of these methods have been scaled up to a preparative context. The reason for this is that resins have only moderate loading capacity and the cost of mobile-phase components and separation matrices are high. In addition, some organic solvents and ion-pairing agents are difficult to remove from the purified oligonucleotides after chromatography and may even require further chromatographic purification [67]. Furthermore, to avoid aggregation of self-complementary or GC-rich oligonucleotides, RP HPLC columns are often placed in a column oven at 60 °C for temporarily destroying secondary structures to improve resolution [65], which can be disadvantageous to maintain the target RNA stability and biological activity.

Therefore, recent investigations have explored the possibility to develop more rigorous and complex IP RP HPLC methods, since great resolving properties of RP columns were evidenced in analytical RNA separation. A recent approach describes the combination of SEC (porous silica with a hydrophilic polymeric coating) and IP RP HPLC (polystyrene-divinyl benzene beads) in a robust multidimensional platform to resolve, isolate and quantify non-coding RNA species in cells or tissues samples from several sources [68]. The method takes advantage of the strengths of two types of HPLC techniques, thus increasing the resolution of non-coding RNA across a wide size range. Many technical issues concerning non-coding RNA preparations were improved, including the analysis speed, the application of non-denaturing conditions, easy quantification, sensitivity, easy automation for fraction collection and use of simple solvent mobile phases that do not contaminate the sample.

In addition, another recent strategy employed IP RP HPLC technique for the generation of a therapeutic mRNA for vaccination [69], which purify long *in vitro*-transcribed mRNA at a preparative scale. Despite the use of some organic solvents in the procedure, the volatility of some ion-pairing reagents allows easy removal from samples through evaporation, and non-denaturing conditions were employed. The successful applicability of the IP RP HPLC

purification technique was characterized by the immunogenicity of the samples, which demonstrated the effectiveness of the purified RNA at an immunological level. The strategy conveys important information about the implementation of future purification strategies suitable for clinical applications [7].

Regardless the success of these chromatographic techniques, the requirement of toxic solvents continues to be their major weakness. The environmental impact and cost that the use of organic compounds entails can be highly inconvenient for a biotechnological industry besides, recovered RNA products might require further treatment to be adequate for therapeutic purposes.

3.2.2. Size exclusion chromatography (SEC)

SEC has also accompanied the advances in RNA purification. This technique is able to distinguish the components of a mixture of RNA on the basis of their molecular size, and is the simplest form of chromatography for oligonucleotides purification. SEC has contributed as a polishing step in removing salts from short oligoribonucleotides that have been purified by HPLC. This extra form of desalting prevents cytotoxic effects from trace synthesis by-products or trace solvents which may carry over from purification [70]. In addition, this technique has been explored as an alternative method to preparative denaturing PAGE in purifying homogeneous-length RNA obtained by *in vitro* transcription [52,53,64]. Puglisi and workers developed SEC-based purification schemes using fast performance liquid chromatography (FPLC) systems that allowed the efficient elimination of unreacted nucleotides, enzymes, short abortive transcripts, and the high molecular weight pDNA template from the desired RNA product. These approaches were performed under non-denaturing conditions, which allows the exclusive separation of monomeric RNA from oligomerized RNA, and avoided harsh precipitation steps that may cause RNA aggregation and degradation [64]. However, SEC-based methods still require several time-consuming preparatory steps, such as phenol/chloroform extractions to remove proteins followed by desalting and sample concentration [20].

3.2.3. Anion exchange chromatography (AEX)

Chromatographic studies on anion exchange matrices have been significantly explored because of the polyanionic nature of RNA molecules. AEC mechanism relies primarily on reversible electrostatic interactions that can be altered by mobile phase ions, typically simple salts and buffers are needed for separation. Furthermore, anion exchange matrices are also described to have some inherent hydrophobicity which favours oligonucleotides separation. AEC selectivity for oligonucleotides was found to be based on molecular size and sequence and the success of separating double and single-stranded nucleic acids relies on the distinct hydrophobic behaviours of the G-C, and A-U base pairs [71]. Additionally, AEC demonstrates simplicity on the operations compared to RP, since the separation is performed in aqueous conditions without the use of high cost eluents, at low to moderate operating pressures and the secondary structures that interfere with resolution are avoided using mild conditions to perform the purification. Hence, oligonucleotide purification during mid- or large-scale manufacturing is typically performed using AEC rather than RP chromatography [72].

Nonetheless, in some AEC strategies the necessity of denaturing conditions either by the use of harsh alkaline conditions or elevated temperatures to accomplish full resolution of oligoribonucleotides persists [73]. As already mentioned, denaturation often leads to misfolded or aggregated material or even leads to RNA degradation, which is unsuitable for further usage. Therefore, these methods can be economically disadvantageous in the preparation of RNA. Example of this is the preparation of double-stranded RNAs (dsRNA), such as siRNA or miRNA. The chromatographic purification is generally performed for the single-strand intermediates rather than the final duplex, under denaturing conditions and therefore further purification of the final siRNA duplex in native form is required and is determined by SEC [73,74]. Despite the successful application of this strategy in the manufacture of virtually all therapeutic duplex oligoribonucleotides [11], the workload and process time to purify the separated single-strands, rather than the final duplex is increased, which also increases process economics.

New developments in AEC purification strategies intended to address large scale duplex preparation under native conditions are required. Good separations had already been achieved in analytical non-denaturing AEC, and recently preparative methods were also described [75]. Therefore, the scale up production of dsRNA has been optimized in two commercial AEC resins using the same quaternary amine as functional group, but differing on matrix chemistry: one material consisted on a polystyrene/divinyl benzene beads and the other on poly-(methyl-methacrylate). Both columns allowed the removal of most non-hybridized strands as well as non-optimal duplexes from the hybridized duplex siRNA, with higher yields than in parallel assays to purify single-strands intermediates before annealing. Thus, subsequent duplex purification enabled the reduction of chromatographic steps, as the final SEC is no longer required, and annealing was significantly simplified.

Furthermore, monolithic columns with a stationary phase based on poly(glycidyl methacrylate-co-ethylene dimethacrylate) functionalized with strong anion exchange quaternary amines were also employed in the development of improved dsRNA purification methods under non-denaturing conditions [60]. Different from chemical synthesis, this work describes the purification of siRNAs from a synthetic pool of functional siRNA produced by the enzymatic generation of long dsRNA molecules followed by digestion with a recombinant Dicer enzyme *in vitro*. Multiple contaminants, such as non-hybridized or unprocessed single-strand RNA, undigested or abortive dsRNA molecules, nucleoside triphosphates, that are obtained in transcription reactions were removed and siRNA was recovered at superior speed and scalability compared to the conventional bead-based AEC columns and traditional stepwise LiCl precipitation. In addition, this study provided some data on dynamic binding capacity, showing that monolithic AE columns have significantly higher binding capacity (~8 mg dsRNA/mL resin) than other commercial non-porous bead-based column (~0.6 mg dsRNA/mL resin). Therefore, monolithic columns were found to be suitable for industrial scale siRNA and dsRNA purification due to the higher flow rates and fast phase transfer kinetics of this approach, allowing fast processing times and high resolution.

With the improvement of synthetic RNAs by chemical modification, other challenges in RNA purification arose. Apart the regular impurities, many isomerizations, including 2′,5′-linkages, phosphoramidate-linked RNA, phosphorothioate-linked, 2′-cyanoethoxymethyl-protected RNA and oligonucleotides harbouring phosphorothioate diastereoisomers, may also be formed during chemical synthesis [76]. Pellicular anion-exchangers (pAE) have been reported in the literature for the ability to analytically resolve those isomers. pAE phases were designed to place the ion exchange sites only on the bead surface in order to overcome the mass transfer limitation of porous beads. While they were capable of very high chromatographic efficiency, they exhibit very low capacity, and they may harbour relatively high nonspecific interactions [77]. Recently, a surface-functionalized monolith coated with pAE nanobeads (latexes) was developed with the intention to enhance purifications of single and dsRNA, aptamers, and nucleic acids that may harbour isobaric linkage isomers, such as phosphorothioate diastereoisomers and similar isobaric oligonucleotide variants [78]. These new resins combine the selectivity and mass transfer characteristics of pAE with the preparative capacity of monoliths in order to use the successful analytical separations of

pAE at a lab-scale. With this strategy it could be possible to evaluate and characterize the oligonucleotides regarding its safety and effectiveness as therapeutic and diagnostic products. However, further efforts are needed to scale up these methods so that the resultant RNA preparations can be readily used as therapeutic products.

AEC has also been used as an alternative methodology to purify large scale *in vitro* transcribed long RNAs in order to circumvent the laborious and hazardous denaturing gel electrophoresis. Commonly weak and strong anion exchangers were reported for this purpose. One method describes a series of three weak anion-exchange diethylaminoethyl (DEAE)-Sepharose FPLC columns that can purify RNA from the direct loaded of crude transcription reactions under non-denaturing conditions using mobile phases of sodium phosphate and sodium chloride in concentration up to 1 M [20]. This technique enables removal of free nucleotides, short abortive transcripts, linearized plasmids, and enzymes from the desirable transcripts that can range in length from 30 to 500 nucleotides. Actually, this method does not purify RNAs with homogeneous 3′ ends often crucial for X-ray crystallographic studies, but it yields structurally stable and natively folded RNA, which is essential for many biochemical and biophysical applications.

Improving upon this strategy, a strong exchanger of quaternary amines (polystyrene/divinyl benzene matrix) was described as a useful tool for transcripts purification [79]. The method was successfully applied to a variety of RNAs with different chain lengths, including $tRNA^{Cys}$, ribozyme transcripts, and 4.5S RNA transcripts. All protocols were performed with only slight variations without the need for special buffer systems, demonstrating the robustness of strong anion-exchange support. When compared to DEAE Sepharose columns, the resolving power of the strong AEC was higher for the separation of RNA transcripts from the short oligonucleotide templates. In addition, the purification by strong AEC led to the enrichment of the functional tRNA from run-off transcripts. Nevertheless, the use of higher salt elution interfered with the RNA folding.

Slow mass transfer and low binding capacity are the major bottlenecks in preparative and industrial purification of large biomolecules [80]. Many efforts have been done to overcome these limitations resulting in the development of new media with large channel or pore diameter, namely monoliths [81]. However, up to date these chromatographic properties have not been extensively focused on RNA purification, and should be addressed.

4. RNA affinity chromatography

The use of affinity chromatography has become a useful platform in the development of therapeutically useful products because of the unique property to simulate and exploit natural biological processes such as molecular recognition for the selective purification of the target molecule [82]. Actually, in general, affinity techniques and concepts have become important tools in bioscience with applications spanning from protein purification, protein interaction mapping, development of biopharmaceuticals and diagnostics, as well as new developments in genomics and proteomics [83].

The major advantage of affinity chromatography relies in its distinctive capacity of establishing a combination of different types of interactions, including electrostatic or hydrophobic interactions, van der Waals forces, or hydrogen bonding, due to the specific biorecognition of the target biomolecule by the specific ligand, which gives the technique an extremely high selectivity and high resolution. Thus, the target biomolecule can be separated from a crude sample in one step with thousands fold of increases in purity and high recovery [84]. The choice of matrix and conditions for purification will depend on the molecular properties of biomolecules and the physiochemical and thermodynamic nature of their molecular interactions. 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 [85]. Overall, affinity methods have the advantages of eliminating additional steps, increasing yields and improving process economics.

One trend that has always been present in affinity chromatography has been the search for more selective, robust, and/or reproducible ligands. Although the design of selective ligands for the purification of biomolecules is complex, time consuming and expensive, their implementation into affinity chromatography processes would result in important economic advantages [86], such as the reduction of downstream steps and the improvement of the product quality, therefore justifying the initial investments. These are the main reasons justifying that affinity chromatography processes have accompanied the growing demands for highly selective, reliable and economical processes to conform to strict quality assurance in production of therapeutic biomolecules [87,88]. Therefore, and in view of RNA peculiar chemical structure and versatility in biological function that includes interaction with many molecules in the cell, as well as, the single-stranded nature of RNA, which is normally involved in RNA recognition due to the high base exposure and availability for interactions [89], affinity chromatography could have a widespread application in RNA preparation (Table 4).

4.1. Oligo(dT) chromatography

Currently, the most widely and successfully used affinity chromatography strategy is the popular oligo(dT) for mRNA purification, based on the nucleotide base pairing specificity of A with T, since a poly(A) tail is found in virtually all eukaryotic mRNA molecules. These strategies allow mRNA purification directly from a biological sample or from previously isolated total RNA. mRNA enrichment is typically achieved using home-made columns or kits with oligo(dT)-cellulose [90], but other oligo(dT)-derived media like biotinylated oligo(dT), streptavidin-coated latex or silica are also used [91,92]. These methods are widely preferred among other isolation procedures because of the simplicity and reliable effectiveness in purifying mRNA molecules with high yields and purity suitable in cGMP production [55]. However, because of particular interactions explored in these affinity techniques, the purification of non-polyadenylated mRNA, as exists in bacteria is not possible [93].

4.2. RNA affinity tags

RNA affinity tags have also emerged as useful tools for the isolation of RNAs from cell extracts in recent years [94,95]. These tags are based on RNA aptamers, which have been selected for affinity against a specific ligand by using the systematic evolution of ligands by exponential enrichment (SELEX) method. A number of RNA aptamers have been used for the purification of RNAs from cellular extracts, including aptamers that bind to immobilized streptavidin or Sephadex. These strategies use competitive elution with biotin or dextran, respectively, in order to recover the RNA of interest [96,97]. Although SELEX permit the selection of practically all oligoribonucleotides sequences with the desired properties to bind to a specific molecule, the cost and availability of appropriate affinity resins where only those aptamers can bind is a huge limitation in the development of purification process based on affinity tags. In addition, several considerations for tagging the RNA to be purified, such as folding, steric blockage, and keeping the tag on the RNA prior to purification is required. Therefore, in some cases more

Table 4
Affinity chromatography methods for RNA Purification (cGMP, current good manufacturing practices; PAGE, polyacrylamide gel electrophoresis; SRP, signal recognition particle; ncRNA, non-coding RNA; rRNA, ribosomal RNA; SPE, solid phase extraction).

Affinity type	Principle	Advantages	Limitations	References
Base pairing	Poly(A) tails present on mRNA molecules specifically interact with immobilized oligo(dT)	High mRNA yield cGMP quality Can be used without intermediate isolation of total RNA	Does not isolate no polyadenylated mRNA	Ruby, et al. [91]
Affinity tags	The RNA of interest is tagged with engineered oligoribonucleotides sequences (aptamers) that bind tightly to Sephadex or streptavidin resins	Allow the isolation of RNA from cells lysates Robust methodologies using affinity tags based on inherent biologic interactions Stability of affinity tags High recovery yields due to the specific selection of the tags with high affinities for the resins	Sequential purifications for high recoveries Use of denaturing condition in recovery Several issue in the insertion of the affinity tag into RNA	Srisawat and Engelke [94] Srisawat et al. [96]
	Immobilized *Tetrahymena thermophile* M4 on a matrix or the commercial Ni^{2+}-affinity column specifically recognizes the affinity tags SRP RNA or MS2 coat protein binding stem–loops incorporated in the RNA of interest, respectively	Effective alternative to preparative PAGE in the purification of RNA prepared by *in vitro* transcription Robust methodologies using affinity tags based on inherent biologic interactions High recoveries	Time-demanding in design considerations Instability of the affinity tag	Kieft and Batey [54], Batey and Kieft [99]
Amino acids-RNA	Multiple interactions occur between immobilized amino acids histidine or arginine and RNA molecules	Isolates a single RNA type Simultaneous isolation of ncRNA and rRNA in a single procedure Effective alternative to phenol/chloroform and SPE purifications of biological total RNA Versatility on RNA purification Stable ligands High recovery yields	Requirement of high salt concentrations (for histidine)	[Martins, et al. [100],Martins, et al. [101]]

than one purification step is required or denaturing conditions are employed in order to achieve higher RNA enrichments [94,96,98].

In line with this, later investigations developed more robust affinity systems when pursuing the improvement of schemes to achieve rapid, large scale and native RNA purification of long constructs produced by *in vitro* transcription methods. These strategies are based on protein–RNA interactions where the affinity tags were attached to the 3′ end of the target RNAs during *in vitro* transcription, and specifically purified using an affinity column with a specific RNA-binding protein immobilized [54,99]. Kieft and Batey described an affinity-based method where the RNA of interest was tagged by a signal recognition particle (SRP) RNA with the ability to bind to the immobilized RNA-binding domain of the SRP protein from *Tetrahymena thermophile*. Purification was achieved by cleavage with a *cis*-acting mutated ribozyme from the *hepatitis delta virus* [54]. In a second strategy, the MS2 coat protein binding stem-loops was used as affinity tags at the 3′ end of the RNA transcript, which binds to a Ni^{2+} affinity column *via* interaction with a hexahistidine-tagged MBP-MS2 coat protein fusion. This RNA tag is preceded by the cis-acting glmS ribozyme sequence which can be activated by glucosamine-6-phosphate to elute the desired RNA product [99]. These robust affinity purification methodologies allow the production of milligramme quantities and the rapid purification of native RNAs with homogeneous 3′ ends, which can be essential for crystallographic application. Although the major advantage of these systems is the broadly applicability to any RNA of interest, these methods are limited to the need of several design issues and binding the tags, which may lead to longer optimization processes. In addition, the use of ribozyme cleavage is not always suitable in purification procedures. The attached ribozyme may form alternative structures with the desired RNA and thus disrupt the correct folding of the ribozyme, with its self-cleaving power disabled [28].

4.3. Amino acid-based affinity chromatography

Recently, the use of amino acids as immobilized ligands for affinity chromatography was exploited and implemented by our research group, as an effective methodology for the purification of RNA molecules [100,101]. These studies were based on the fact that many different specific interactions exist between proteins and nucleic acids in biological systems, involving in particular basic amino acids such as histidine or arginine [102]. Moreover, several atomic and molecular recognition studies performed on RNA–protein interfaces have predicted preferential interactions occurring between particular amino acids and nucleotide bases [103–105].

The use of less selective, but at the same time more robust, small molecular ligands (also known as pseudo bioaffinity ligands) was first introduced by Vijayalakshmi and coworkers for the purification of immunoglobulins and a wide variety of proteins, which has confirmed its exceptional potential [106]. The concept of using these ligands was then applied by our research group for the first time to pDNA purification and their ability to isolate supercoiled (sc) pDNA proved the presence of specific interactions occurring between nucleic acids and the amino acid based matrices [87,102].

Meanwhile, several studies are already available demonstrating the advantages of the amino acid-based affinity chromatography in the purification process of sc pDNA preparations intended for DNA vaccination or other gene therapy approaches [107–109].

The potential of amino acids-based affinity chromatography in obtaining RNA preparations under their native state and with high integrity and purity was proved in various biological RNA molecules from prokaryotic and eukaryotic cells rather than synthetic oligoribonucleotides (Figs. 2 and 3). Although the use of biological RNAs has been diminished in many applications because of the improvements in oligoribonucleotides synthesis, most of the native RNAs have post-transcriptional modifications [29] that may not be reproduce under chemical or enzymatic synthesis and some of the modifications are quite important for their structure and function. Therefore, efficient methods for extraction and purification of RNA molecules from biological matrices are needed for the study of cellular mechanisms, as well as, in pharmacokinetic and pharmacodynamics analysis and biomarker evaluations of regulatory non-coding RNA [110].

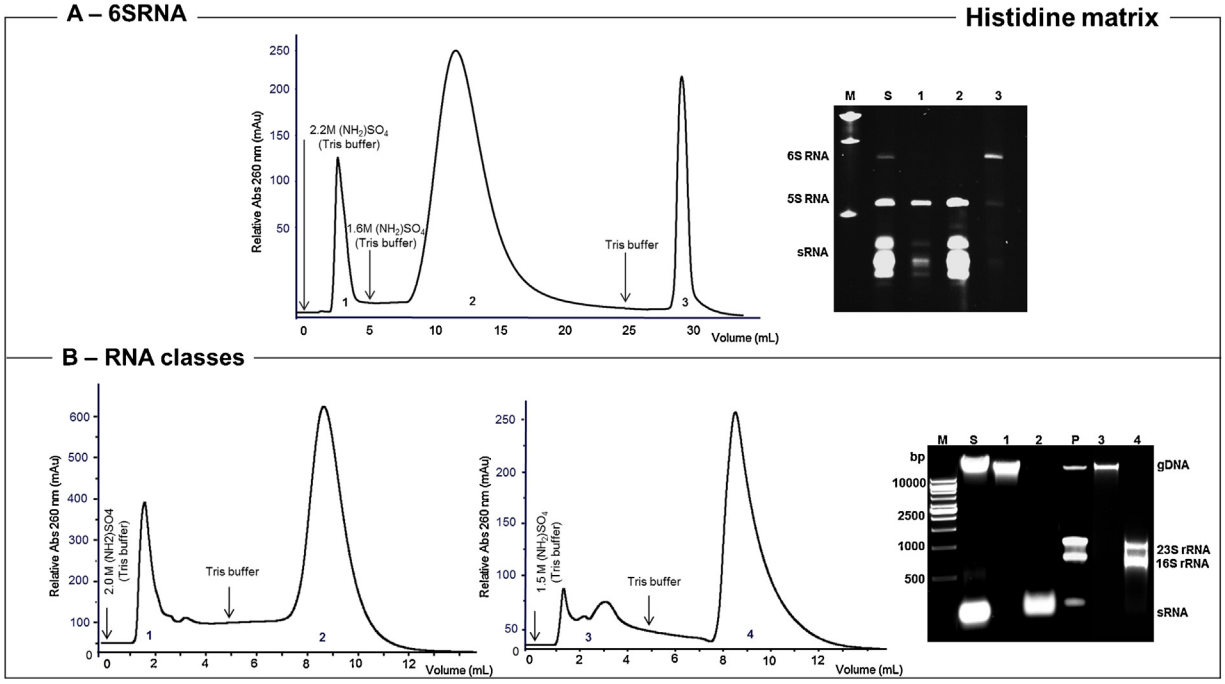

Fig. 2. Purification of RNA molecules from *Escherichia coli* cells using histidine affinity chromatography. (A) 6S RNA was selectively purified from other sRNA populations by stepwise decreasing $(NH_4)_2SO_4$ concentration in the buffer from 2.2 to 1.6 M, finishing with ammonium sulphate-free 10 mM Tris–HCl (pH 7.0) buffer, as indicated by the arrows. Collected fractions corresponding to peaks (1), (2) and (3) were analyzed by polyacrylamide gel electrophoresis and are shown in lanes 1–3. Lane M, low molecular weight marker; lane S, sRNA sample injected onto the column; sRNA, prokaryotic small RNAs. (B) RNA classes were purified in two strategies employing a stepwise decreasing $(NH_4)_2SO_4$ concentration. For sRNA class, the binding was promoted at 2.0 M $(NH_4)_2SO_4$ in 10 mM Tris–HCl (pH 8.0) buffer while for rRNA class 1.5 M $(NH_4)_2SO_4$ in Tris buffer was used. Their purification from host impurities (genomic DNA and proteins) was achieved removing the salt from the buffer. Samples collected at the column outlet were analyzed by agarose gel electrophoresis. Fractions corresponding to peaks 1–4 are shown in lanes 1–4, respectively. Lane M, high molecular weight marker; lane S, impure samples containing sRNA molecules; lanes 1 and 3, gDNA; lane 2, sRNA molecules; lane P, impure sample containing rRNA species; lane 4, rRNA molecules 16S and 23S. In all strategies elution was performed at 1.0 mL/min.

4.3.1. Histidine affinity chromatography

The potential applicability of histidine-based affinity chromatography in the purification of RNA molecules was first demonstrated in the separation of 6S RNA, a small RNA (sRNA) of the prokaryotic *E. coli* that has a relevant regulatory function in the transcription process (Fig. 2A). Because of the aromatic imidazole side chain of histidine amino acid, and considering the fact that at the working pH (7.0 and 8.0) histidine ($pK_a = 6.5$) is not significantly protonated [111,112], the interactions between RNA and the matrix are suggested to be mainly ring stacking/hydrophobic interactions and histidine–RNA direct hydrogen-bonding. Accordingly, a three stepwise decreasing gradient of ammonium sulphate was employed to histidine matrix that revealed a specific recognition for 6S RNA, allowing its accurate purification from a complex

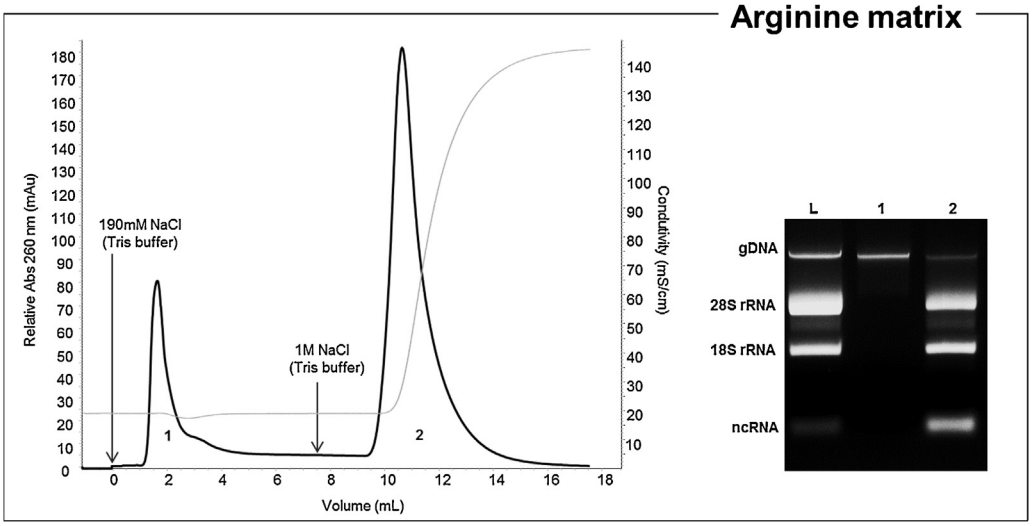

Fig. 3. Purification of total RNA using arginine affinity chromatography. Arginine matrix was used to purify eukaryotic total RNA from cell lysates by stepwise increasing NaCl concentration in the eluent from 190 mM to 1 M, as represented by the arrows. Conductivity represented by the lighter line was followed in order to strictly control chromatographic condition. The nucleic acid content of peaks (1) and (2) are shown in lanes 1 (gDNA) and 2 (high-quality total RNA) of agarose gel electrophoresis. Elution was performed at 1.0 mL/min. Lane L, cell lysate sample injected onto the column; gDNA, genomic DNA; rRNA, ribosomal RNA; ncRNA, eukaryotic non-coding RNA.

mixture of sRNA molecules (Fig. 2A) [100]. Subsequently, the simultaneous isolation of sRNA and ribosomal RNA (rRNA) from *E. coli* cell lysates was also attained by an histidine-based method but in a single step gradient [101] (Fig. 2B). In this study, both RNA classes were separated from host impurities (gDNA and proteins) show similar purity and recovery to conventional phenol-based methods, but with the advantage of avoiding the use of toxic chemicals during the isolation process.

The versatility of histidine matrix in the purification of both single 6S RNA molecule and non-coding RNA and rRNA classes suggested that the underlying mechanism involves not only hydrophobic interaction, but also a bio-recognition of RNA bases by histidine [100].

While the RNAs of these preparations need further functional characterization to prove their applicability in RNA-based studies, histidine methodology represents a major advance in the ability to accurately purify RNA molecules. In fact, this affinity technique is able to selectively distinguish a single RNA molecule or to promote the simultaneous isolation of sRNA and rRNA in a single step. However, the requirement for high salt concentration can be a disadvantage, especially with regard to biotechnological application, because the use of salt is associated with higher costs and environmental impact.

4.3.2. Arginine affinity chromatography

The use of arginine as the immobilized ligand could improve the previous techniques. In fact, arginine-base interactions have been recognized as the most prevalent interactions in several protein-RNA complexes [105]. As arginine is a positively charged amino acid, interactions could be exploited for RNA purification using only mild elution conditions. Thus, a stepwise increasing gradient of sodium chloride up to 1 M allowed the isolation of total RNA from impurities of eukaryotic cell extracts (Fig. 3) [113]. In order to demonstrate the high-quality RNA obtained by arginine affinity purification, the integrity of total RNA samples was evaluated by ribosomal band intensity, gDNA impurity was quantified by real-time PCR and protein contamination was assessed by a commercial available kit. Total RNA purified by arginine-based chromatography showed a high integrity (28S:18S ratio = 1.96) as well as a good purity, demonstrated by the scarce detection of proteins and gDNA. Arginine support showed an exceptional ability to interact with all functional classes of RNA, despite their structural diversity and different folding states as they are in their native state. These strong and selective interactions with the affinity matrix may result from the multiplicity of arginine side chain, which can promote multi contact with the RNA backbone or RNA bases, according to RNA folding. Although electrostatic interactions between RNA phosphate groups and arginine ligands can be playing an important role on RNA retention, the base contacts are also involved and modulate some favoured interaction and specificity found in arginine–agarose chromatography. Hence, this process resulted in a high recovery yield of RNA and quality control analysis showed a high integrity in RNA preparations as well as good purity, demonstrated by the low protein and genomic DNA. The efficiency and applicability of this technique was attested by gene expression analysis, where the total RNA samples were used as template in real-time PCR and the expression of two common housekeeping genes was successfully measured [113]. Therefore, this arginine-based procedure can be an alternative method for the purification of total RNA pursuing gene expression analysis.

Taken together, the results on RNA isolation and purification from a complex biological mixture using amino acids-based affinity chromatography techniques showed several improvements over the currently phenol/chloroform or SPE isolation methods, as it simplified the workflow integration and miniaturizes sample handling process, making them useful for the development of a RNase and organic solvents free methodology, particularly important for several structural and functional studies and clinical investigation.

In fact, these purification schemes can a be valuable tool in recent emerged strategies to produce stable structured RNA *in vivo* using recombinant approaches as an alternative to *in vitro* transcription or chemical synthesis. Since synthetic methods can often be costly and laborious and have their drawbacks with respect to sequence requirements, variations in yield, non-templated nucleotide additions and/or the maximum length of the oligonucleotide, tRNA-scaffolds have been described with some success as general method for producing a large variety of recombinant RNA using *E. coli* as a host, providing an inexhaustible source of RNA [95]. At the end of the culture, cells are pelleted, lysed and total RNA is isolated by direct phenol extraction and the recombinant RNA is purified by AEC or alternatively, since the recombinant RNA molecules are equipped with a Sephadex aptamer, by affinity chromatography using Sephadex G-200, avoiding phenol-based purification. Finally, the product RNA is separated from the tRNA-scaffold on preparative denaturing PAGE [114]. The use of amino-acid based methods to purify recombinant RNA would definitely improve the present of purification strategies, since the phenol/chloroform extraction as well as the preparative PAGE and all their disadvantages can be eliminated, maintaining the chemical stability and biological competence of RNA molecules.

In biopharmaceutical industry, the use of an appropriate recombinant expression system together with robust liquid chromatographic techniques is a well-established method for the production of therapeutic bioproducts, such as recombinant proteins or pDNA [115,116]. Accordingly, the recombinant production of RNA in *E. coli* and the subsequent purification employing an amino acid-based affinity chromatography method could be a promising biotechnological process for the production of RNA bioproducts in large scale. Fig. 4 schematize a generic procedure for the preparation of recombinant RNA bioproduct based on amino acid-based affinity chromatography technique. This approach can be economically feasible and easily adopted by biopharmaceutical industries.

Whether the implementation of such method is feasible for the preparation of therapeutic RNA molecules, new high-throughput analytical tools that can readily identify and quantify RNA are required, since several flaws still exist in the current RNA quantification techniques, as the lack of selectivity for RNA [117]. With the success of amino acids as affinity ligands in the purification of RNA, an analytical technique based on arginine affinity chromatography for quantification and quality verification of total RNA was also developed and validated, according to international and European legislation for bioanalytical methods. The versatility of the methodology was demonstrated by its applicability in the quantification of RNA from different eukaryotic cells and in crude samples of chemically synthetized RNA, which can have a potential multipurpose applicability in molecular biology RNA-based analysis and RNA therapeutics [117].

More efforts into RNA purification are being developed with amino acid-based matrices, in particular with arginine-based matrix. The new goal is to exploit their applicability in purifying mRNA molecules not from cells, but from synthetic crudes of *in vitro* transcription, pursuing mRNA vaccination for cervical cancer. So far, mRNA molecules encoding Human Papillomavirus (HPV) 16 E6 and E7 proteins were successfully purified from the impurities of pDNA template, enzymes, nucleotides, salts or buffer. In this work, arginine-based chromatography is also demonstrating its singular ability in improving purification processes, showing the advantages of eliminating additional steps and improving global economics of the production process (unpublished results).

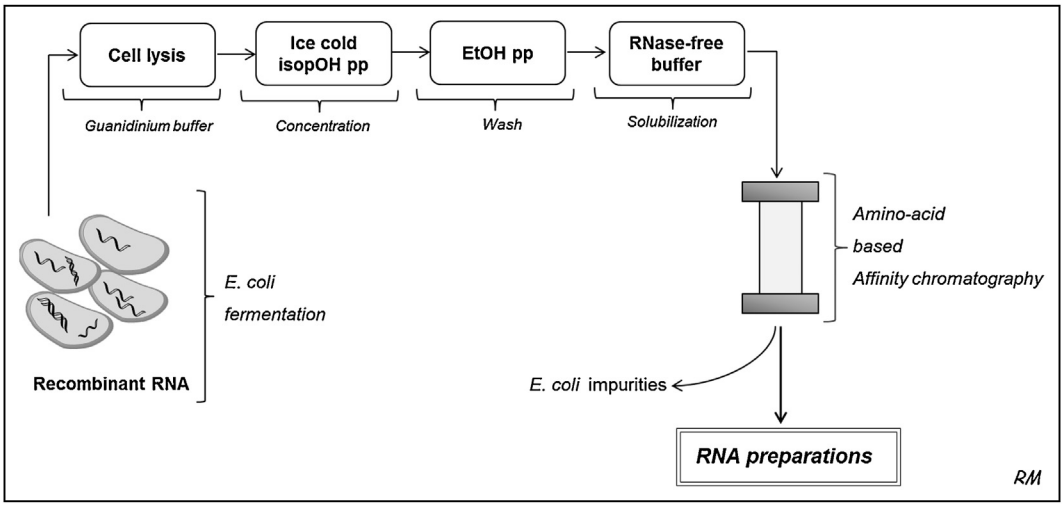

Fig. 4. Projected biotechnological process for RNA preparation in large scale. The downstream processing is based on previously reported amino acid-based affinity chromatography works [101,113]. After amplification in *E. coli*, the recombinant RNA are lysed with a guanidinium-based buffer, followed by a concentration, wash and solubilization steps in order to change to a simple buffer free of RNases, and the final step of amino acid-based affinity chromatography to isolate and purify RNA from impurities. *E. coli*, *Escherichia coli*; RNases, Ribonuclease; IsopOH pp, isopropanol precipitation; EtOH pp, ethanol precipitation.

5. Conclusion and future trends

To attain large quantities of RNA with the demanded quality for structural and functional studies as well as for application in medical therapies, more selective, reliable and efficient isolation and purification processes are required.

The difficulty of successfully handling RNA in the laboratory, because of the structural susceptibility to RNases present in cell extracts and in the environment, and the impurities present in cell samples or synthetic preparations, result in numerous challenges for the selective purification of RNA molecules and in higher costs for laboratories and biopharmaceutical industries. The current methodologies used in RNA preparations circumvent some of those challenges, however many difficulties still existing in order to obtain an innocuous, selective and economically feasible procedure to purify RNA. Recent investigations in chromatographic bioseparation are providing reliable data in process development both for preparative-scale and for large-scale applications. In particular, affinity chromatography stands out because of combining the selectivity of a naturally occurring biological interaction with the simplicity of a single small molecule. Moreover, the contribution of novel affinity approaches based on histidine and arginine as amino acid ligands provided new insights in the way that the diverse RNA molecules can be highly purified. Histidine matrix specifically recognized 6S RNA, allowing its purification from the mixture of other *E. coli* sRNA and also attained the simultaneous isolation of sRNA and rRNA from an *E. coli* cell lysates, eliminating host impurities. On the other hand, arginine matrix enabled the purification of high-quality total RNA from eukaryotic cell extracts. These methodologies can be integrated in recombinant RNA production platforms, allowing the development of robust biotechnological processes. This will certainly be profitable for the industrial production of RNA. Although affinity chromatography represents a particularly attractive option for RNA purification, there are limitations regarding capacity of supports and availability of suitable affinity ligands, which remain to be solved. However, with the increase understanding on RNA synthetic synthesis and chemical modification, the design of highly selective and stable synthetic affinity ligands may be more easily achieved. In addition, recent chromatographic technologies as monolithic supports that combine the advantages of conventional chromatographic columns packed with porous particles in terms of separation power, capacity and sample distribution and those of membrane technology in terms of convective mass transport, could be used with immobilized affinity ligands. These approaches largely benefit the industrial production of RNA biotherapeutics.

The combined efforts that are expected to occur in this field should help to accomplish enhanced purification strategies with positive effects on basic and applied RNA research.

Conflict of interest statement

The authors declared no conflict of interest.

Acknowledgments

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