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Research Article

A new strategy for RNA isolation from eukaryotic cells using arginine affinity chromatography

The relevance of RNA in many biological functions has been recognized, broadening the scope of RNA research activities, from basic to applied sciences, also aiming the translation to clinical fields. The preparation and purification of RNA is a critical step for further application, since the quality of the template is crucial to ensure reproducibility and biological relevance. Therefore, the establishment of new tools that allows the isolation of pure RNA with high quality is of particular importance. New chromatographic strategies for RNA purification were considered, exploiting affinity interactions between amino acids and nucleic acids. In the present study, a single arginine-affinity chromatography step was employed for the purification of RNA from a total eukaryotic nucleic acid extract, thus eliminating several steps compared with current RNA isolation procedures. The application of this process resulted in a high RNA recovery yield of 96 ± 17% and the quality control analysis revealed a high integrity (28S:18S ratio = 1.96) in RNA preparations as well as a good purity, demonstrated by the scarce detection of proteins and the reduction on genomic DNA contamination to residual concentrations. Furthermore, the performance of the new RNA isolation method was tested regarding the applicability of the isolated RNA in modern molecular biology techniques. Hence, this new affinity approach will simplify the isolation and purification of RNA, which can bring great improvements in biomedical investigation.

Keywords: Affinity chromatography / Arginine / Gene expression / Real-time PCR / RNA isolation
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1 Introduction

The increasing awareness that RNA is one of the central molecules in cellular processes is broadening the RNA-based research [1]. Understanding the role of the various types of RNA present in cellular events is critical to develop new methods of diagnosis and treating diseases [2]. On the other hand, RNA is also the starting material in numerous molecular biology procedures involving the characterization of known messenger RNA (mRNA), the identification of unknown genes, and the assignment of function to several proteins. Moreover, in applications such as complementary DNA (cDNA) library construction, Northern blot analysis, reverse transcription (RT), and *in situ* hybridization analysis, a major factor determining the rate of success is the quality of initial RNA. Although RNA purification is a first critical step of a number of preparative and analytical methods, the commonly used isolation techniques present several limitations and have changed little in the past years [3]. To overcome this issue, the development of new tools that allows the isolation of biological and chemically stable RNA is of particular importance. The mainly used procedure for total RNA isolation employs a combination of denaturing agents, acid phenol chloroform extraction followed by precipitation of the nucleic acids [3, 4]. This procedure has the disadvantages of using hazardous products and of being very time consuming and highly operator dependent.

Liquid chromatography, especially HPLC, has been largely applied in attempting to overcome the limitations on RNA purification [5–9]. In these cases, the RNA is prepared through linear plasmid DNA (pDNA) templates for large-scale *in vitro* transcription and the HPLC methods, mainly reversed-phase, size-exclusion, or anion-exchange chromatography, are used to separate the desired RNA oligonucleotide from the transcription mixture. More recently, a robust affinity-purification protocol via ribozyme-cleavable RNA affinity purification tags was also proposed

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Abbreviations: AC, affinity chromatography; **BCA**, bicinchoninic acid; **cDNA**, complementary DNA; **Ct**, threshold cycle; **DEPC**, diethylpyrocarbonate; **gDNA**, genomic DNA; **hGAPDH**, human glyceraldehyde 3-phosphate dehydrogenase; **ncRNA**, noncoding RNA; **PC-3 cells**, human prostate cancer cell line; **pDNA**, plasmid DNA; **qPCR**, real-time PCR; **RT**, reverse transcription; **rRNA**, ribosomal RNA; **RNases**, RNA-degrading enzymes; **STD-NMR**, saturation transfer difference-nuclear magnetic resonance; **SPR**, surface plasmon resonance; **sc**, supercoiled; **tRNA**, transfer RNA

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[10]. In those applications, *in vitro* RNA transcripts are used as substitutes for native RNAs. However, most of the native RNAs have posttranscriptional modifications [11] and some of the modifications are quite important for their structure and function. Thus, isolation of intact RNAs from cells is essential for their study and application.

In this work, affinity chromatography (AC) with immobilized arginine is considered a potential technique for RNA isolation from a biological source, because of its unique characteristic of using a biospecific ligand to purify biomolecules, on the basis of their biological function or individual chemical structure. In fact, the use of amino acids as immobilized ligands for AC has been recently exploited and implemented, by our research group, as an effective methodology for nucleic acids purification [12–14]. Histidine [15, 16] and arginine [17–19] have been used as amino acid ligands, and their ability to isolate supercoiled (sc) pDNA proved the presence of specific interactions occurring between pDNA and the amino acid based matrices. Furthermore, histidine AC was also applied in the purification of RNA [20, 21]. As a result, histidine matrix showed a specific recognition for 6S RNA, allowing its purification from a complex mixture of *Escherichia coli* regulatory noncoding RNA (ncRNA) molecules [20]. Subsequently, the simultaneous isolation of ncRNA and ribosomal RNA (rRNA) was also accomplished using histidine AC. In this strategy, both RNA classes were accurately purified from *E. coli* impurities (genomic DNA (gDNA) and proteins) [21].

On the other hand, the application of arginine matrix showed some improvements in pDNA purification over other chromatographic techniques [13]. The arginine-based support allowed the efficient separation of plasmid isoforms, revealing the presence of a specific recognition for sc isoform [22]. Moreover, the different interactions of arginine ligands with pDNA, RNA, and gDNA suggested its potential application for the selective recovery of any nucleic acid. Additionally, it was found that the simplified purification process achieved with this support had a significant impact on sc pDNA stability, enhancing its biological function [18].

Building on the interesting results obtained, we will explore the possibility of using arginine AC to selectively isolate total RNA with high quality, in view of the application in molecular biology procedures, namely for gene expression analysis. This work intends to aid in the development of new procedures for RNA isolation and purification, which are generally recognized to be crucial for the overall success of RNA-based analyses [23, 24]. Therefore, it is expected that this new affinity protocol for RNA isolation can offer advantages over other less-selective and time-consuming multistep procedures and can improve process economics.

2 Materials and methods

2.1 Materials

Human caucasian prostate adenocarcinoma cell line (PC-3, ECACC 90112714) was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cell culture reagents, namely RPMI 1640, and trypsin/EDTA were purchase from Sigma (St. Louis, MO, USA). Fetal bovine serum was obtained in Biochrom (Berlin, Germany) while penicillin/streptomycin solution was obtained from Invitrogen (Carlsbad, CA, USA). All the chemicals used in the cell lysis buffer were obtained from Sigma. Arginine–Sepharose 4B gel was obtained from GE Healthcare Biosciences (Uppsala, Sweden). The compounds used for chromatographic experiments were sodium chloride purchased from Panreac (Barcelona, Spain) and Tris base from Fisher Scientific (Leicestershine, UK). Glycogen was obtained from USB (Cleveland, OH, USA). All solutions were freshly prepared using 0.05% diethylpyrocarbonate (DEPC)-treated water from Fluka (Sigma) and the elution buffers were filtered through a 0.20-μm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically. The DNA molecular weight marker, HyperLadder I, was obtained from Bioline (London, UK). All the experiments were performed with RNase-free disposables.

2.2 Methods

2.2.1 Cell culture and lysis

PC-3 cells were initially cultured in 25-cm^2 flasks in RPMI 1640 at 37°C in a humidified incubator in 95% air/ 5% CO_2. The culture medium was supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. The PC-3 cells were routinely passaged at 90–95% confluence to 75-cm^2 flasks. In order to obtain total nucleic acid extracts, PC-3 cells from passages number 19, 21, and 22 were collected by washing and detaching with 0.25% trypsin/EDTA. The trypsin solution was neutralized with RPMI 1640 supplemented culture medium and the cells were spun down with a centrifuge (Sigma 3K18C, Bioblock Scientific; Sigma Laboratory Centrifuges) at 1500 rpm for 5 min at room temperature, and resuspended in culture medium. The cells spin and resuspension was repeated to ensure complete removal of trypsin. The recovered cells were resuspended in 1 mL of PBS and the total cells were counted using a Neubauer chamber. Next, approximately 10×10^6 cells were spun down and the pellets were resuspended in 2 mL denaturing cell lysis solution (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 4.0; 0.5% (m/v) *N*-laurosylsarcosine; and 0.1 M β-mercaptoethanol). After 5-min incubation at room temperature, 2 mL of ice-cold isopropanol was added. The lysate solution was homogenized by inversion and incubated on ice for 5 min. The precipitated molecules were recovered by centrifugation at 16 000 × *g* for 20 min at 4°C. The pellet was washed with 1 mL of 75% ethanol in DEPC-treated water and incubated at room temperature for 10 min, followed by a 5-min centrifugation at 16 000 × *g* (4°C). The air-dried pellet was dissolved in 240 μL of 0.05% DEPC-treated water and incubated for 5 min at room temperature to ensure

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complete solubilization. The concentration of total nucleic acid preparation was estimated using NanoPhotometer (IMPLEN, Munich, Germany).

Besides the preparation of total nucleic acid extracts, total RNA was also isolated by using a conventional procedure. Thus, approximately 10^7 PC-3 cells from the same cell passages mentioned above were treated with commercial TRI reagent (Ambion, Carlsbad, CA, USA), according to manufacturer's instructions.

2.2.2 Preparative arginine–agarose chromatography

Chromatographic experiments were carried out using an ÄKTA Avant system with UNICORN 6 software (GE Healthcare). It was used a commercial arginine–Sepharose 4B gel characterized by the manufacturer as a cross-linked 4% beaded agarose matrix with a 12-atom spacer and an extent of labeling between 14 and 20 μmoL/mL. The stationary phase was packed in a 10 mm diameter × 20 mm long (~2 mL) column. Temperature was maintained at 7°C during the experiments by a circulating water bath.

The stationary phase was equilibrated with 190 mM NaCl in 10 mM Tris-HCl (pH 8.0) buffer at a flow rate of 1 mL/min. Conductivity was controlled at 19 mS/cm. With relation to the sample application, 200 ng/μL of total nucleic acid preparation, from passages 19, 21, and 22, was injected onto the column using a 100-μL loop at the same flow rate. After washing out the unbound material with 190 mM NaCl in 10 mM Tris-HCl (pH 8.0) buffer, the ionic strength of the buffer was increased to 1 M NaCl in 10 mM Tris-HCl (pH 8.0) buffer. The different peaks were monitored with a UV detector at 260 nm. Each peak was automatically collected in a climate-controlled fraction collector chamber and was concentrated by addition of 20 μg glycogen and four volumes of 100% ethanol. After a 2-h incubating period at –80°C, the fractions were recovered by centrifugation at 16 000 × g for 20 min at 4°C. Pellets were air-dried for 15 min and reconstituted in DEPC-treated water. After chromatographic runs, the column was cleaned with three column volumes of 0.2 M NaOH.

For the identification of eluting species and evaluation of RNA integrity, the samples were resolved on a 1.2% native agarose gel in TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH 8.0) prepared in DEPC-treated water. The gels were stained with ethidium bromide (0.5 μg/mL), and photographed. The assessment of RNA quality and downstream analysis were further performed as described below.

2.2.3 RNA quantification

ÄKTA Avant system (GE Healthcare) with arginine–agarose column was also used to quantify the RNA present in each sample recovered from the chromatographic purification. A calibration curve was prepared with RNA standards (10–80 μg/mL) purified with TRI reagent (Ambion). The standard experiments were undertaken in triplicate. The experiments were performed by injecting 100 μL of RNA standards onto arginine–agarose matrix after an equilibration step with 190 mM NaCl in 10 mM Tris-HCl (pH 8.0) buffer, using a flow rate of 1 mL/min. The elution buffer of 1 M NaCl in 10 mM Tris-HCl (pH 8.0) buffer was immediately applied to the column in order to favor entire RNA elution. The areas of the peaks obtained in the ÄKTA histogram were calculated using UNICORN 6 software. A standard curve was obtained by a linear fit between the sample concentration and peak integration area, with a correlation coefficient of 0.998. The RNA quantification in other samples was accomplished by comparing their peak areas with the respective standard curve.

2.2.4 Protein analysis

Protein residual contamination in RNA samples, either collected from the purification with arginine–agarose support or isolated by TRI reagent, was assessed by using the micro-BCA (bicinchoninic acid) assay (Thermo Fisher Scientific Inc., Rockford, IL, USA), according to manufacturer's instructions. Briefly, the calibration curve was prepared using BSA standards (0.01–0.1 mg/mL). A total of 50 μL of each standard or RNA samples was added to 200 μL of BCA reagent in a microplate and incubated for 30 min at 60°C. Absorbance was measured at 570 nm in a microplate reader.

2.2.5 RT-PCR and real-time PCR (qPCR)

All RNA samples in study were amplified by RT-PCR in a thermo cycler (Biometra, Goettingen, Germany). cDNAs synthesis was performed using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific Inc.), according to manufacturer's instructions. A total of 1 μg of total RNA was used to initiate cDNA synthesis. PCR reactions were carried out using 1 μL of the synthesized cDNAs in a 25 μL reaction containing 1 × Taq DNA polymerase buffer (Xnzytech, Lisboa, Portugal), 500 μM deoxynucleotide triphosphates (Amersham Biosciences, Uppsala, Sweden), 3 mM of magnesium chloride (Fermentas, Thermo Fisher Inc.), 300 nM of each primer, and 0.125 U of Taq DNA polymerase (Xnzytech). The used human glyceraldehyde 3-phosphate dehydrogenase (hGAPDH) primers were provided by RevertAid First Strand cDNA Synthesis Kit, which amplify products of 496 bp. The cycling conditions were used in accordance to the instructions of cDNA synthesis kit in Control PCR amplification section. To confirm the presence and purity of amplicons, RT-PCR products were analyzed by 1% agarose gel electrophoresis.

gDNA contamination and gene expression was evaluated using total RNA purified with the arginine matrix or TRI reagent in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Specific primers to hGAPDH and 18S rRNA were used in gDNA and cDNAs amplification according to Table 1. The qPCR efficiency was determined for all primer sets using serial dilutions of cDNA samples (1:1, 1:10, 1:100, and 1:1000). A standard curve was generated by serial dilution of PC-3 gDNA (purified with the Wizard gDNA purification kit; Promega,

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Table 1. Technical features of the specific primers used in qPCR analysis

Primer	Sequence		Amplified fragment (bp)	Annealing temperature (°C)
	Sense	Antisense		
hGAPDH_intron[a)]	5′-CCCACACACATGCACTTACC-3′	5′-CCCACCCCTTCTCTAAGTCC-3′	176	60
hGAPDH_74 hGAPDH_149	5′-CGCCCGCAGCCGACACATC-3′	5′-CGCCCAATACGACCAAATCCG-3′	75	
18S _980 18S _1128	5′-AAGACGGACCAGAGCGAAAG-3′	5′-GGCGGGTCATGGGAATA-3′	148	

a) The hGAPDH primers inside the first gene intron were used for gDNA amplification.

Madison, WI, USA) in the range of 0.8–800 ng/μL. qPCR reactions were carried out using 1 μL of gDNA standards or synthesized cDNA in a 20 μL reaction containing 10 μL Maxima SYBR Green/ROX qPCR Master mix (Fermentas, Thermo Fisher Scientific Inc.) and 300 nM hGAPDH or 18S primers. All reactions were performed in 96-well plates for PCR heat-sealed with heat sealing film (Bio-Rad Laboratories). After an initial denaturation at 95°C for 5 min, cycling conditions were as follows: 35 cycles consisting of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 10 s. The amplified PCR fragments were checked by melting curves: reactions were heated from 55 to 95°C with 10 s holds at each temperature (0.05°C/s). Samples were run in triplicate for each assay. Results were analyzed using iQ5 optical system software version 2.0 after manual adjustment of the baseline and fluorescence threshold. Fold differences were calculated following the mathematical model proposed by Pfaffl using the formula $2^{-\Delta Ct}$ [25].

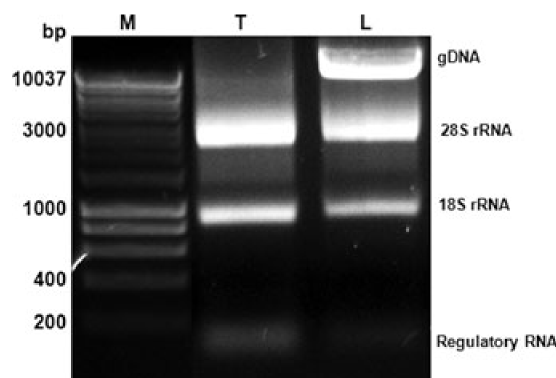

Figure 1. Agarose gel electrophoresis of the nucleic acids in PC-3 total nucleic acid extract recovered by guanidinium-based lysis (lane L) and in samples isolated with TRI reagent (lane T). Lane M, DNA molecular weight marker. Data are representative of three independent experiments with three cell passages.

3 Results and discussion

3.1 RNA purification from PC-3 total nucleic acid extracts

In this work, the applicability of a method based on arginine AC for the isolation of RNA from PC-3 cells, a well-established prostate cancer cell line [26], was evaluated.

Total nucleic acid preparations were obtained by chemical lysis with guanidinium buffer, which due to its chaotropic effect disrupts the plasma membrane and induces organelle lysis. This process liberates heterogeneous nuclear RNA and gDNA from nucleus and mitochondria, all of which are recovered with the cytoplasmic RNA. In addition, guanidinium buffer causes efficient unfolding of proteins, by which RNA-degrading enzymes (RNases) tertiary structure is distorted, inhibiting their activity [27]. Therefore, it is not necessary to add additional RNase inhibitors.

Figure 1 shows the electrophoretic profile of the nucleic acids present in a PC-3 total nucleic acid preparation after chemical lysis (Fig. 1, lane L). This sample contains four distinct nucleic acid species, which correspond to gDNA, 28S rRNA, 18S rRNA, and low molecular weight RNA species, from the slower to faster migrating biomolecules. Total RNA isolated by TRI reagent was also loaded onto the gel as a control (Fig. 1, lane T). The electrophoretic analysis indicates an accurate banding profile for RNA molecules, with a clear definition of 28S and 18S rRNA species. The greater fluorescence of the 28S rRNA demonstrates the integrity of the samples. All transfer RNA (tRNA) and the low molecular weight 5S and 5.8S rRNA species comigrate and appear at the bottom of the gel. Because electrophoretic analysis was performed under native conditions, a resultant smear can be occasionally visualized. The smear is not due to sample degradation but instead it reflects persistent RNA secondary structure [27]. Therefore, this qualitative analysis suggests that RNA in total nucleic acid extracts is chemically intact.

Total RNA isolation with arginine-chromatography was achieved after several optimizing experiments using different salt concentrations, buffer types, and temperatures in order to select the best conditions for RNA binding and elution (results not shown). Therefore, mild chromatographic conditions using low NaCl concentrations in Tris buffer pH 8.0 were used while maintaining the temperature at 7°C to prevent RNA degradation. Total RNA retention was achieved with NaCl concentrations between 150 and 200 mM and its elution occurred when using 1 M NaCl. Curiously, when no salt was present in the Tris buffer, gDNA and rRNA were

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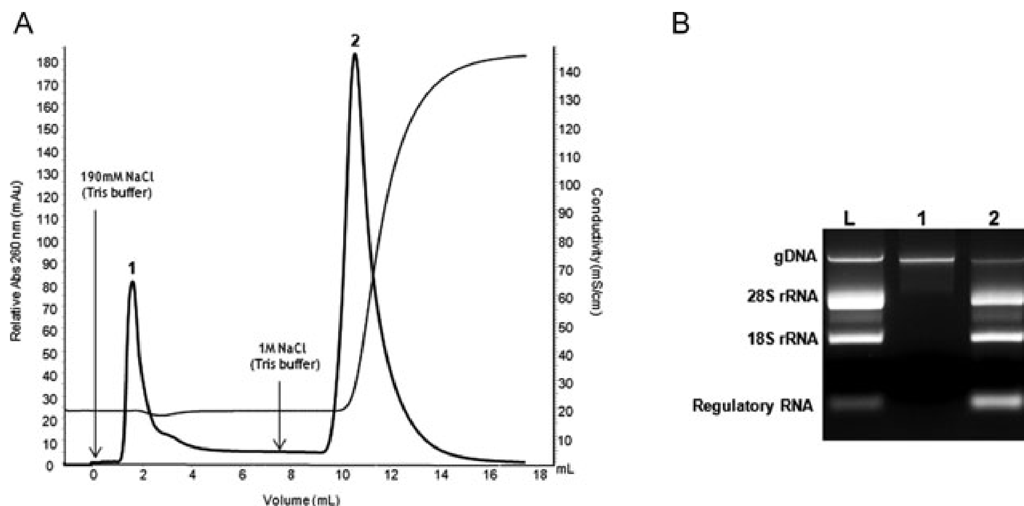

Figure 2. (A) Chromatographic profile of the purification of total RNA from PC-3 total nucleic acid extract by arginine–agarose chromatography. Elution was performed at 1.0 mL/min by stepwise increasing NaCl concentration in the eluent from 190 mM to 1 M, as represented by the arrows. The conductivity was followed along the chromatographic purification as indicated by the dashed line. (B) Agarose gel electrophoresis of the samples collected at the column outlet. Fractions corresponding to peaks (1) and (2) are shown in lanes 1 and 2, respectively. Lane L, total nucleic acid extract injected onto the column. Data are representative of three independent experiments with three cell passages.

observed to promptly elute, while sRNAs were retained. During these experiments, the need for strict control of chromatographic conditions was verified in order to maintain the reproducibility since a slight variation in conductivity (salt concentration and/or temperature) affected total RNA retention.

Figure 2A shows the chromatographic profile obtained after the injection of total nucleic acid preparation on the arginine support. The chromatographic run was initiated at low ionic strength with 190 mM NaCl in 10 mM Tris buffer (pH 8.0). Under these conditions, it was observed a flow-through peak containing unbound species. The elution of highly bound species was then achieved by increasing the ionic strength of the buffer to 1 M NaCl. The presence of two different peaks in the chromatogram is a consequence of the different interaction that nucleic acids exhibit with arginine–agarose matrix. The agarose gel electrophoresis was used to identify the different nucleic acid species from each peak (Fig. 2B). The electrophoretic profile presented in lanes 1 and 2 corresponds to the samples pooled from the respective peaks in the chromatogram. The total nucleic acid preparation injected on arginine matrix (Fig. 2B, lane L) was also run in the gel for comparative purposes. Hence, electrophoretic analysis showed that the first peak of unbound species corresponds to gDNA (lane 1), while the second peak mainly refers to RNA species (lane 2). These results suggest that the different functional classes of RNA present a stronger interaction with the arginine matrix than gDNA.

Although the second peak mainly includes RNA species, a slight band of gDNA is still visible in the electrophoretic analysis (lane 2). Thus, it is important to determine the extent of gDNA contamination on RNA samples. Further experiments on the quantification of gDNA by qPCR were performed and they are discussed in the next section of RNA quality characterization.

Additionally, it was attempted to improve the quality of RNA preparations by performing some changes in the elution condition on arginine AC.

In AC, the elution of a target solute that is bound to the affinity ligands can be achieved through addition of a competing agent in the elution buffer rather than changing the ionic strength, pH, or polarity of elution buffer. In this study, the competitive studies were performed by adding 250 mM of arginine to the elution buffer in linear or stepwise gradients (data not shown). Arginine was used as competing agent to exploit specific elution of RNA from the column and therefore to evaluate the possibility to reach higher purification factors. However, the experiments did not result in an improvement in RNA purification, because higher quantity of gDNA was recovered in RNA fractions. These results suggest that the presence of arginine in the elution buffer has also an effect in gDNA elution (data not shown).

In AC, the interactions occurring between a target biomolecule and its specific ligand are responsible for the high selectivity achieved in this technique. Those interactions are similar to the contacts described in many biological systems [28]. Thus, the binding mechanism is suggested to involve phenomenological interactions, such as biorecognition, between the amino acid and RNA, including, electrostatic, hydrophobic interactions, multiple hydrogen bonds, dipole–dipole forces, or cation–π interactions [29]. However, depending on the environmental conditions established, some interactions can be more favored than others, becoming more evident under those conditions. Since RNA is negatively charged due to the phosphate groups in its backbone, it is reasonable to predict a favored electrostatic interaction between RNA

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phosphate groups and arginine ligands. In fact, in some molecular recognition studies, arginine is reported as a preferential amino acid to contact with RNA when the overall negative charge of RNA is considered [29, 30]. Additionally, saturation transfer difference-nuclear magnetic resonance (STD-NMR) spectroscopy and surface plasmon resonance (SPR) biosensor techniques recently reported that adenine, cytosine, and guanine polynucleotides bind to arginine–agarose support mainly through the sugar-phosphate backbone [31].

On the other hand, gDNA was observed to be the less-retained nucleic acid, revealing a not so strong affinity for the arginine–agarose support as in case of RNA species. These findings are in agreement with some previous studies focused on the purification of pDNA by arginine chromatography [18, 32]. According to the authors, the negative charge of the biomolecules is important for their interaction with arginine, nonetheless the nucleotide bases exposure is also suggested to have a crucial role in nucleic acid retention [18, 32]. Thus, despite the negative charge of gDNA, its double-stranded structure causes the coverage of the nucleotides bases, disabling gDNA interactions. In line with this, the bases exposure on RNA species should play an important role in the favorable interaction found with arginine–agarose.

Furthermore, structural diversity of RNAs was recently described to be of significant importance in protein–RNA interactions because RNA can exhibit different moieties according to its folding state [1, 33]. Bioinformatics predictions showed that amino acids complexes with mRNA and tRNA, exhibiting less-compact secondary structures, have a greater number of base-specific contacts and fewer backbone contacts, while the amino acids complexes with rRNA (more compact secondary structures) have less base-specific contacts [33].

In this study, the purification approach described enabled the separation of gDNA and RNA. Moreover, the arginine support has shown ability to interact with all RNA classes even with different conformational rearrangements. The multi-position interaction of arginine with RNA sites [34, 35] can explain this result. The multiplicity of interaction sites can occur because arginine has two different polar centers with which RNA can strongly associate: at α-carbon group and the side chain guanidinium [29]. Thus, it is reasonable to suppose that the retention of all functional classes of RNA in arginine–agarose matrix is due to arginine side chain, which can promote multicontact with RNA backbone or RNA bases, according to RNA folding. Overall, it is suggested that although electrostatic interactions could play an important role on RNA retention, the bases contacts are also involved and modulate some favored interaction and specificity found in arginine–agarose chromatography.

3.2 RNA quality characterization

Fundamental criteria for extraction and purification procedures of total RNA concerning molecular biology application, in particular qPCR, have been reported [36, 37]. Accordingly, the RNA preparation should be free of proteins and gDNA, especially if the target is an intronless gene, should be undegraded (28S:18S ratio should be roughly between 1.8 and 2.0), free of enzymatic inhibitors for RT and PCR reactions, free of any substance that complexes essential reaction cofactors, such as Mg^{2+} or Mn^{2+}, and free of nucleases [36, 37].

Table 2. Total RNA quantification in different PC-3 total nucleic acid preparations injected onto arginine column

Total nucleic acid preparation injected (μg)	Integrated peak area	Total RNA (μg)
30	247.91	23.35
35	257.49	24.35
40	273.15	25.97
50	355.02	34.49
155		108.20

To validate these criteria, RNA samples obtained by arginine affinity purification were quantified, the RNA integrity was evaluated by ribosomal band intensity, and the presence of gDNA and proteins was assessed. Total RNA control samples obtained by using the commercial TRI reagent were also included in the measurements.

The results of RNA quantification are shown in Table 2 that presents the concentration of total RNA in different amounts of PC-3 nucleic acid extracts, which were applied to arginine chromatography. These results indicated that arginine–agarose matrix allowed the quantitation of different RNA contents in the complex mixture. In addition, the mass reduction relative to total nucleic acid extract corresponds to gDNA that is separated from total RNA by arginine–agarose matrix. Total RNA quantification by peak integration provided a reliable and accurate method because spectrometric methods often fail in sensitivity and are highly variable leading to over- or underestimation of the real RNA concentration [37, 38]. Regarding the recovery yields obtained, it was verified that from the chemical lysis of around 10^7 PC-3 cells, it was possible to obtain about 282 μg of nucleic acids. In Table 2, it can also be seen that from 114 $\pm$ 19 μg of total RNA present in the nucleic acids extract, 108 $\pm$ 11 μg of pure RNA was obtained after arginine AC purification, achieving a recovery yield of 96 $\pm$ 17% (Table 3). This RNA recovery is similar to the one obtained with TRI reagent, which enables the recovery of an RNA averaging amount of 157 $\pm$ 17 μg from the same starting number of cells.

The RNA integrity was assessed by agarose gel electrophoresis using UVIband–1D gel analysis software (Uvitec, Cambridge, UK) to determine the intensity of ribosomal bands. The proportion of the ribosomal bands (28S:18S) is crucial to guarantee RNA integrity, followed by the absence of low molecular weight RNA-degradation products. Figure 2B lane 2 shows the electrophoretic analysis of the RNA sample obtained after arginine AC isolation and Fig. 3 shows the semiquantitative analysis of the intensity of ribosomal bands.

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Table 3. Quantitative analysis of purity and recovery yield of RNA isolated by arginine–agarose chromatography. The correlation coefficients of gDNA and protein calibration curves were 0.995 and 0.993, respectively. Data are presented as means with SD ($n = 3$). TNA, total nucleic acid extract; Arg, arginine

Method	Sample	Volume (μL)	Nucleic acids (μg)		Recovery yield (%)	Proteins (ng/μL)	
			gDNA	RNA		200 TNA	400 TNA
Arg–AC	TNA extract	120	155 ± 21			18 ± 6	42 ± 4
			41 ± 29	114 ± 19			
	Peak 2 (RNA)		1 ± 0.27	108 ± 11	96 ± 17	ND[b]	5 ± 2
TRI[a]	RNA	120	0.39 ± 0.44	157 ± 17	____	ND[b]	4 ± 2

a) Control sample.
b) ND, not detected by micro-BCA assay.

The ratio of 1.96 indicates that the intensity of 28S rRNA band is two times higher than the intensity of 18S rRNA, demonstrating a good RNA integrity. In fact, the integrity of the RNA molecules is a key factor for the overall success of further application. Furthermore, RNA is one of the most difficult materials to separate under chromatographic conditions. One reason for this is that RNA is degraded very quickly in nature and its stabilization is very difficult. Hence, the final RNA integrity will depend on maintaining the stability of the sample before separation, throughout the purification process, and also during the recovery of RNA fractions when the separation has been completed. Our results demonstrate that the isolation steps involved in the affinity procedure allowed maintaining the RNA stability. As previously discussed, the chaotropicity of guanidinium buffers inhibits RNase action, preventing RNA degradation. Additionally, the application of arginine AC can also be strongly associated with the preserved integrity observed in RNA samples since arginine, owing to its multiplicity for interactions, has been largely associated with stabilizing effects on RNA conformations [35, 39, 40].

With relation to the residual contamination of gDNA, it is an inherent problem during RNA purification due to the similar physicochemical properties of RNA and DNA. Quantification by qPCR indicated that the residual concentration

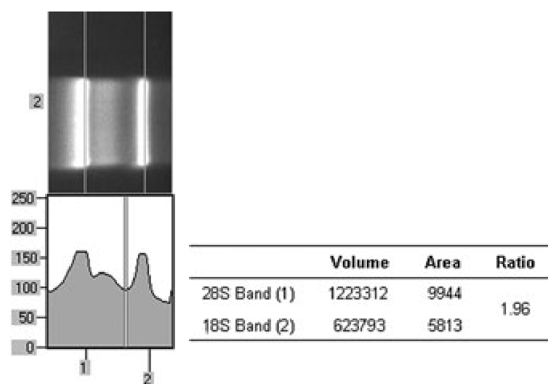

Figure 3. Semiquantitative analysis of the intensity of rRNA bands using UVIband–1D gel analysis software. 28S:18S ratio was calculated using the peak volumes. Data are representative of three independent experiments.

of gDNA was of 8 ± 0.27 and 3 ± 0.44 ng/μL in RNA samples after arginine AC and in total RNA samples isolated by TRI reagent, respectively (Table 3). Nevertheless, arginine AC allowed a significant decrease in gDNA levels in total RNA preparations. As mentioned above, the double-stranded structure of gDNA does not favor the interaction with the arginine matrix, so reduced gDNA content on total RNA fraction would be expected.

Some of the concerns associated with the presence of gDNA in RNA preparations are related to the interfering effect that it can promote on several molecular biology analyses, particularly in qPCR analysis. However, the impact of gDNA contamination on qPCR signals is often dependent on primer design strategy [41].

The protein analysis (Table 3) performed by micro-BCA method showed that when 200 ng/μL of total nucleic acid preparation was injected on arginine–agarose column, no protein was detected in total RNA or gDNA pools, indicating that protein content is inferior to the detection limit of the method (<5 ng/μL). However, a residual protein concentration of 5 ± 2 ng/μL was determined in total RNA samples, when the injection of total nucleic acid preparation was increased to 400 ng/μL. These results may indicate that proteins have a propensity to elute steadily throughout the gradient, which is probably due to the heterogeneous proteins content, promoting different interactions with arginine–agarose matrix. In line with these results, total RNA samples isolated with TRI reagent demonstrated undetectable proteins in concentrations of RNA up to 400 ng/μL, while above this concentration the residual protein level was of 4 ± 2 ng/μL.

Overall, regarding the described criteria for an accurate RNA extraction and purification method [36, 37], the results obtained so far demonstrate that the arginine affinity based protocol described here is a potential methodology for RNA isolation. The chemical lysis with guanidinium buffers used in this method is extensively described in literature for RNA extraction, including in many commercial kit and reagents, with no significant implications in downstream applications [27, 42]. Moreover, total nucleic acid extract is purified by AC controlled by an automatic system that improves reproducibility. In addition, arginine chromatography makes use

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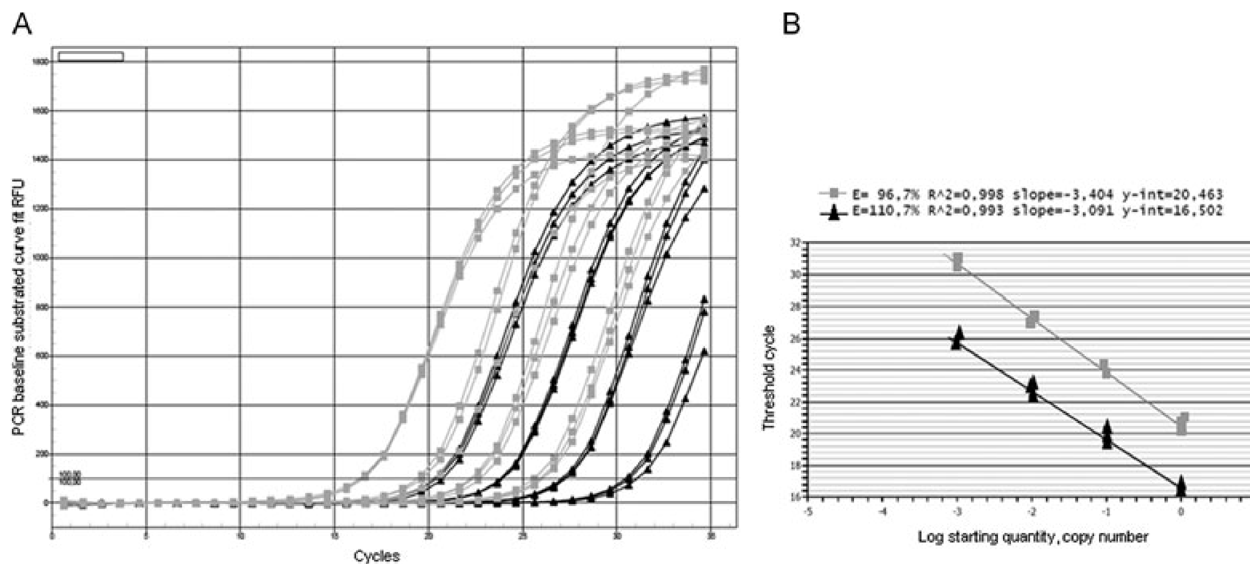

Figure 4. qPCR output showing amplification plots of hGAPDH expression (A) and PCR efficiencies (B). The squares and triangles lines represent hGAPDH expression profile from total RNA samples purified by arginine AC or TRI reagent, respectively. Data in (A) and (B) are representative of three separated experiments with total RNA samples isolated from three cell passages using both methods and were confirmed in other three experiments with 18S gene.

of low NaCl concentrations, rather than organic or toxic compounds, and the use of enzymes is not necessary. Finally, RNA concentration step employs ethanol precipitation in the presence of glycogen, which is used as a carrier agent to increase the recovery of RNA and to help in the visualization of the pellet. Ethanol is easily removed by centrifugation and evaporation and the use of 20 μg of glycogen is described not affecting downstream analysis [43]. Furthermore, total RNA samples were found with good integrity, low gDNA content, and the protein content was negligible.

Although chromatography is used more often in RNA isolation methodologies [6, 7, 10, 44], this is the first attempt to purify total RNA from eukaryotic cells using AC in an agarose support. Thus, it is considered that the implementation and optimization of this methodology can bring new insights to RNA purification.

3.3 Evaluation of total RNA isolated by arginine AC

With the aim to characterize arginine affinity based method for RNA isolation, total RNA samples obtained were used as template in qPCR, which is one of the most widely used techniques in modern molecular biology. Since TRI reagent is extensively used to isolate RNA for gene expression analysis, total RNA samples extracted by TRI reagent were used as a reference group.

First, the cDNA synthesis was successfully carried out using total RNA isolated by arginine column or TRI reagent. The quality of cDNAs was evaluated by conventional PCR, which enabled the amplification of hGAPDH fragments with 496 bp in both RNA samples (data not shown).

In order to evaluate if there is significant differences in detection of gene expression levels between RNA samples, the mRNA expression of hGAPDH and 18S genes was analyzed because they are two common housekeeping genes often used as endogenous references in qPCR [45, 46].

Figure 4 shows the qPCR plots (A) and PCR efficiency slops (B) for hGAPDH expression profiling from cDNAs synthesized from total RNA samples isolated by arginine affinity based method or TRI reagent. The amplification plots allowed obtaining the threshold cycle values (Ct), while PCR standard curves demonstrate the primers efficiencies. The spaced amplification curves from each sample produced a linear standard curve with reaction efficiencies between 90 and 110%, which is indicative of a good efficiency [47].

The Pfaffl method ($2^{-\Delta Ct}$) is one of the most recurrent approaches for calculating relative gene expression and it gives the possibility to present the expression data as "fold variation" [48, 49].

Table 4 indicates the mean of Ct values obtained for each sample and the fold variation between the two methods used

Table 4. Ct values obtained from the qPCR plots of each housekeeping gene and calculations of fold variation in expression between the two methods used for total RNA isolation. Data are presented as means with SD ($n = 3$)

Gene	Method	Ct	$2^{-\Delta Ct}$
GAPDH	Arg–AC	21 ± 3	16
	TRI	17 ± 2	
18S	Arg–AC	12 ± 4	4
	TRI	10 ± 3	

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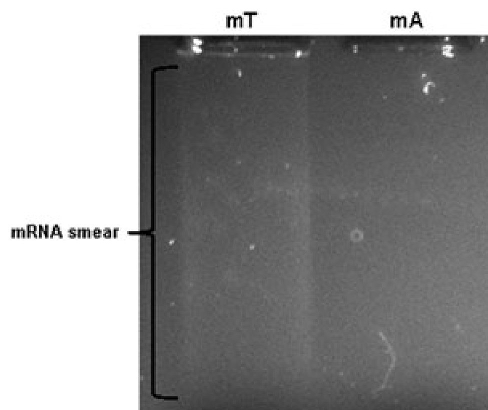

Figure 5. Electrophoretic analysis of mRNA samples isolated by Oligotex mRNA Mini Kit from total RNA purified by arginine AC and isolated by TRI reagent. Lane mT, mRNA from total RNA samples isolated by TRI; lane mA, mRNA from total RNA samples isolated by arginine–agarose column. Data were confirmed in three independent experiments with total RNA samples isolated from three cell passages using both methods.

to extract total RNA. The hGAPDH or 18S gene expression was found to be diminished in samples where total RNA was isolated by arginine affinity method, showing different fold variations between genes. The number of hGAPDH mRNA molecules in total RNA samples isolated by arginine AC is decreased by 16-fold relatively to hGAPDH mRNA molecules isolated by TRI reagent, while the expression of 18S rRNA is decreased by fourfold. These expression differences might be due to the fact that the methods may isolate different proportion of RNA molecules, leading to a higher concentration of rRNA comparatively to mRNA in total RNA samples.

Thus, it was supposed that Ct values obtained by qPCR were probably affected by the quantities of the starting material, since the same amounts of RNA should be used when comparing different samples using qPCR [37]. Therefore, mRNA was isolated from total RNA samples (Oligotex mRNA Mini Kit, Qiagen Hilden, Germany) obtained either by arginine column or TRI reagent, in order to guarantee that the same initial concentration of molecules was present when performing qPCR. The mRNA concentration and quality were measured using NanoPhotometer and through agarose gel electrophoresis (Fig. 5). Curiously, no smear was visualized in mRNA sample obtained from arginine affinity based method (Fig. 5, lane mA), contrarily to what was observed in the other sample (lane mT). Next, hGAPDH amplification was quantified by qPCR in both mRNA samples, which revealed a similar fold variation comparatively to the experiments using total RNA samples isolated by arginine AC or TRI reagent (data not shown). These findings sustain the hypothesis that the expression differences can be due to different amounts of starting molecules. This is supported by the fact that mRNA molecules from arginine AC purification were not visualized in agarose gel, suggesting that the real RNA concentration was not the one measured in the spectrophotometer. Considering the 16-fold variation between the two methods for hGAPDH expression, the mRNA injected in the agarose gel was indeed less than the expected and it was below the detection limit of electrophoresis technique. It is possible that the mRNA quantified spectrophotometrically has been overestimated due to the presence of contaminants in the sample. Regarding the gDNA contamination in total RNA samples, a higher gDNA level in total RNA obtained by arginine affinity method than in TRI samples was found (Table 3). The primers used to detect hGAPDH did not amplify gDNA because the primers are located in different exons. On the other hand, 18S primers can amplify contaminating gDNA. Our results suggest that the residual gDNA in total RNA samples did not affect the gene amplification because the Ct values obtained from arginine affinity protocol are higher than the Ct values determined in total RNA extracted by TRI reagent. This means that more PCR amplification cycles in samples from arginine AC method were necessary than in TRI samples, in order to detect the presence of RNA molecules. If qPCR was affected by gDNA contamination, Ct values in the samples from arginine AC method would be lower than in TRI samples.

Nevertheless, the overall fold variation (<16) for the housekeeping genes used are considered minor differences in RNA concentrations [24], which encourage the use of arginine affinity based method to prepare RNA samples to be applied in downstream analysis aiming gene expression studies. Yet, the use of total RNA isolated by the arginine AC should be taken cautiously in qPCR analysis of genes with low-expression levels. Moreover, the accuracy of the amplification plots and PCR efficiencies obtained in qPCR quantification attest the performance of arginine affinity based method to isolate total RNA with high quality, since degraded or impure RNA can limit the efficiency of the RT reaction and reduce yield [23].

4 Concluding remarks

In the present study, a new affinity approach for total RNA isolation from PC-3 total nucleic acid extracts using arginine AC is introduced. Amino acid based AC has shown a great potential for the purification of nucleic acids, because it combines the selectivity of a naturally occurring biological interaction with the simplicity of a single small molecule used as a ligand. The exploitation of specific affinity interactions between RNA and arginine allowed obtaining total RNA preparations with high integrity and purity, which were attested by qPCR analysis.

The results of control analysis and performance indicated that the chromatographic separation is a promising strategy for total RNA isolation. Nevertheless, some experimental setting should be reevaluated in order to render arginine–agarose chromatography viable for the purification of total RNA pursuing gene expression analysis.

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