UNIVERSIDADE DA BEIRA INTERIOR
Ciências

Ionic liquids for the purification and stabilization of nucleic acids

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Dissertação para obtenção do Grau de Mestre em
Biotecnologia
(2º ciclo de estudos)

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Covilhã, outubro de 2019

Acknowledgments

For the accomplishment of this work I had the unconditional support of several people, to whom I am deeply grateful.

First, I want to thank Doctor Fani Sousa and Doctor Mara Freire for the excellent follow-up throughout this year. During this time, I have learned that work is done in many ups and downs, and when we are down there is always a way around, no matter how difficult it may seem. Thank you for all your unconditional support, effort, trust, patience and scientific knowledge, which were fundamental for my development as student, person and professional worker.

A big thank you to Doctor Patrícia Pereira and Doctor Augusto Pedro for their excellent knowledge transmitted, even not for so long as I would wish, was an indispensable help for the development of this work. Also want to deeply thank Tiago Santos for helping me in any kind of situation, you were always willing to support me even if you could not. To Doctor Márcia Neves for welcoming me so well in a new environment for me, and for all her effort and knowledge transmitted through my brief stay in Aveiro.

A deep thanks to my so special friend, Micaela Riscado, to whom I am forever grateful. You taught me so much, at so many levels, professionally and personally. There really are not enough words to describe what you meant to me throughout this turbulent year, and I know that without you, I would never have done it. I really love you with all my heart. You are my perfect story book.

A very special thanks to Bruno Baptista, for all his incredible patience and unconditional support. Thank you for always being someone I could count on in many situations, and for making me grow both professionally and personally. Without you this journey would never have been the same.

To all my friends, Diana Pereira, Raquel Neves, Adriana Pinto, Rosa Sequeira, Rita Proença, Mariana Flor, Diana Gomes, Diana Esteves and Duarte Rocha, who are very important to me, you made this year so much better and even more special. Also, to my most recent laboratory colleagues Pedro Ferreira, Pedro Vicente and Sofia Oliveira. All of you are like family to me.

I also want to thank all the people I have worked with in CICS, specially Jorge Ferreira, and to all Path members. A special thanks to Liliana Azevedo, Maria João Santos, Johanna Simões, Catarina Almeida and Jéssica Bairos for making my stay in Aveiro so amazing and memorable.

Last, but not least, I want to thank my parents, Maria do Carmo Carapito and Carlos Carapito, as well as my dearest brother, Luís Carapito, for all their incredible patience and support through everything in my life.

Resumo Alargado

As terapias baseadas em ácidos nucleicos têm surgido como excelentes abordagens no que diz respeito ao tratamento de diversas doenças como cancro e doenças neurodegenerativas. Contudo, para que estas biomoléculas possam ser usadas como biofármacos elas devem ser obtidas com elevados graus de pureza, apresentando atividade biológica e integridade estrutural. Sendo estes os maiores desafios na obtenção de biofármacos, é necessário desenvolver estratégias de purificação que assegurem a qualidade dos mesmos. Os líquidos iónicos (ILs) são sais líquidos à temperatura ambiente que apresentam propriedades muito interessantes, nomeadamente a possibilidade de criar inúmeras combinações entre catiões e aniões, adequando assim as estruturas aos objetivos pretendidos.

Neste âmbito, o presente trabalho tem como principal objetivo o desenvolvimento de uma nova estratégia de purificação de ácidos nucleicos usando suportes funcionalizados com diferentes ILs. Paralelamente, pretende-se também avaliar a capacidade de estabilização de RNA que determinados ILs apresentam, assim como a sua toxicidade em linhas celulares humanas, de forma a averiguar a biossegurança destas substâncias.

Inicialmente, diferentes ILs foram imobilizados covalentemente em suportes de sílica esférica, usada como fase estacionária, dos quais resultaram os seguintes materiais: [Si][C3C1Im]Cl, [Si][N3222]Cl, [Si][N3444]Cl, [Si][N3888] e [Si][N3114]Cl. Considerando estruturalmente os ILs selecionados, destaca-se a utilização como catião de um composto heterocíclico, um baseado em uma amina assimétrica e três com aminas simétricas que variam no tamanho da cadeia carbonada (contendo 2, 4 ou 8 carbonos). Para além da caracterização estrutural dos suportes, cada um deles foi empacotado em colunas de bancada e foram realizados ensaios de *screening* de ligação e eluição com amostras de RNA. Nestes ensaios, foi possível verificar que todos os ligandos tinham a capacidade de estabelecer interações electroestáticas e hidrofóbicas com o RNA, isto porque todos possuem grupos de carácter iónico e hidrofóbico, demonstrando assim a possibilidade de explorar um comportamento multimodal. Contudo, os resultados mais promissores foram obtidos com os suportes de [Si][N3222]Cl e [Si][N3114]Cl, pelo que foram estes os materiais usados nos ensaios seguintes. Neste contexto, a etapa seguinte foi de avaliação do potencial destes suportes para separar DNA e RNA presentes em amostras de lisado, considerando também o efeito do pH (entre 6 e 8) nos perfis de retenção e padrão de seletividade entre estas biomoléculas. Ambos os suportes demonstraram essa capacidade de separação, apesar de [Si][N3114]Cl ter apresentado melhor seletividade que [Si][N3222]Cl, sendo por isso mais promissor para outros estudos de separação.

Adicionalmente, foram realizados ensaios de estabilização de amostras de RNA com quatro diferentes ILs, nomeadamente [N1111]Cl, [N2222]Cl, [N3333]Cl e [N4444]Cl. Com este estudo foi observado um efeito negativo na estabilidade do RNA consoante o aumento do tamanho da cadeia carbonada do catião, tendo-se apenas obtido aumento da estabilidade térmica do RNA com o IL [N1111]Cl. Por fim, e de forma a averiguar a toxicidade dos suportes e dos ILs análogos, foram realizados ensaios de citotoxicidade numa linha celular humana, em que os materiais e

ILs foram colocados em contacto com as células até um período de tempo de 48 horas. Verificou-se que os suportes com os ILs imobilizados na superfície não demonstraram qualquer toxicidade para estas células, ao contrário do observado com os ILs análogos aos usados nos suportes. Estes demonstraram significativa toxicidade, que também se verificou ser dependente do tamanho das cadeias carbonadas e da concentração de IL aplicada, apresentando assim uma vantagem dos ILs imobilizados em suportes, comparativamente ao uso de ILs no estado líquido. Desta forma, foi possível demonstrar a importância de vários ILs na purificação e estabilização dos ácidos nucleicos, tratando-se de uma área em constante crescimento.

Palavras-chave

Líquidos Iónicos; Cromatografia; Purificação de ácidos nucleicos; Estabilidade de RNA

Abstract

Nucleic acids-based therapies have emerged as excellent approaches to treat various diseases such as cancer and neurodegenerative disorders. However, for these biomolecules to be used as biopharmaceuticals they must be obtained with high purity, presenting biological activity and structural integrity. As these are the main challenges in obtaining biopharmaceuticals, it is necessary to develop purification strategies that ensure their quality. Ionic liquids (ILs) are liquid salts at room temperature that have very interesting properties, namely the vast combination of cation and anion that can be made, thus tailoring the structures to the intended purpose.

This work has as main objective the development of a new strategy for the purification of nucleic acids using functionalized supports with different ILs. In addition, we also intend to evaluate the RNA stabilizing capacity of certain ILs as well as their toxicity in human cell lines in order to ascertain the biosafety of these substances.

Initially, different ILs were covalently immobilized onto spherical silica supports, used as a stationary phase, resulting in the following materials: [Si][C3C1Im]Cl, [Si][N3222]Cl, [Si][N3444]Cl, [Si][N3888]Cl and [Si][N3114]Cl. In addition to the structural characterization of the supports, and as a screening procedure, the binding and elution of low molecular weight RNA molecules was tested under ionic and hydrophobic conditions with all the synthesised supports, in order to select the most promising ligand(s) for nucleic acids purification. [Si][N3222]Cl and [Si][N3114]Cl were the chosen supports for further separation procedures between genomic DNA and RNA. Both supports showed ability for separating these two species, although [Si][N3114]Cl displayed better selectivity, thus becoming more promising for future separation assays. Additionally, RNA stabilization assays were performed with four different types of ILs, analogues to the ligands, namely [N1111]Cl, [N2222]Cl, [N3333]Cl and [N4444]Cl. Only [N1111]Cl showed to enhance RNAs thermal stability, verifying by this, a negative contribution of the alkyl chains lengths in the stabilization of this biomolecule. Besides, cytotoxicity assays with the two chosen supports and these four ILs were performed. IL-immobilized supports did not present any cytotoxicity, while liquid ILs were discovered to largely compromise cell viability. In this sense, the usage of ILs immobilized onto solid supports appears to be safer than using bulk ILs. Thus, was possible to demonstrate the importance of various ILs in purification and manipulation of nucleic acids, becoming a continuously growing area.

Keywords

Ionic Liquids; Chromatography; Nucleic acids purification; RNA Stability

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List of Acronyms

A	Adenine
ABS	Aqueous Biphasic System
AC	Affinity Chromatography
C	Cytosine
CD	Circular Dichroism
circRNA	Circular RNA
DEPC	Diethyl Pirocarbonate
DNA	Deoxyribonucleic Acid
dsRNA	Double-stranded RNA
E. coli	*Escherichia coli*
G	Guanine
GB-ILs	Good's Buffers ILs
gDNA	Genomic DNA
hFIB	Human Fibroblasts
HIC	Hydrophobic Interaction Chromatography
HIV	Human Immunodeficiency Virus
HPLC	High-Performance Liquid Chromatography
IEC	Ion Exchange Chromatography
IL	Ionic Liquid
lncRNA	Long non-coding RNA
mcDNA	Minicircle DNA
miRNA	Micro RNA
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ncRNA	Non-coding RNA
NMR	Nuclear Magnetic Resonance
pDNA	Plasmid DNA
PILs	Poly(ionic liquids)
piRNA	Piwi-interacting RNA
pre-miRNA	miRNA precursor
pri-miRNA	Primary miRNA
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy

SILs	Supported Ionic Liquids
siRNA	Short-interfering RNA
snRNA	Small Nuclear RNA
T	Thymine
T_m	Melting Temperature
tRNA	Transfer RNA
U	Uracil
VOS	Volatile Organic Solvents

Scientific Communications

Oral Presentation - Award for "Best short-communication"

Rita Carapito, Márcia Neves, Patrícia Pereira, Augusto Pedro, João A. Queiroz, Mara G. Freire, Fani Sousa. Ionic liquids-based supports for the purification of RNA. XIV Anual CICS-UBI Symposium. 4-5 July 2019, Covilhã, Portugal.

Poster Presentation (Accepted)

Rita Carapito, Márcia Neves, Patrícia Pereira, Augusto Pedro, João A. Queiroz, Mara G. Freire, Fani Sousa. Ionic liquids-based supports for the separation of nucleic acids. III International Congress in Health Sciences Research: Towards Innovation and Entrepreneurship - Trends in Aging and Cancer. 14-16 November 2019, Covilhã, Portugal.

CHAPTER 1 – Introduction

1.1. Nucleic Acids

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), which are the molecular storage of genetic information in cells (Nelson & Cox, 2011), are constituted by nucleotides, which also have a variety of roles in cellular metabolism. Nucleotides are composed by three characteristic elements: a nitrogen-containing base, a pentose, and a phosphate group. The common bases and pentoses of the nucleotides are heterocyclic compounds. The base of a nucleotide is covalently attached by an N-ß-glycosyl bond to the 1' carbon of the pentose ring, and the phosphate is esterified to the 5' carbon (Figure 1). Relatively to nitrogen bases they can be classified as purines, which include adenine (A) and guanine (G), and pyrimidines, which include cytosine (C), thymine (T) and uracil (U) (Figure 2). Purines are constituted by two rings of five and six atoms, while pyrimidines are constituted only by one ring of six atoms (Nelson & Cox, 2011; Quintas *et al.*, 2008).

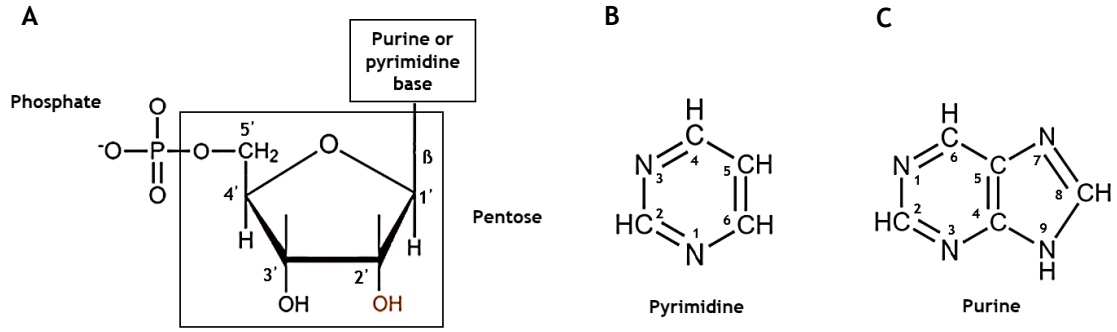


Figure 1: Structure of nucleotides. A represents the general structure of a nucleotide, with the convention numbering of the pentose ring. In this case, it is shown a ribonucleotide, due to the presence of the –OH group on the 2' carbon (in orange), which is replaced by –H in deoxyribonucleotides. B and C represent the general structures of pyrimidine and purine bases of nucleotides, with the respective numbering conventions (Adapted from Nelson & Cox, 2011).

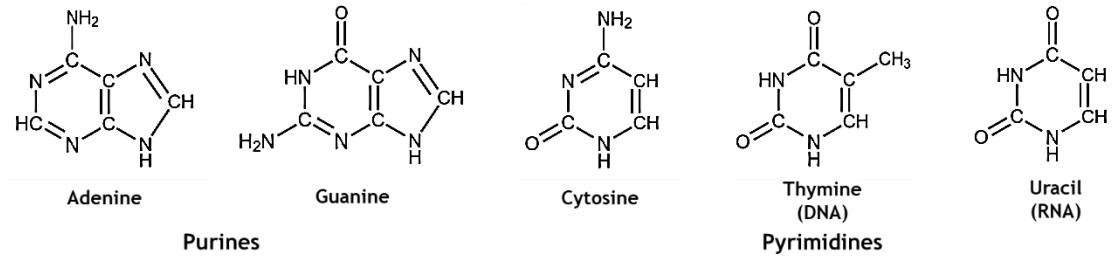


Figure 2: Structure of the major purines and pyrimidines that constitute nucleic acids (Adapted from Nelson *et al.*, 2011).

Both DNA and RNA contain the two purines, adenine and guanine, and the pyrimidine cytosine. However, they differ in one pyrimidine, because DNA has thymine in its constitution, while RNA has uracil. Only rarely thymine occurs in RNA or uracil in DNA (Nelson & Cox, 2011; Quintas *et al.*, 2008).

Nucleotides in nucleic acids are successively linked by a covalent bond through phosphate-group "bridges", in which the 5'-phosphate group of one nucleotide is linked to the 3'-hydroxyl group of the next nucleotide, and so creating a phosphodiester linkage. This molecules organization confers a moderate hydrophilic nature to both DNA and RNA. The phosphate groups, with a pK_a near to 0, are completely ionized and negatively charged at pH 7, and the negative charges are generally neutralized by ionic interactions with positively charged groups on proteins, metal ions and polyamines (Nelson & Cox, 2011).

1.1.1. Ribonucleic Acid (RNA)

Regarding the role of RNA in cells, for long time it was only seen as a simple intermediate in the transference of genetic information from DNA to proteins, with no further functionalities in the complex gene expression process. However nowadays, it is known that different types of RNA play a pivotal role in several essential biological processes, including epigenetic control, gene transcription, translation, RNA turn over, chromosomal organization, genome defense, among others. Examples of classes of RNA involved in the catalysis and genetic regulation are ribozymes and micro RNAs (miRNAs), respectively. Meanwhile, new roles for RNA continue to be discovered, thus proving the tremendous importance of this macromolecule in cell biology and consequently, in the potential diagnosis and treatment of many diseases (Guil & Esteller, 2015; Martins *et al.*, 2014; Sharp, 2009; Quintas *et al.*, 2008).

RNA is a polymer with a long chain of ribonucleotides, that often presents a single-stranded structure and is quite flexible. This biomolecule resembles to DNA in many ways, except for one nucleotide, as mentioned above. Besides this, each nucleotide in RNA has a 2'-OH group in the pentose ring (ribonucleotide), contrary to DNA, that has only a hydrogen atom in the same position (deoxyribonucleotide). This characteristic confers DNA a greater resistance to alkaline hydrolysis, and so RNA is less stable due to its 2'-OH group making the 3'-phosphodiester bond susceptible to nucleophilic cleavage, being easily hydrolyzed by hydroxide ions (Martins *et al.*, 2014). Therefore, RNA is quite unstable *in vivo* given that ribonucleases (RNase) are always present in serum and in cells (Burnett & Rossi, 2012). Though RNA displays usually a single-stranded conformation, when short regions within the nucleotide strand have complementarity, they can pair and form secondary structures. In contrast, since DNA is already constituted by two complementary strands, is inherently more restricted in the range of secondary structures that can assume. RNA secondary structures are often designated as hairpins (or hairpin-loop or stem-loop structures). Once these secondary structures are determined by the base sequence of the nucleotide strand, many different types of structures can take place, and since their structures dictate their function, RNA molecules have the potential of performing a tremendous variety of functions (Pierce, 2016). These comparison between DNA and RNA characteristics is summarized in Table 1.

Table 1: Features comparison between RNA and DNA (Adapted from Pierce, 2016).

Features	DNA	RNA
Composed by nucleotides	Yes	Yes
Sugar type	Deoxyribose	Ribose
Presence of 2'-OH group	No	Yes
Bases	A, G, C and T	A, G, C and U
Double or single stranded	Usually double	Usually single
Secondary structure	Double helix	Many types
Stability	Stable	Easily degraded

1.1.1.1. Coding and Non-coding RNA

Coding RNA refers to the RNA that encodes for a protein, which is messenger RNA (mRNA). The mRNA contains the information needed to produce a protein, which means that the nucleotides sequence on the mRNA dictates the sequence of amino acid residues in the polypeptide chain resulting from the translation process (Pierce, 2016; Quintas *et al.*, 2008).

The first step of gene expression is transcription, in which an mRNA molecule is formed based on the information of a DNA template, by the enzyme RNA polymerase in the nucleus. The product of transcription of DNA is a single-stranded RNA, which tends to assume a right-handed helical conformation dominated by base stacking interactions, and when there are any self-complementary sequences in the molecule, more complex structures take place (Nelson & Cox, 2011). Following some modifications, the mature mRNA is then used in the translation process to obtain the encoded protein.

Non-coding RNA (ncRNA) refers to RNA that does not encode a protein but modulates cell function by controlling gene expression programs through many different mechanisms (He *et al.*, 2018; Idda *et al.*, 2018). ncRNAs are divided into two groups: housekeeping ncRNAs and regulatory ncRNAs. The housekeeping ncRNAs are constitutively and ubiquitously expressed and play crucial roles in diverse cellular activities; they include transfer RNA (tRNA), ribosomal RNA (rRNA) and small nuclear RNA (snRNA). The tRNA acts like an adaptor molecule during protein synthesis, being the carrier of amino acids and acting as the physical linkage between mRNA and the amino acid sequence of the protein. In its turn, rRNA is one of the structural components of the ribosome and is the predominant product of transcription, constituting about 80-90% of total mass of cellular RNA. In prokaryotes there are three types of rRNA: 16S, 23S, and 5S, while in eukaryotes exist 4 types: 18S, 28S, 5.8S, and 5S. Lastly, snRNAs comprise a small group of non-polyadenylated non-coding transcripts that act in the nucleoplasm (Quintas *et al.*, 2008; Matera *et al.*, 2007).

On the other hand, regulatory ncRNAs are expressed in specific cell types and act in response to developmental cues, internal conditions, and environmental stimulations. Thus, regulatory

ncRNAs include short-interfering RNA (siRNA), piwi-interacting RNA (piRNA), long non-coding RNA (lncRNA), circular RNA (circRNA) and micro RNA (miRNA) (Idda *et al.*, 2018; Quintas *et al.*, 2008). The siRNAs (20-25 nt) are a class of double-stranded RNAs (dsRNAs) designed specifically to silence the expression of target genes (Gallas *et al.*, 2013). The piRNAs (24-32 nt) are short single-stranded ncRNAs produced via DICER-independent mechanism. By interacting with piwi-proteins, these RNAs regulate gene expression programs through transposon silencing, gene transcription, as well as mRNA turn over and translation (Ponnusamy *et al.*, 2017). LncRNAs (>200 nt) consist in pivotal molecules in the regulation of gene expression, once they bind to the mRNA with few or no protein-coding capacity. This type of RNAs can be located within nuclear or cytosolic fractions, and have some structural features similar to mRNA, including 5' capping, polyadenylated 3' tails and undergo through alternative splicing to give origin to the final product (Pereira *et al.*, 2016 (b)). Regarding circRNAs (50-2000 nt), that were more recently discovered as a special novel type of endogenous ncRNAs, they present covalently closed loop structures with neither 5' or 3' polarities, and non-polyadenylated tails, unlike linear RNAs (Qu *et al.*, 2015). This specific structure allows this class to not be affected by exonuclease RNase R. Although circRNAs in humans are considered to belong to ncRNAs class, some of them are experimentally verified to encode protein products (Bagchi, 2018).

In respect to miRNAs (20-30 nts), they are the most intensively studied type of ncRNAs due to their great potential for the development and application in therapeutic strategies for diseases that are caused by gene defections, since Human Immunodeficiency Virus (HIV) infection, to various types of cancer, and even Alzheimer's disease (AD). This therapeutic effect can be accomplished because miRNAs, depending on the degree of complementarity, are capable of binding to mRNA and directly repress the expression of the protein encoded in that same mRNA. This regulation is made through a series of successive biological steps, at the post-transcriptional level, involving a miRNA precursor (pre-miRNA), and consequently, a mature miRNA that will be incorporated into the RNA-induced silencing complex (RISC) in order to regulate the target mRNA. This biochemical mechanism is more detailed in Figure 3 (Pereira *et al.*, 2017; Pereira *et al.*, 2016 (b); Gomes *et al.*, 2013; Burnett & Rossi, 2012;).

There are two different types of therapeutic strategies based on miRNA. One approach goes through a miRNA replacement, where a specific miRNA is under expressed in a disease, and therefore, a small double-stranded RNA molecule is delivered into the cells attempting to mimic the activity of the absent or downregulated miRNA. On the other hand, the other strategy undergo miRNA targeting, where antisense oligonucleotides bind to endogenous miRNAs to restrain their interaction with targets (Vidigal & Ventura, 2015; Guo *et al.*, 2011). Since this targeting process requires the presence of such short-conserved sequences, experimental evidence has shown that under or overexpression of a miRNA in cultured cells results in the regulation of hundreds of genes (Vidigal & Ventura, 2015).

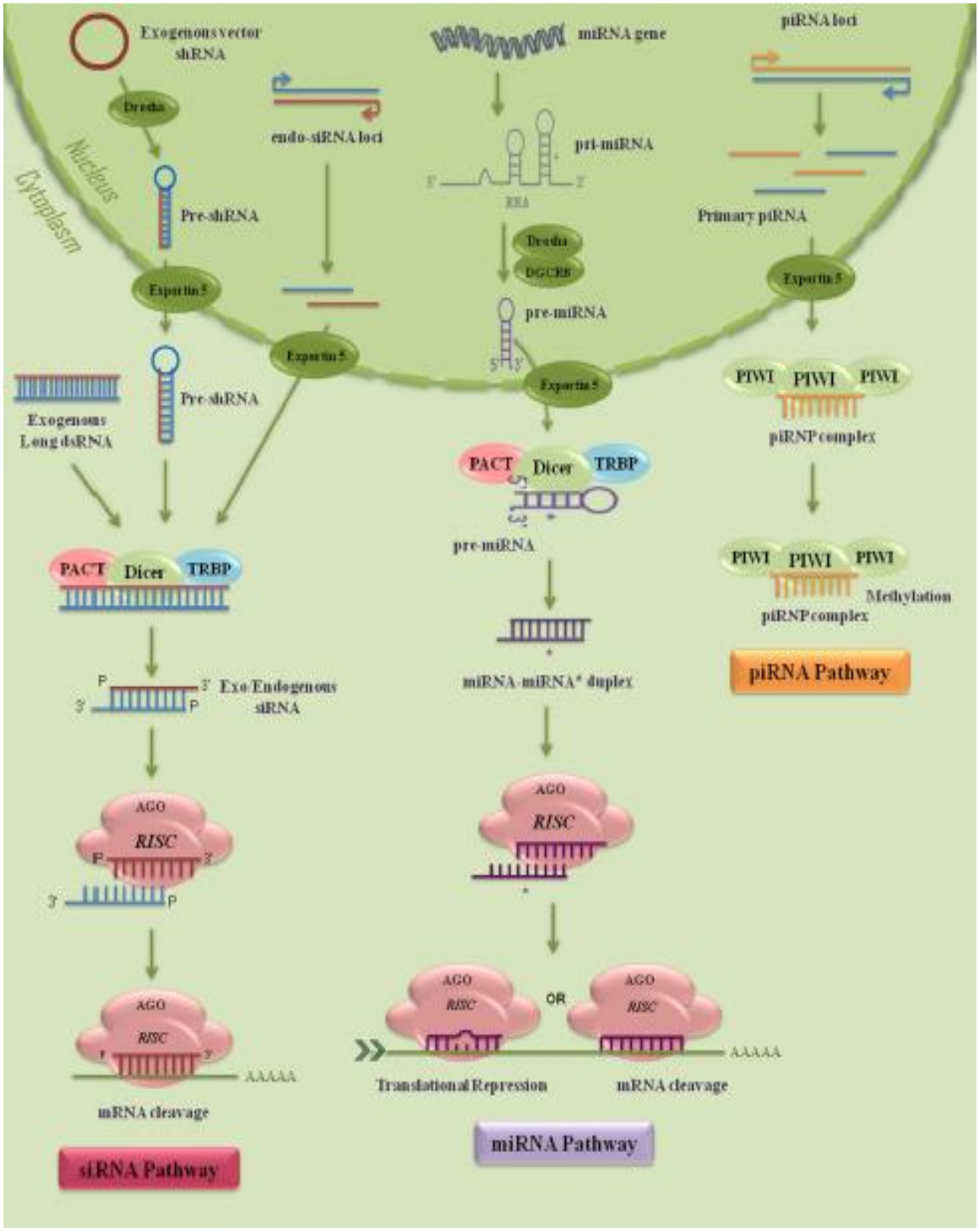


Figure 3: siRNA, miRNA and piRNA silencing pathways and their essential components (Pereira *et al.*, 2016 (b)).

1.1.1.2. ncRNAs: Role and Applications

In Table 2 are summarized the main functions of each ncRNA presented in the previous topic, as well as their corresponding applications in many different research areas.

Table 2: Different types of ncRNAs with their corresponding lengths and main applications in various investigation areas.

ncRNAs	Length	Function	Applications	References
tRNA	76-90 nt	Helps to incorporate amino acids into polypeptide chain	*In vitro* kinetics studies of translation rate	Pierce, 2016; Ledbetter *et al.* , 2018
rRNA	Variable	Structural and functional components of the ribosome	Structural and functional studies of mechanism features of translation process	Pierce, 2016; Weisser & Ban, 2019
snRNA	~150 nt	Splicing and other functions	Understanding many human diseases	Martins *et al.*, 2014; Matera *et al.*, 2007
siRNA	20-25 nt	mRNA cleavage and translation repression	Therapeutic tool for many diseases, including cancer	Martins *et al.*, 2014; Hajiasgharzadeh *et al.*, 2018
piRNA	24-32 nt	Supresses the transcription of transposable elements in reproductive cells	Studies of molecular mechanisms, associated to various diseases, specially cancer	Öner, 2019; Pierce, 2016
lncRNA	> 200 nt	Transcriptional and post-transcriptional regulation of gene expression	Maintain cell physiology and are involved in a range of human diseases including AD, cardiovascular disorders and cancer	Gomes *et al.*, 2013; Idda *et al.*, 2018
circRNA	50-2000 nt	Regulate gene expression by acting as a miRNA sponge, RNA binding protein sponge and translational regulator	Novel therapeutic approaches to treat various diseases from neurological disfunctions to cancer	Sekar & Liang, 2019; Zhang *et al.*, 2018
miRNA	20-30 nt	mRNA cleavage and translation repression	Already shown promising results for treatment of cancer, degenerative disorders and viral infections	Martins *et al.*, 2014; Saliminejad *et al.*, 2018

Besides the applications referred above, it must be noted that siRNA, lncRNA, piRNA, circRNA and miRNA are believed to be potential biomarkers for various diseases, thus developing an important role in the diagnosis and prognosis of these diseases (Fattahi *et al.*, 2019; Öner, 2019; Sekar & Liang, 2019; Hajiasgharzadeh *et al.*, 2018; Saliminejad *et al.*, 2018). By this, we can infer the great importance of preparing these biomolecules in order to be applied in a large range of studies.

1.2. Production of RNA

RNA is gaining more and more relevance as an increasing number of functions, including regulatory and enzymatic functions, are being discovered. In order to understand RNA functions and perform biochemical, biophysical and genetic studies, sample preparation is required, and most techniques are still in development (Baronti *et al.*, 2018; Beckert & Masquida, 2011). Several available techniques are adequate for molecular biology procedures, but if it is intended the preparation of RNAs for therapeutic purposes, other challenges must be overcome, and the procedures must be improved in accordance with the product requirements. Most miRNAs used in the development of new therapeutic strategies have been obtained by chemical synthesis, enzymatic synthesis, or recombinant production (Pereira *et al.*, 2017 (a)).

Chemical synthesis is the method of choice to produce oligonucleotides shorter than 10 nucleotides, being the upper size limit of approximately 80 nucleotides. The synthesis reaction proceeds from the 3' end to the 5' end, and comprises four steps, that include 2'-OH and 5'-OH protecting groups of phosphoramidite monomers and successive reactions of deblocking-coupling-capping-oxidation. The whole cycle can be repeated until the desired length of the oligonucleotide polymer is achieved. Therefore, it is a fast method and possible to modify, however, it requires expensive equipment, it has a limited chain length, and modified phosphoramidites are very expensive (Baronti *et al.*, 2018; Hogrefe *et al.*, 2013; Caruthers *et al.*, 2011).

In the other hand, enzymatic synthesis, or *in vitro* transcription, allows template-directed synthesis of RNA molecules with any sequence, since short oligonucleotides to those of several kilobases. An RNA molecule is synthesized by *in vitro* transcription using the promotor components of bacteriophage systems, being the most frequently used the T7 system that requires only Mg^{2+} as a co-factor to perform RNA synthesis. Although this method can produce RNA molecules on a microgram scale, large-scale production is more difficult by the necessity of high quantities of RNA polymerases, which are very expensive. Besides that, the method often results in low yields and multiple enzymatic and purification steps are required in order to remove impurities associated to the process. In fact, the presence of some impurities can lead to non-targeted gene silencing, which unable these produced RNAs to be applied onto preclinical or clinical studies (Baronti *et al.*, 2018; Pereira *et al.*, 2017 (a); Beckert & Masquida, 2011).

However, considering that there is a need for rapidly produce promising biopharmaceuticals, and even achieve sustainable production procedures at large scale, recombinant production

emerges as the more promising method for this purpose. Recombinant RNA techniques using *in vivo* agents are more capable to retain the structure, function and safety properties of natural RNAs. The principle of this production strategy consists in the introduction of the target RNA coding sequence into a vector, and the resulting plasmid is inserted into the host cells, usually *Escherichia coli* (*E. coli*). Posteriorly, the cell machinery will produce the target RNA that will be accumulated in the cytosol. The recovery of RNA depends on the lysis of these cells, and finally the RNA of interest must be purified mainly by chromatographic techniques. Thus, recombinant approaches offer a cheap and simple alternative to *in vitro* synthesis, being capable of achieving high yields in a shorter period of time (Baronti *et al.*, 2018; Pereira *et al.*, 2017 (a) ; Pereira *et al.*, 2017 (b); Ponchon & Dardel, 2011).

The possibility of using different expression hosts has been considered by Pereira and co-workers, when using *Rhodovulum sulfidophilum* as an expression system. In that work, it was shown that, even though *E. coli* production could lead to higher yield within a shorter period of time, the production in *R. sulfidophilum* could be advantageous because of its ability to secrete the RNA to the extracellular medium, which greatly simplifies the downstream purification and limits protein contamination (Pereira *et al.*, 2016 (a)).

1.3. Chromatography for RNA purification

Usually, RNA molecules are obtained from complex mixtures, being of great importance the establishment of a robust purification process in order to obtain the target molecule with total integrity, stability, and purity. In this sense, chromatography techniques arise as the method of choice for the purification of biomolecules, since they are capable of isolating different products with high intrinsic value (Scott, 2003).

Chromatography consists in a very robust, efficient and versatile technique, in which with only a single step, it can be possible to separate a mixture into its individual components and simultaneously, have a quantitative characterization of each component. This differential separation of the mixture is achieved by the co-existence of two main phases, the stationary phase and the mobile phase of the system. The stationary phase is always composed by a solid phase, or solid support with ligands or a layer of a liquid adsorbed on its surface, while the mobile phase is always a liquid or a gaseous component (Coskun, 2016; Scott, 2003). The constituents of the mobile phase that interact more with the stationary phase are more retained in the system than those that are distributed selectively in the mobile phase. As a result, solutes are eluted from the system in the increasing order of affinity to the column, this is, the more interacting solutes will be eluted at the end, while the least interacting will be eluted at the beginning of the run (Grob, 2004). The elution process can be controlled by different conditions, including ionic strength, temperature, pH, various additives such as detergents, reducing agents and metals. Therefore, by appropriately adjusting the buffer composition, the conditions of binding and elution of the target biomolecule can be optimized (Janson, 2011).

As a matter of fact, a chromatographic process occurs in five main stages: 1) An equilibration of the two phases, adjusting de column to the binding conditions; 2) Sample injection; 3) A washing or elution of non-binding molecules; 4) Elution of the target biomolecule by altering the buffers composition; 5) A regeneration/re-equilibration step, in which all the remaining bound material and impurities are eluted, and de column is ready for another chromatographic process. An ideal matrix should be chemically and physically stable so that it can be resistant to extreme conditions usually used in regeneration and sterilization steps between chromatographic assays. It should also be rigid enough to allow high linear flow rates (5 cm/min or more), and the matrix substance should allow the formation of gels with a wide range of controllable porosities (Janson, 2011).

1.3.1. Size exclusion chromatography (SEC)

Size exclusion chromatography (SEC) or gel filtration consists of a separation technique that discriminates different compounds by their size or molecular weight. So, in the mobile phase, larger molecules, with higher molecular weight, flow through the column without being retained because they do not fit into the pores of the stationary phase. Therefore, these bigger compounds are the first ones to be eluted. Meanwhile, smaller particles that can fit into de smaller pores, reside longer in the stationary phase, being eluted later. Thus, in SEC, components of the mobile phase elute by decreasing order of their size (Coskun, 2016; Janson, 2011; Grob, 2004).

SEC is a separation process often chosen when a sample is already clarified, having the purpose of evaluating the presence of high-order impurities such as aggregates or degradation products (Shimoyama *et al.*, 2017; Sousa *et al.*, 2008). Regarding nucleic acids purification, SEC can be applied in a mixture containing genomic DNA (gDNA) or plasmid DNA (pDNA) that elute firstly in the run due to their high molecular weight, and other impurities like RNA, proteins and endotoxins, with lower molecular weights that elute later. It should be noted that this effective separation between DNA and RNA can only be accomplished by choosing an appropriate support. As a matter of fact, inherent disadvantages are associated with this type of chromatography, namely low resolution and the need for higher dilutions of the sample. Thus, SEC is mainly seen as a final step in the downstream processing of nucleic acids (Sousa *et al.*, 2009; Fani Sousa *et al.*, 2008).

In a recent study, a novel minicircle DNA (mcDNA) purification method was established by Almeida and co-workers. In here, it was possible to successfully isolate mcDNA from RNA, parental plasmid and miniplasmid by size exclusion chromatography with a Sephacryl S-1000 SF matrix, achieving 66.7% of mcDNA recovery with 98.1% of purity (Almeida *et al.*, 2019).

1.3.2. Hydrophobic interaction chromatography (HIC)

Hydrophobic interaction chromatography (HIC) is a well-established bioseparation technique at the laboratory and industrial scales. HIC explores hydrophobic interactions between immobilized hydrophobic ligands and non-polar regions of the biomolecules surface, having few solvent requirements. In this method, the target biomolecule retention occurs at relatively high salt concentrations (usually ammonium sulfate), due to a displacement of ordered water molecules around the solute leading to an increase of the entropy. Then, desorption of bound solutes is achieved by performing a stepwise or linear gradient elution with buffer containing low salt concentration, thus weakening the hydrophobic interaction occurring between the biomolecules and chromatographic ligands. It is one of the simplest chromatographic separation methods in terms of operation mode, and although the interaction mechanism is complex, a minimal structural damage occurs to the biomolecule, preserving its biological activity (Guerrero-Germán *et al.*, 2011; Janson, 2011; Freitas *et al.*,2009; Sousa *et al.*, 2009; Sousa *et al.*, 2008).

Nevertheless, the need for high concentrations of salt in the binding step constitutes a major disadvantage, regarding the industrial application of this method, because the use of salt is directly associated with higher costs and negative environmental impact (Sousa *et al.*, 2009).

In what concerns to nucleic acids purification, HIC has been applied more properly on preparative purification of pDNA. In this case, it takes place a negative chromatography, in which the aim is to bind impurities (RNA, gDNA, oligonucleotides, denatured pDNA) instead of binding the target biomolecule. Since the majority of impurities possess more hydrophobic character than the target molecule, they bind easily to the column in the presence of salt, while the double-stranded pDNA elutes in the first place. The single-stranded hydrophobic impurities can then be eluted by decreasing the ionic strength of the eluent (Sousa *et al.*, 2009; Diogo *et al.*, 2003).

Diogo and co-workers studied the capacity of the support Sepharose CL-6B treated with 1,4-butanediol diglycidyl ether to separate nucleic acids by exploring hydrophobic interactions. It was observed that, when a nucleic acids-containig mixture obtained from *E. coli* cells was loaded onto the column, it was possible to separate single stranded from double stranded nucleic acids. RNA and gDNA were more retained in the column, due to interactions occurring with the hydrophobic exposed bases of these biomolecules, while pDNA was eluted in the beginning since nitrogenous bases are less exposed (Diogo *et al.*, 2002). These results allow to infer that HIC supports can also be used for RNA purification, exploiting its ability to bind and taking advantage from the different interaction strengths between ligands and other retained species.

1.3.3. Ion-exchange chromatography (IEC)

In ion-exchange chromatography (IEC), the separation is based on the reversible electrostatic interaction between a charged solute and an oppositely charged chromatographic column. The strength of the interaction depends upon the overall net charge of the analyte, and the competition from other ions for the charged groups of the ion exchanger (Janson, 2011). This separation technique can be performed in two distinct ways. When the matrix is positively charged, the solutes that are negatively charged are going to be adsorbed onto the column, being this designated as anion-exchange chromatography. On the other hand, when the matrix is negatively charged, the solutes that are positively charged are going to be adsorbed, being this the cation-exchange chromatography (Coskun, 2016). When oligonucleotides are meant to be separated, it is anion-exchange chromatography that is used, and elution is achieved through competition of the binding site with an increasing gradient of anions in the mobile phase, and so nucleic acids elute in the order of an increasing overall net charge (McGinnis *et al.*, 2012; Sousa *et al.*, 2008). Nucleic acids, which are polyanionic molecules due to the presence of phosphate groups on their backbone structures, are conveniently captured on a support functionalized with positively charged groups. IEC offers many different advantages, such as a rapid separation and analysis, does not need the use of organic solvents, it is possible to perform sanitization with sodium hydroxide and there are many commercial stationary phases available in the market. However, there are some drawbacks, because in some cases RNA can co-elute with pDNA, and has a relatively low resolution between pDNA and endotoxins (Fani Sousa *et al.*, 2008; Eon-duval & Burke, 2004). Easton and co-workers have described a fast method for RNA oligonucleotides purification using anion-exchange liquid chromatography with suitable quality for structural and biochemical studies. By using a diethylaminoethyl sepharose support it was possible to separate the desired RNA product from other RNA species like T7 RNA polymerase, small abortive transcripts and the plasmid DNA template (Easton *et al.*, 2010).

1.3.4. Affinity chromatography (AC)

Affinity chromatography (AC) is the most powerful and versatile type of chromatography, even when the target biomolecule is a minor component of a complex mixture. The separation of sample components and the recognition of the target biomolecule is based in specific interactions, as the ones found for example between antigen-antibody, enzyme-substrate or nucleic acid-protein. So, these different types of interactions that naturally occur in many biological systems are extrapolated to AC, by immobilizing one of the members of the pair in the stationary phase (ligand), while the other member passes through the column in the mobile phase, being adsorbed in the solid phase (Janson, 2011). Nevertheless, the biological nature of the ligands can represent a limitation to this purification technique, since these ligands tend to be fragile and present low binding capacities. Hence, there is a growing interest on the design of synthetic ligands that would mimic the biologic ones, presenting the same selectivity, as well as high capacity, durability, robustness, and reproducibility (Pereira *et al.*, 2016 (b); Sousa *et al.*, 2008).

The interactions occurring in AC can be of many different natures, like electrostatic and/or hydrophobic interactions, hydrogen bonds, van der Waals forces, dipole-dipole forces, and π-π interactions. The selectivity results from the conjugation of multiple interactions. However, some interactions can be preferentially established depending on the chromatographic conditions used, namely in the mobile phase, which confers high selectivity and resolution to this technique. Hence, the elution step can be performed in two distinct ways: specifically (biospecific elution), where a competitive agent is applied in the mobile phase that can either bind to the retained target or to the immobilized ligand, depending on their characteristics, or non-specifically, where chemical changes are made in the mobile phase by altering ionic strength, type of salt, pH or polarity depending on the matrix and the chemical features of the biomolecule. It should be noted that the binding strength between ligand and target molecule should not be too weak, or there will be no adsorption, nor be too strong, in order to be possible to perform elution in moderate conditions. Thus, it is crucial that the complex ligand-biomolecule forms a reversible bonding in order to preserve the good quality and biological activity of the target biomolecule (Pereira *et al.*, 2016 (b); Sousa *et al.*, 2009; Janson, 2011).
Due to the high nucleotide bases exposure, RNA becomes very accessible for the establishment of many interactions with the ligand chosen for the separation. Therefore, different types of AC have already been tested and proven to succeed in the purification of ncRNAs, taking advantage of properties such as its size, charge and hydrophobicity, namely boronate AC, RNA affinity tags and amino acid-based AC, being their respective advantages and disadvantages discriminated in Table 3 (Wei *et al.*, 2014; Pereira *et al.*, 2014; Flores-jasso *et al.*, 2013; Srivastava *et al.*, 2012). It should be noted that our research group developed a successful purification method of pre-miR29 from other small RNA molecules through the use of an Arginine-Sepharose 4B gel, with high purity levels and structural integrity (Pereira *et al.*, 2014).

Table 3: Affinity Chromatography in the bioseparation of ncRNAs (Adapted from Pereira *et al.*, 2016).

Methods	Target RNA	Advantages	Disadvantages
Boronate Affinity Chromatography	• tRNAs	✓ Isolation from complex cell extracts ✓ No pre-treatment of the sample is needed ✓ High purity and quality ✓ Efficient elimination of gDNA ✓ Very fast process	x Sometimes, the column must be washed with 6M urea prior to washing with the regeneration buffer
RNA Affinity Tags	• shRNA • miRNA • miRNA-mRNA complex RNA oligonucleotides	✓ Reliable, fast and robust method ✓ Broadly applicable to any RNA of interest ✓ High recovery and purity	x Chemical modifications during the process can lead to structural perturbations x Contamination of the target RNA with tag can occur x Use of proteases requires additional purification steps
Amino Acid-based Affinity Chromatography	• pre-miRNA • rRNAs • tRNAs • total RNA	✓ Isolation from complex cell extracts ✓ Robust method using natural small ligands ✓ High purity and quality of purified RNA ✓ High reproducibility ✓ Efficient elimination of endotoxin, proteins, gDNA and single/double stranded RNAs ✓ Occurs under non-denaturing conditions	x High salt concentrations can be required for the elution of the target RNAs x Low recovery yields x Lower durability of supports

1.4. Ionic Liquids: Brief History

In the last few years, ionic liquids (ILs) have attracted the interest of many researchers, due to their versatility and many different applications in several areas. They can be used on almost all kinds of chemical transformations, and they present some exceptional properties, which will be discussed below (Kuchenbuch & Giernoth, 2015).

The first IL was discovered by Paul Walden in 1914 when he was searching for molten salts that were liquid at temperatures at which he could use his equipment without special adaptations. Walden's interest in these molten salts was the relation of their molecular size and their conductivity, however, and unfortunately, the potential of this breakthrough went unnoticed for a long time. Almost 40 years later, in 1951, another group recognized the potential benefits of lower melting points of the molten salts (Welton, 2018). Nowadays, ILs are extensively employed in various fields, like, organic, inorganic, physical, and biological chemistry (Shukla *et al.*, 2018).

1.4.1. General Properties

ILs are pure salts that, in contrast with common electrolytes, are liquid at room temperature, because their melting point is below 100 ºC. They are constituted by a large and unsymmetrical organic cation and an organic or inorganic anion. This fact contributes to their enormous versatility since there are numerous possible cation and anion combinations, with the possibility of designing task-specific ILs, and because of that, they are designated as "Designer Solvents" (Ventura *et al.*, 2017; Freire *et al.*, 2012). Due to their ionic character, ILs present some outstanding features, including low volatility, non-flammability, variable viscosity, high ionic conductivity, wide electrochemical potential window, high solvation ability, as well as excellent chemical, thermal and electrochemical stability (Sintra *et al.*, 2017; Ventura *et al.*, 2017; Freire *et al.*, 2012). The first two characteristics contribute to the classification of ILs as "Green Solvents", and as a result, these fluids have been viewed as good alternatives to replace dangerous and volatile organic solvents (VOSs) recurrently used in a wide range of processes. Theoretically, this replacement would eliminate these solvents' losses to the atmosphere, and consequently reduce the harmful effects to the environment and human resources, making it possible to develop "greener" processes (Taha *et al.*, 2017; Freire *et al.*, 2012). ILs are also usually recognized by their excellent solvation capacity for a wide range of compounds, as well as a good stabilizing media for proteins, nucleic acids, and others (Ventura *et al.*, 2017). In Figure 4 are represented the structures of commonly used IL cations and anions and in Table 4 their general advantages and some disadvantages.

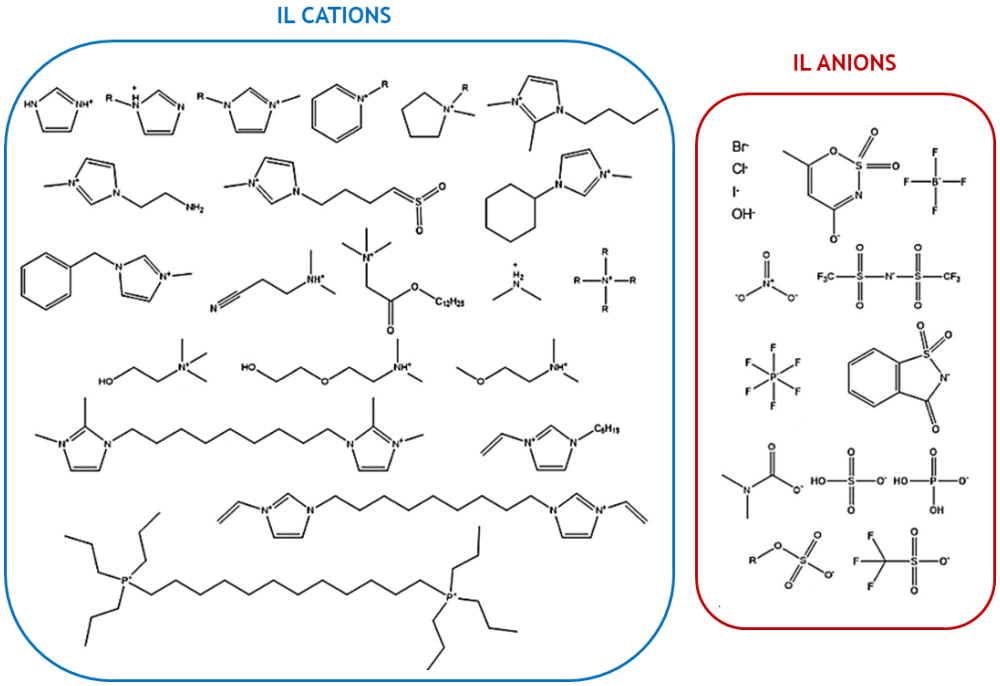


Figure 4: Chemical structure of some IL cations and anions described in literature (Adapted from Soares *et al.*, 2016).

Table 4: General advantages and disadvantages of ILs (Adapted from Kunz & Häckl, 2016).

Advantages		Disadvantages	
Number of imaginable solvents	High number, up until 10^{18} possible combinations	**Cost**	Commonly 5-20 times more expensive than molecular solvents
Tunability	Tunable by varying functional groups or alkyl chain length ("Designer Solvents")	**Viscosity**	Often very high viscosity of pure ILs
Vapour pressure	Negligible vapour pressure at normal conditions, with almost no emission to the atmosphere	**Vapour pressure**	Low vapour pressure limits distillative solvent separation
Flammability	Usually non-flammable	**Synthesis**	In many cases costly multi-step synthesis
Detachability	Vaporable compounds can easily be separated	**Sustainability**	Green image, but often toxic, non-biodegradable and non-sustainable in their way of synthesis
Stability	Thermo and electrochemically stable in a wide potential range	**Corrosiveness**	Often significant to very high corrosiveness

However, the fact that ILs have a negligible vapor pressure is not enough to assure that these fluids can be considered as environmentally harmless solvents, making it imperative to determine their environmental, health and safety impact (Sintra *et al.*, 2017; Petkovic *et al.*, 2011). The ionic character of ILs makes most of them soluble in water, even the most hydrophobic ILs, which can lead to an environmental problem if they happen to be toxic to the

aquatic environment and organisms living in it. The main challenges in using ILs for manipulation of biomolecules are the significant uncertainty of their toxicity, both eco and cytotoxicity, biodegradability and the eventual effect of the ILs on biomolecules (Sivapragasam *et al.*, 2016). Due to that, researchers are more concerned about their potential impact on the aquatic and terrestrial environments, and since ILs are constituted by an anion and a cation, those two parts must be considered in this issues (Amde *et al.*, 2015; Kudłak, 2015; Jordan & Gathergood, 2015). From the data hitherto reported, several studies have been done, in various cell types, like bacteria, fungi, algae, mammalian cell lines, vertebrates, and others (Sintra *et al.*, 2017). It was found that the toxic effects of ILs vary considerably according to their type, test conditions and morphology of the model organisms (Amde *et al.*, 2015). For example, it is well established that cation toxicity highly depends on the alkyl side chains length, due to increasing hydrophobicity and consequently, lipophilicity. This is, ILs with long alkyl chains in the cation, are significantly more toxic than ILs with shorter chain versions (Mikkola *et al.*, 2015). In addition to the strong side-chain effect, the chemical structure of the cationic group also has a significant effect on toxicity, this is, imidazolium, pyridinium and quinolinium groups display stronger toxicological effects even when they have shorter side chains linked (Sivapragasam *et al.*, 2016). Regarding the effect of the anion part, studies have shown that this counterpart presents less toxicity effect than changing the alkyl chain substituent in the cation. However anions impact in toxicity should not be underestimated (Kudłak, 2015; Pham *et al.*, 2010). Hence, it is of great importance to perform a complete ecotoxicological characterization before assuming ILs as environmentally compatible, as well as cytotoxic assays (Santos *et al.*, 2015).

There is also an agreement on the fact that functionalized cations tend to form less toxic ILs when compared with non-functionalized counterparts since they are made more hydrophilic, and that the cation is the main driver of toxicity (Santos *et al.*, 2015). So, it was observed that incorporation of functional polar groups like ether, hydroxyl and nitrile groups to the side chains reduces toxicity when compared to those with “simple” alkyl side chains, and additionally increases the biodegradation efficiency to some extent (Pham *et al.*, 2010). Considering their “designer solvent” character becomes possible to tailor ILs by combinaing suitable funtional groups on their structure, which will lead to a more environmental friendly compound, thus ensuring its safe application in biotechnological processes.

1.4.2. Applications of Ionic Liquids

It should be noted that ILs are not only limited to the role of solvents, on the contrary, due to their enormous versatility and beneficial features, in the last two decades, they could be extended to many different fields of industry like chemistry, electrochemistry, nanotechnology, medicine, material production, power engineering, biotechnology, among others (Kudłak, 2015). In the next topics we will focus mainly in the application of ILs in stabilization, extraction, and purification of different types of biomolecules.

1.4.2.1. Biomolecules Stabilization

ILs have recently been studied as versatile solvents and additives in biotechnology field, particularly as stabilizers of proteins, enzymes and nucleic acids. In this industry it is of extreme importance the formulation of stable biopharmaceuticals, therapeutic proteins and vaccines, which have revolutionized the treatment of various diseases, as mentioned above. Thus, becomes very important to assure the preservation of these therapeutic biomolecules in long-term storage, maintaining their integrity and biological function (Clark *et al.*, 2018). Proteins storage in water limits their application in many fields since their thermal stability decreases. Most of the preservation methods used to overcome this problem, like chemical modification, immobilization and stabilizers addition do not prevent their irreversible thermal denaturation. By this, numerous studies have shown that some ILs are efficient in maintaining or even increasing proteins solubility and stability. Once proteins solubilization in ILs is favoured, it is less likely to occur protein aggregation, consequently improving their stability and activity (Sivapragasam *et al.*, 2016). Taha and co-workers have discovered that stability of bovine serum albumin (BSA) in aqueous solutions of cholinium-based Good's buffers ILs (GB-ILs) was increased when compared to other aqueous solutions (Taha *et al.*, 2015).

Regarding DNA, it also has been proven many advantages of using ILs in their storage, including enhanced solubility and excellent stability. Nucleic acids are highly susceptible to nuclease cleavage, mainly RNA since it is less stable, however most stabilization studies with ILs have been more focused on DNA (Pedro *et al.*, 2018; Clark *et al.*, 2018). For example, in a study, 25% (w/w) of DNA was solubilized in 2-hydroxyethylammonium formate, which is an ammonium-based IL, and a long-term chemical and structural stability was verified upon storage under ambient conditions for at least one year. This was indicative of a great nucleic acid preservation medium (Singh *et al.*, 2017). Another study performed by Tateishi-Karimata and co-workers, proved that solutions with high concentrations of choline dihydrogen phosphate induced an increased stability of G-quadruplex and i-motif DNA (Karimata *et al.*, 2015). A more recent study with RNA, performed by our research group, has proven that some GB-ILs like [Ch][HEPES] and [Ch][MES] would also increase this biomolecule thermal stability, preserving its structural characteristics. Besides this, these specific ILs also shown no cytotoxicity to human cell lines, which is extremely relevant when envisaging the use of RNA as biopharmaceutical (Pedro *et al.*, 2018).

1.4.2.2. Extraction of Biomolecules by Aqueous Biphasic Systems

The ability of ILs to create biphasic splitting makes them promising candidates for Aqueous Biphasic Systems (ABS). ABS are a softer and greener alternative to traditional liquid-liquid extraction techniques, which generally make use of hazardous VOSs and aim for the efficient separation of biomolecules.

ABS consist of two immiscible aqueous rich phases based on combinations of polymer-polymer (for example polyethylene glycol and dextran), polymer-salt, or salt-salt. Both solutes are water soluble, however, above a given concentration, they separate into two coexisting phases:

one of the aqueous phases is rich in one solute, whereas the other phase is enriched with the other polymer/salt. These systems have been recognized as an economical method, exhibiting low-energy consumption, high performance, high biocompatibility and suitability for scale-up. Furthermore, IL-based ABS have many advantages when compared to conventional liquid-liquid extractions, such as their low viscosity, which leads to quick phase separation, and the ability to tune their polarities and affinities (Shukla *et al.*, 2018; Taha *et al.*, 2017; Freire *et al.*, 2012). This last advantage is one of the most interesting, which can be accomplished by proper manipulation of the cation/anion design and their combinations. Moreover, this aspect is usually viewed as a tremendous limitation to polymer-based ABS, given that the polarity range is limited, making the application of ILs even more advantageous (Freire *et al.*, 2012). ABS are used for the extraction of various products like proteins, enzymes, antibodies, and nucleic acids, some of which are described in Table 5.

Table 5: Extraction of various interest compounds using IL-based ABS.

Product type	Example	Separation process	Reference
Protein	BSA	Extraction of BSA with ABS formed with polypropylene glycol and GB-ILs (almost 100% extraction efficiency)	Taha *et al.*, 2015
Enzyme	Extremozymes	Enzyme extraction with ABS formed by ammonium sulphate salt and IL-rich phase (96% extraction efficiency)	Gutiérrez-Arnillas *et al.*, 2015
Antibodies	IgG	Extraction of IgG antibody with polypropylene glycol and biobased ILs (100% extraction recovery)	Mondal *et al.*, 2016
Nucleic acids	RNA	Extraction of RNA with ABS formed by amino-acid-based ILs (AA-ILs) and polypropylene glycol	Quental *et al.*, 2019

Despite their advantages, these systems are not capable of good selectivity, comparatively to chromatography, that can achieve great selectivity and better purity levels, which are required for biopharmaceuticals. Thus, extraction techniques are mainly used as a capture, clarification or pre-purification procedure, that can be combined with a chromatographic step to complete the purification process.

1.4.2.3. ILs in chromatography

The purity level of the target compounds is a crucial parameter to be considered when attempting the development of novel purification and separation processes (Ventura *et al.*, 2017). The usage of bulk ILs in the liquid state has some drawbacks, such as high cost derived from large quantities required, low diffusion coefficients and sometimes inherent difficulties in product purification and solvent recycling. On the other hand, the concept of IL immobilization onto solid supports can overcome some of these disadvantages, since fewer IL quantities are needed, their unique physicochemical properties can be transferred to substrates (mainly their "designer solvent" capacity) and can be achieved higher recovery yield and purity levels (Xin & Hao, 2014). During the last two decades, ILs have been used in liquid chromatography in three distinct ways: as additives of the mobile phase, as stationary phase itself or as ligands of the stationary phase (Wang *et al.*, 2006; Zhang *et al.*, 2004).

1.4.2.3.1. ILs as mobile phase additives

Several studies demonstrated that the use of ILs in separation techniques, namely in high-performance liquid chromatography (HPLC) as additives in mobile phases, can contribute to a decrease in the amount of organic solvents used, as well as to a decrease in the energy consumption by increasing the speed of analysis without compromising the analytical performance or even improving it (Soares *et al.*, 2016). Since ILs work as low concentration additives they were found to be more useful than organic modifiers in HPLC. So, the primary aim of adding ILs to the mobile phases is to shield the acidic silica surface (common support for HPLC) in order to obtain better peak shapes and reduce peaks broadening. The retention mechanism of ILs as additives is complex, once both the cation and anion can be adsorbed on the stationary phase, creating a bilayer. This justifies the alteration in the retention behaviour and peak shape (García-alvarez-coque *et al.*, 2015; Sun & Armstrong, 2010). However, implementation of ILs in mobile phases requires also an extensive knowledge of their potential hazardous environmental impact, and only being much less harmful than organic solvents would justify their replacement.

1.4.2.3.2. ILs as stationary phase

ILs-based stationary phases can be synthesised using ILs that display mechanical and physical properties of a polymer. Poly(ionic liquids) (PILs) consist in many IL species connected through a polymeric backbone that forms a macromolecular framework, which are being explored as novel separation phases in gas separation membranes, gas chromatography and solid-phase microextraction. In this last one, it is possible to extract specific components from complex systems, such as gas, metal ions and even pharmaceuticals inorganic pollutants. In a very recent study, PILs have been used as separation matrices for purifying M13 phages, that are a possible antimicrobial agent for treatment of bacterial infections. This method enabled the usage of PILs as anion exchanger as a fast and simple method for recovering phage M13 with over 70% recovery. However, it should be noted, that PILs have some limitations due to structural

instability under certain circumstances and there is still a need of understanding their physical properties (Jacinto *et al.*, 2018; Tomé & Marrucho, 2016; Yuan *et al.*, 2013).

1.4.2.3.3. ILs as ligands of the stationary phase

Usually, ILs are immobilized on the surface of solid supports by covalent bonding of their cations or anions (Shi *et al.*, 2015). By this, there are five distinct ways to perform the IL immobilization onto the supports, as shown in Figure 5.

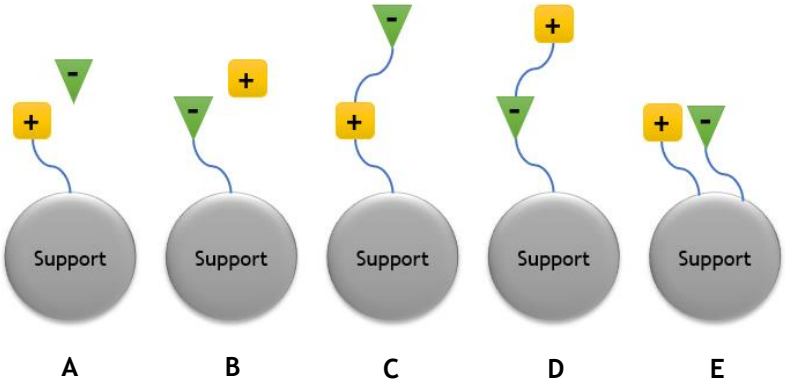


Figure 5: Illustration of distinct ways of IL immobilization onto a support. In A and B only the cation or anion is immobilized on the supports, while the counterions are free. In C and D are immobilized zwitterionic ILs, where the cation and anion are linked through a covalent bond. In E the cation and the anion of the IL are co-immobilized on the support (Adapted from Shi *et al.*, 2015).

In Figure 5, A, IL cation is covalently attached to the solid support while the anion acts as a free counterion. This case is relatively easy to prepare and has the advantage that free anions can be easily replaced, making regenerations processes simpler, or even exchanged, enabling slightly modifications by the conversion of various anions (Shi *et al.*, 2015). Spherical porous silica is often used as a stationary phase matrix for HPLC, in which an imidazolium ring (most commonly used IL for covalent modification) is immobilized with a spacer arm. This type of stationary phase has already proven to be efficient in the separation of alkaloids, inorganic anions and cations (Qiu *et al.*, 2009), xylose and glucose (Bi *et al.*, 2010). Besides that, this solid phase shows capacity of exploring different types of interactions, demonstrating hydrophobic and ionic properties, and hence, multi-mode separation. Usually, the cation is anchored on the silica by a small spacer arm, and different lengths of these spacers may influence selectivity of the support by alteration of hydrophilicity (Shi *et al.*, 2015; Zhang *et al.*, 2014; Qiu *et al.*, 2012). Moreover, in a study performed by our research group, where a macroporous support was functionalized with an IL (1-methylimmidazole), it was also proven the establishment of different types of interactions between the ILs and biomolecules like gDNA and RNA, enabling the effective separation of these two species. Several aspects point to the potential of ILs as truly multimodal ligands, considering their ability to interact with analytes through different mechanisms, including hydrophobic, electrostatic, hydrogen bonding, π-π, and dipole-dipole interactions, owning to their unique structure that comprises both hydrophobic and ionic moieties.

On the other hand, anions are rarely immobilized on the supports and the cations are free (Figure 5, B), since in a particular study by Qiu and co-workers, demonstrated that in this case of stationary phases the counterions where easily exchanged by the ionic species present on the mobile phases during usage (Qiu *et al.*, 2013). In Figure 5, C and D are the examples of zwitterionic ILs which can avoid the previous issues, once the cation and anion are covalently bond to each other, varying between which one is immobilized onto the solid support. Qiao and co-workers developed a zwitterionic stationary phase with a positively charged imidazole ring and a negatively charged sulfonate group, which exhibited good selectivity and favourable retention for a wide range of polar solutes, including nucleosides and nucleic acid based (Qiao *et al.,* 2013). Lastly, in Figure 5, E, ILs are co-immobilized on the solid supports which can improve the stability of these ligands during the use of different types of buffers in mobile phase, influencing selectivity by the distribution of polar groups (Qiu *et al.*, 2014; Zhang *et al.*, 2014).

1.4.2.3.4. ILs to modify other chromatographic supports

New ligands screening is clearly recommended to be performed with cheaper supports, like silica support. However, this type of support is more suitable for analytical chromatography (HPLC) rather than preparative chromatography. In this sense, it is very important to transpose these ILs-based ligands to other types of chromatographic supports that can display better separation and purification performance and binding capacity. Examples of novel and promising supports in this area are sepharose, bigger spherical silica particles, macroporous matrices and even monoliths. These types of supports can stand higher pressure, flow rates, better robustness and packing characteristics. Together with the advantages of ILs, like the numerous combinations between cation and anion, these supports functionalized with ILs can demonstrate great potential in what concerns to good selectivity for the analyte, as well as a good chromatographic performance and capacity (Liu *et al.*, 2015; Shi *et al.*, 2015).

CHAPTER 2 – Global Aims

Purification of biomolecules through chromatographic processes is a field highly established but also in continuous need of evolution, particularly when considering challenging molecules as biopharmaceuticals. Actually, with this technique it is possible to obtain these valuable therapeutic molecules, highly pure, active, and presenting suitable quality to be used for the treatment of numerous diseases. Nucleic acids have been discovered through the years that can regulate cell function and pathological pathways, therefore working as a great tool for treatment of many diseases caused by gene defects, like cancer, Parkinson's Disease, Alzheimer's Disease, among others.

Ionic liquids (ILs) have aroused interest in this area as they can be involved in the development of new alternative purification strategies which turn biopharmaceuticals obtention more viable. ILs display unique physicochemical features that make them extremely versatile for application such in recovery or purification processes. Beyond numerous cation/anion combinations that can be explored, they also present less negative environmental impact than other organic solvents usually used in biotechnological processes.

In this sense, the main aim of this work is to test the capacity of ILs immobilized onto a silica support, to bind and reversibly elute nucleic acids, and consequently display some selectivity between different nucleic acids species. Through ILs structure it is expected that they can both display electrostatic and hydrophobic interactions, since the immobilized cation has a charged and a hydrophobic moiety. By this, these will be the two conditions explored for the screening chromatographic assays. Additional stabilization and cytotoxicity assays will be performed with ILs analogues to the ones used in the immobilization process, in order to explore their safety.

In a general way, this work aims for the development of a new and eco-friendly purification process that allows the preparation of purified and intact nucleic acid fractions in order to be suitable for use as biopharmaceuticals.

CHAPTER 3 – Materials and Methods

3.1. Materials

For the activation of silica, it was used Silica gel spherical with a particle size of 75-200 µm from Supelco and hydrochloric acid (purity 37%) from Sigma-Aldrich (St. Louis, Missouri, EUA). For the functionalization of the SIL materials, the reagents used were toluene (purity 99.98%), ethanol (purity 99.99%) both from Fisher Scientific, methanol (purity < 98%) and triethylamine (HPLC grade) from Fisher Chemical (Waltham, EUA), (3-chloropropyl)trimethoxysilane (purity 98%), 1-methylimidazole (purity 99%), tributylamine (purity 99%) all provided by Acros Organics (Geel, Belgium), trioctylamine (purity < 98%) from Fluka (United Kingdom) and N,N-dimethylbutylamine (purity 99%) from Aldrich (St. Louis, Missouri, EUA). For the cytotoxicity assays the ILs used were tetramethylammonium chloride (purity 97%), tetrabutylammonium chloride (purity 97%) from Sigma-Aldrich (St. Louis, Missouri, EUA) and tetraethylammonium chloride (purity 98%) and tetrapropylammonium chloride (purity 98%) from Thermo Fisher Scientific Inc. (Waltham, USA).For the culture of bacterial cells, the reagents used were tryptone and yeast extract both from Bioakar (Beauvais, France), glycerol from Himedia, dipotassium hydrogenphosphate (K_2HPO_4) from Panreac (Barcelona, Spain), potassium dihydrogen phosphate (KH_2PO_4) from Sigma-Aldrich (St. Louis, Missouri, USA), "Luria-Broth Agar" from Pronalab (Mérida, Yucatán, Mexico) and kanamycin from Thermo Fisher Scientific Inc. (Waltham, USA). For the extraction of nucleic acids, the reagents used were guanidine thiocyanate, N-Lauroylsarcosine sodium salt, sodium citrate and isoamyl alcohol all from Sigma-Aldrich (St. Louis, Missouri, USA), isopropanol from Thermo Fisher Scientific Inc. (Waltham, USA), and ß-mercaptoethanol from Merck (Whitehouse Station, USA). In the chromatographic assays it was used sodium chloride (NaCl) and ammonium sulphate ($(NH_4)_2SO_4$) both commercialized by Panreac (Barcelona, Spain) and tris(hydroxymethyl)aminomethane (Tris) from Merck (Darmstadt, Germany). All solutions were prepared with Milli-Q water treated with 0.01% of diethyl pyrocarbonate (DEPC) from Sigma-Aldrich (St. Louis, Missouri, USA). The buffers were then filtered through membranes with a pore size of 0.20 µm and sonicated for about 15 minutes before each utilization. The molecular weight marker and Green-Safe were purchased from Grisp (Porto, Portugal). For the cytotoxicity assay it was used the Cell Proliferation Kit I (MTT) from Merck (Darmstadt, Germany). For cell culture of human dermal fibroblasts (hFIB) the following reagents were used: "Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12" (DMEM-F12) from Sigma-Aldrich (St. Louis, Missouri, USA), fetal bovine serum (FBS) from Gibco, Life Technologies (EUA), and penicillin-streptomycin from Grisp (Porto, Portugal).

3.2. Methods

3.2.1. Ligand immobilization in silica

For the functionalization of the silica support, three steps are required. The first step consisted in the activation of silica gel (5g) with hydrochloric acid (HCl) 37% for 24 hours. After this, the activated silica was washed with large volumes of water until pH reached approximately 7, and then dried at 100 ºC in an incubator. In the second step, 5 g of activated silica are added to 60 mL of toluene (solvent of the reaction) and 5 mL of (3-chloropropyl) trimethoxysilane, which is the binding agent to the -OH groups in the activated silica. The suspension is refluxed at 95 ˚C, and 500 rpm for 24 h. After this, the material is filtered and washed with 100 mL of toluene, 200 mL of ethanol/H_2O (1:1), 500 mL of H_2O and 100 mL of methanol and dried at 60 ºC. Then in the third, 5 g of SilPrCl are added to 60 mL of toluene and 5 mL of the compound that will originate the cation of interest, being the reaction carried out at 110 ºC, and 500 rpm for 24 h. The resultant material is filtrated and washed with 100 mL of toluene, 350 mL of methanol, 300 mL of H_2O and 150 mL of methanol, and then dried in the incubator at 60 ˚C. This process is summarized in Figure 6, where the synthesis of the six different supported ionic liquid materials are described.

3.2.2. Support characterization

All the synthesized materials were analysed by elemental analysis, solid state ^{13}C nuclear magnetic resonance (NMR) and scanning electron microscopy (SEM) in order to verify the functionalization with the ligands of interest. By elemental analysis, using the equipment Truspec 630-200-200, carbon, and hydrogen percentages were obtained, after the sample burned at 1348 K, by absorption at infra-red radiation and nitrogen quantification by thermal conductivity. For the measurements, approximately 2 mg of material was used. For solid state ^{13}C NMR, about 100 mg of each material were analysed with the equipment Bruker Avance III 400 MHz (DSX model) using 4 mm BL cross-polarization magic angle spinning (CPMAS) VTN probes at 100.6 MHz, at room temperature. In order to verify the effect on the morphology, of ILs binding to the supports, some SEM images were collected with the equipment Hitachi S4100 at 25 kV, with magnifications of 100x, 250x and 1000x. For this, and to obtain electrical conductivity, the samples were placed in an appropriate support and coated with a carbon thin film

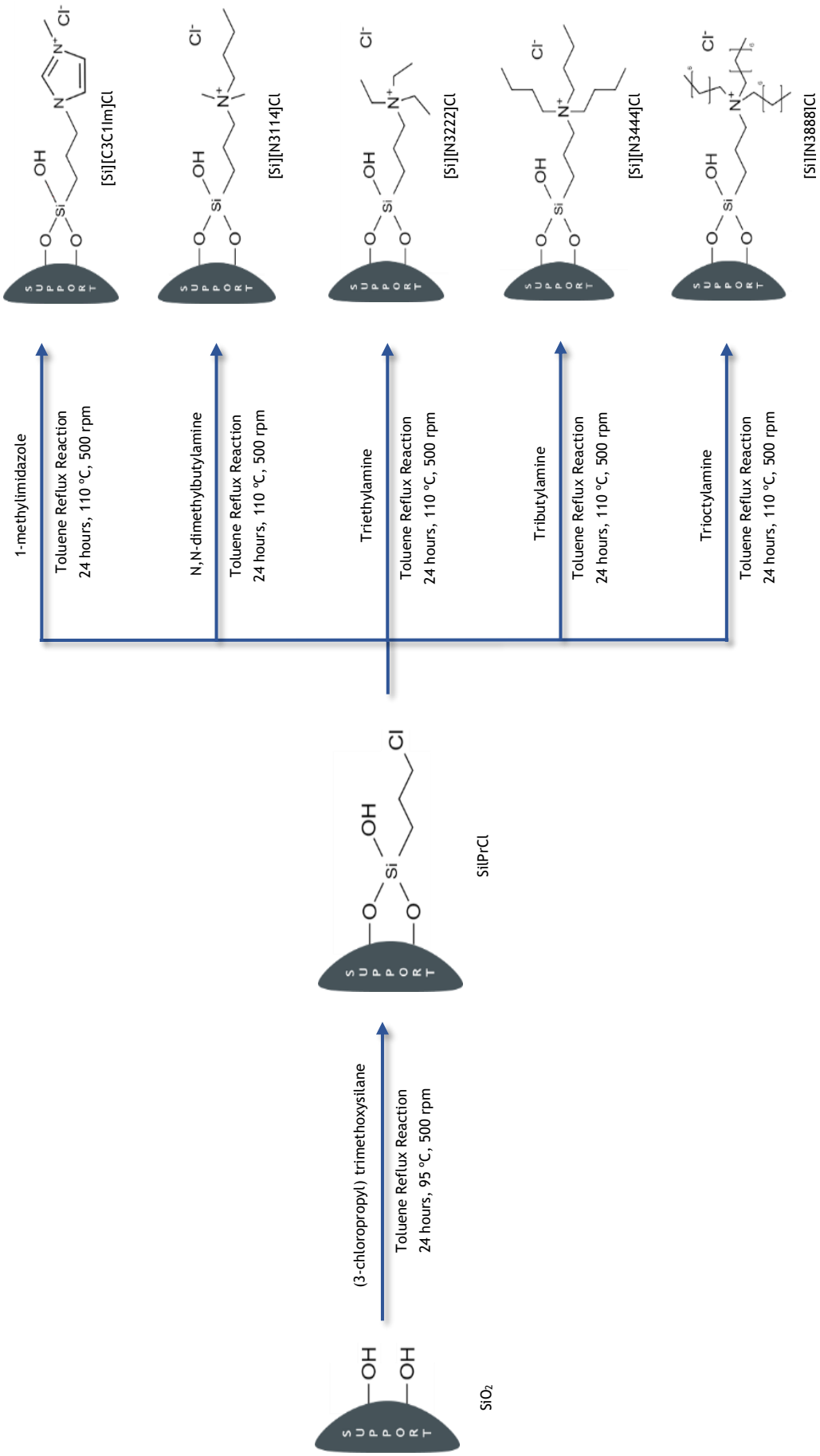


Figure 6: Summarized scheme of the reactions performed for the functionalization of the silica supports with ionic liquids.

3.2.3. Nucleic acids production in *Escherichia coli* DH5α

Production of RNA was performed in a strain of *Escherichia coli* DH5α (*E. coli*) previously transformed with the plasmid pBHSR1-RM containing the sequence of human pre-miRNA29b. Firstly, *E. coli* was cultured in a plate with solid medium "Luria-Broth Agar" (LB-Agar) supplemented with 50 μg/mL of kanamycin, overnight at 37 ˚C. The pre-fermentation and fermentation were performed in "Terrific Broth" medium (TB) with the following composition: 12 g/L of tryptone, 24 g/L of yeast extract, 5.5×10^{-5} M of glycerol, 0.017 M of KH_2PO_4 and 0.072 M of K_2HPO_4. Pre-fermentation medium was then inoculated with *E. coli* from the plaque and incubated at 37 ˚C in an orbital shaker at 250 rpm. Optical Density (OD) was frequently measured at 600 nm, until it reached 2.6. The volume of pre-fermentation needed for de fermentation to start with an OD of 0.2 was calculated by the following equation:

$$\mathbf{V_{to\ take\ from\ pre-fermentation}} = \frac{\left(\mathbf{V_{pre-fermentation}} + \mathbf{V_{fermentation}}\right) \times \mathbf{OD_{fermentation}}}{\mathbf{OD_{pre-fermentation}}}$$

During the process the OD was measured in the spectrophotometer Pharmacia Biotech Ultraspec 3000 UV/Visible (Cambridge, England). Fermentation was kept for 8 hours in order to obtain low molecular weight RNA. For the lysates the fermentations were kept for about 16 hours. The medium was then centrifuged at 3900 g and 4 ˚C for 10 minutes, being the resultant pellets stored at -20 ˚C. This process is summarized in Figure 7.

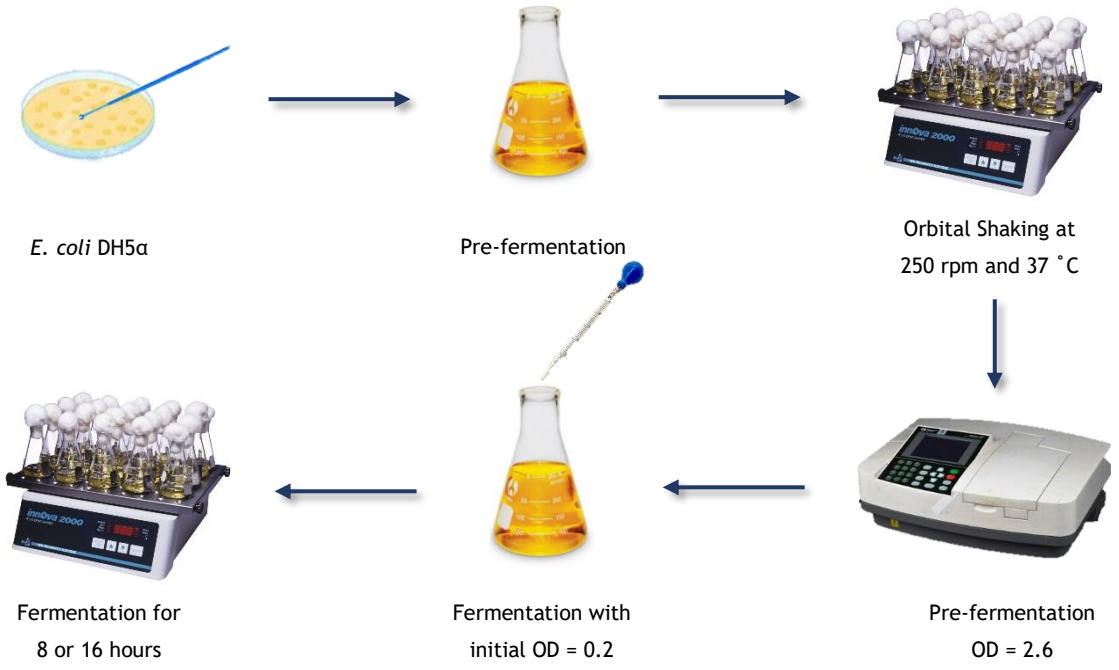


Figure 7: Summarized scheme of the *E. coli* fermentation process.

3.2.4. Low molecular weight RNA extraction

RNA extraction was performed by the method of acid guanidinium thiocyanate-phenol-chloroform. Firstly, the pellets of *E. coli* stored at -20 ˚C were thawed and resuspended in 0.8 % NaCl, followed by centrifugation at 6000 g for 10 minutes at 4 ˚C. Supernatant was discarded and the resultant pellets were resuspended with 5 mL of D Solution (4 M guanidinium thiocyanate, 0.025 M sodium citrate pH 7, 0.5 % sodium N-lauroylsarcosinate and 0.1 M ß-mercaptoethanol) and incubated in ice for 10 minutes. After this, 0.5 mL of 2 M sodium acetate pH 4 and 5 mL of phenol were added to the suspensions, being carefully homogenized at each step. Then, 1 mL of a mix of chloroform/isoamyl alcohol (49:1) is added followed by vigorous shaking and incubation in ice for 15 minutes. The suspensions were centrifuged at 10000 g for 20 minutes at 4 ˚C. Two aqueous phases are formed, being the upper phase enriched in RNA while the bottom phase is enriched in DNA, so the upper phase must be very carefully transferred to new lysis tubes, avoiding DNA contamination. To these new tubes, were added 5 mL of isopropanol in order to precipitate the RNA and centrifuged at 10000 g for 20 minutes at 4 ˚C. After discarding the supernatant, RNA pellets were dissolved in 1.5 mL of D Solution, and then 1.5 mL of isopropanol, followed by centrifugation at 10000 g for 10 minutes at 4 ˚C. Supernatant was discarded, and resultant pellets were resuspended in 2.5 mL of 75% ethanol in DEPC water, incubating the samples at room temperature for 10-15 minutes, followed by a centrifugation at 10000 g for 5 minutes at 4 ˚C. Pellet was then dried for 5-10 minutes at room temperature. Finally, RNA pellets were dissolved in 1 mL of DEPC treated water and incubated at room temperature for 10-15 minutes. The concentration of RNA was measured in the Nano Photometer (IMPLEN, United Kingdom) and integrity of the samples was verified by agarose gel electrophoresis, being the samples stored at -80 ˚C.

3.2.5. Nucleic acids extraction

For the extraction of nucleic acids from *E. coli* cells, the protocol was initiated with chemical lysis. Pellets were resuspended in 5 mL of D Solution (4 M guanidinium thiocyanate, 0.025 M sodium citrate pH 7, 0.5 % sodium N-lauroylsarcosinate and 0.1 M ß-mercaptoethanol), allowing the disintegration of the membrane cells. Then, the lysis tubes were incubated in ice for 10 minutes, following a centrifugation at 16000 g for 30 minutes at 4 ˚C. To the supernatant, 5 mL of isopropanol were added to each tube for the precipitation of nucleic acids. The tubes were incubated in ice for 30 minutes and then centrifuged at 16000 g for 20 minutes at 4 ˚C. After discarding the supernatant, 2.5 mL of 75 % ethanol-DEPC were added to each tube followed by room temperature incubation for 10 minutes. A centrifugation at 16000 g for 5 minutes at 4 ˚C was performed and the supernatant was discarded, and pellets were air-dried for 10 minutes. Pellets were suspended in 2 mL of DEPC treated water and incubated in a 60 ˚C water bath for 10 minutes, and finally centrifuged at 16000 g for 30 minutes at 4 ˚C, being the supernatant recovered. The concentration of nucleic acids was measured in the Nano Photometer (IMPLEN, United Kingdom) and integrity of the samples was verified by agarose gel electrophoresis, being the samples stored at -80 ˚C.

3.2.6. Agarose gel electrophoresis

Analysis of the extracted nucleic acids and peaks recovered from chromatographic assays was performed with horizontal electrophoresis in 1 % agarose gel. For the visualization of nucleic acids, the gel was prepared with 0.012 µL/mL of Green Safe (Porto, Portugal). Electrophoresis was performed at 120 V for 30 minutes in TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0). The gels were revealed using ultraviolet (UV) light exposure in the Uvitec Cambridge Fire-Reader equipped with a UV chamber (UVITEC Cambridge, Cambridge, United Kingdom).

3.2.7. Chromatographic assays

3.2.7.1. Screening of RNA binding/elution with different supports

For the screening of RNA behaviour in terms of binding and elution to the different supports, each one of the synthesised materials was packed in an empty column. For this, each support was dispersed in Milli-Q water and then added to de column until it reached 2 cm of height, being extremely important to perform this with constant running water. All the supports were washed with large volumes of water and then closed with a filter, keeping them always hydrated.

Chromatographic conditions that mainly favour electrostatic interactions:

Equilibrium stage was performed by applying 10 mL of 10 mM Tris-HCl pH 8 to the packed column allowing it to empty by gravity flow, followed by injection of approximately 30 µg of low molecular weight RNA. Binding step was favoured by applying 10 mL of 10 mM Tris-HCl pH 8, collecting fractions of 1 mL for further analysis. Elution step was then favoured by adding 10 mL of 1.5 M NaCl in 10 mM Tris-HCl pH 8, also collecting the fractions of 1 mL for further evaluation. At the end of each assay, columns were washed with DEPC treated water, and the absorbance of the fractions was measured in the Nano Photometer (IMPLEN, United Kingdom).

Chromatographic conditions that mainly favour hydrophobic interactions:

Equilibrium stage was performed by applying 10 mL of 2 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl pH 8 to the packed column allowing it to empty by gravity flow, followed by injection of approximately 30 µg of low molecular weight RNA. Binding step was favoured by applying 10 mL of 2 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl pH 8, collecting fractions of 1 mL for further analysis. Elution step was then favoured by adding 10 mL of 10 mM Tris-HCl pH 8, also collecting the fractions of 1 mL for further evaluation. At the end of each assay, columns were washed with DEPC treated water, and the absorbance of the fractions was measured in the Nano Photometer (IMPLEN, United Kingdom).

3.2.7.2. Separation between nucleic acids

Chromatographic assays to test the separation of different nucleic acids in the supports that showed better results in the screening experiments, were performed in the equipment AKTA Avant with the software UNICORNTM 6.3 (GE Healthcare Biosciences Uppsala, Sweden). The supports under evaluation were in this case two functionalized silicas, namely [Si][N3114]Cl and [Si][N3222]Cl. The supports were packed in columns with 10 mm diameter x 20 mm of height. For each chromatographic assay, the column was equilibrated with a solution previously filtered and sonicated, of 10 mM Tris-HCl (pH 8), using a flow rate of 1 mL/min. After equilibration, samples of RNA or lysate were independently injected in a 200 µL loop. Completed the binding step, it was applied a linear gradient of increasing salt concentration up to 1 M NaCl in 10 mM Tris-HCl (pH 8), in order to analyse different retention patterns and eventual species separation. A final step with higher salt concentration was performed, whenever it was needed, to guarantee the total elution of the sample. All experiments were performed at room temperature. Absorbance of eluted species was continuously monitored at 260 nm. The fractions of the elution peaks were recovered and further desalted with concentrators Vivaspin 10.000 KDa (Vivascience) until reaching 100 µL being lastly analysed by agarose gel electrophoresis.

3.2.8. Column regeneration

To guarantee the reproducibility between experiments and maintain the performance of these supports, it is imperative to stablish an effective regeneration protocol, that allows the elution of any specie strongly retained. For that, a solution of 0.2 M of sodium hydroxide (NaOH) was added to the support in order to remove any residues of sample followed by a solution of 0.5 M of HCl for replacement of the counterion (Cl^-) in the matrix. After every regeneration protocol, the supports were washed with large volumes of DEPC treated water.

3.2.9. Circular Dichroism (CD) spectroscopy

Circular Dichroism (CD) experiments were performed in a Jasco J-815 spectropolarimeter (Jasco, Easton, MD, USA), using a Peltier-type temperature control system. CD spectra were acquired at a constant temperature of 20 °C using a scanning speed of 50 nm/min, with a response time of 1 second over wavelengths ranging from 200 to 320 nm. The recording bandwidth was of 1 nm with a step size of 1 nm using a quartz cell with an optical path length of 1 mm. Three scans were measured *per* spectrum to improve the signal to-noise ration and the spectra were smoothed by using the noise-reducing option in the operating software. CD melting experiments were performed in the temperature range from 20 to 100 °C, with a heating rate of 1 °C/min, by monitoring the ellipticity at 265 nm.

3.2.10. Eukaryotic cell culture and cytotoxicity assay (MTT)

Normal human dermal fibroblasts (hFIB) were used for the cytotoxicity evaluation assays. Cells were cultured in DMEM-F12 medium (Sigma-Aldrich) supplemented with 10 % (w/v) FBS heat inactivated and 1 % (w/v) penicillin-streptomycin. The cellular cytotoxicity effect of the different supports and analogues ILs was evaluated using the Cell Proliferation Kit I (MTT) assay. To this end, hFIB cells at passages 10-20 were seeded at a density of $1x10^4$ cells per well in a 96-well plate and 24 h after, the cell culture medium was replaced by new medium and the different supports, in different quantities namely 25 µg, 50 µg and 100 µg, were placed in contact to the cells. For the ILs different percentages were applied in each well, namely 0.5 %, 1 % and 2 % (w/w). In both assays, MTT was performed at different time points (24 and 48 h). Hence, the medium was replaced by a mixture of 100 µL of medium and 10 µL of MTT reagent, and cells were incubated during 4 hours at 37 ˚C in a humidified atmosphere containing 5 % CO_2. Following incubation, the medium was removed, and 100 µL of dimethyl sulfoxide (DMSO) were added to each well and the plate was placed in agitation for about 15 minutes for crystals solubilization. The absorbance measurements were performed in a microplate reader at 570 nm. All experiments were repeated three times and for positive control for cytotoxicity cells were treated with 70 % ethanol.

3.2.11. Statistical analysis

All cytotoxicity experiments were repeated three times using independent culture preparations. The data are expressed as mean ± standard error. Quantitative data were statistically analysed by One-Way Analysis of Variance (ANOVA), followed by pair-wise comparisons using Dunnett's test. "*" indicate significant difference versus untreated cells.

CHAPTER 4 – Results and Discussion

Immobilization of ILs onto solid supports, and consequently their use as chromatographic ligands can become an interesting field in downstream processing due to the potential of ILs to establish different types of interactions with biomolecules. Hence, it is expected that these multiple interactions can contribute to achieve greater selectivity in the purification of nucleic acids. For this, in this work, different ILs were immobilized onto a solid support, namely the spherical silica, proceeding to an initial test of binding and elution profiles of RNA samples in order to choose the most promising ligands for the separation/purification of nucleic acids. Silica was the chosen support for this initial screening, because it is robust, cheaper than other chromatographic supports and the synthesis protocols with ILs are well stablished, becoming a good choice for the screening of new chromatographic ligands.

4.1. Characterization of functionalized supports

The functionalization of SilPrCl with 1-methylimidazole, triethylamine, tributylamine, trioctylamine and N,N-dimethylbutylamine originated five Supported-Ionic Liquids (SILs): [Si][C3C1Im]Cl, [Si][N3222]Cl, [Si][N3444]Cl, [Si][N3888] and [Si][N3114]Cl respectively. After the synthesis of the SILs it was important to analyse and verify if the different ligands were correctly bound to the silica support, through different techniques. Besides analysing the SILs, it was also analysed activated silica (SiO_2) and the intermediate support with the spacer arm (SilPrCl) as controls (Figure 8).

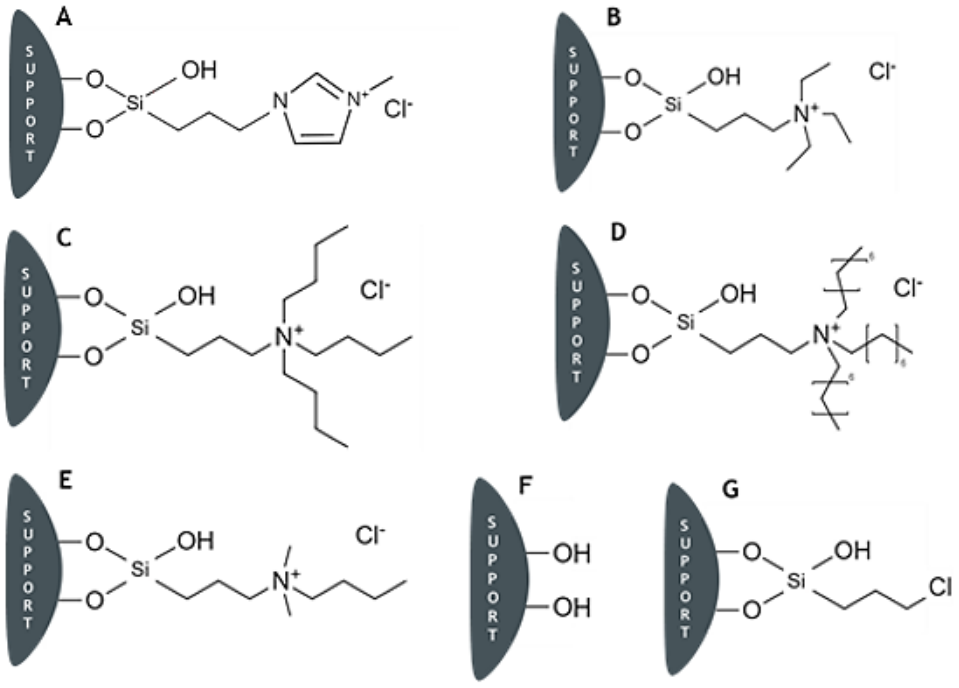


Figure 8: Representation of the chemical structures of functionalized silica with the different ILs. A - [Si][C3C1Im]Cl; B - [Si][N3222]Cl; C - [Si][N3444]Cl; D - [Si][N3888]Cl; E - [Si][N3114]Cl; F - SiO_2 e G – SilPrCl.

One of the ligands is based in a heterocyclic compound (Figure 8, A) which has already been proven to be a good ligand for the separation of gDNA and RNA in a previous work from our research group. Meanwhile, the other ligands are tetraalkylammonium-based cations which vary mainly in the length of the alkyl chain and in the symmetry, being three of the ligands symmetrical (Figure 8, B, C and D) and one asymmetrical (Figure 8, E). It was clearly observed that with the increasing of the alkyl chain length, the dispersion capacity in water decreased, indicating that the hydrophobicity increases with the increase of the alkyl chain length of the cation, as expected.

4.1.1. Elemental Analysis

The first method used for the characterization of the new supports was elemental analysis (Table 6). Neither the activated silica nor the SilPrCl present a content in nitrogen as expected, but by elemental analysis we confirmed the presence of nitrogen in the SILs materials. These results proved that the studied ILs were efficiently immobilized on the silica. In Table 6 are presented the results of elemental analysis for each support, in which the real percentage (RS%) of carbon, hydrogen and nitrogen measured in the support samples are compared to the theoretical percentage (TS%) of these compounds taking into account the chemical structure of each ligand and respective molar mass. The aim of this study is to verify if the real percentage of elements comes close to the theorical percentage after functionalization reactions.

Considering the [Si][N3114]Cl, it was obtained 7.64% as total of real element percentage in the sample, which represents 100%, and corresponds to the sum of 5.64% carbon, 1.29% of hydrogen and 0.71% of nitrogen. Thus, if 7.64% correspond to 100%, consequently, we have in the sample 73.83% of carbon, 16.83% of hydrogen and 9.35% of nitrogen. Regarding theoretical molar mass of the three analysed elements in the support, it was obtained a total of 143.27 g/mol (100%) in which 108.10 g/mol are carbon (75.45%), 21,17 g/mol are hydrogen (14.77%) and 14.01 g/mol are nitrogen (9.78%). In case of real sample percentage (RS%) divided by theoretical sample percentage (TS%) comes close to 1, it indicates a correct functionalization of the support with the IL.

Trough analysis of Table 6, we can verify that [Si][C3C1Im]Cl is correctly functionalized. Immobilization of this kind of IL is the most described in literature, and here we could see that all RS%/TS% value for each element come close to 1 (Carbon = 0.94, Hydrogen = 1.47 and Nitrogen = 0.97), thus proving an effective binding of this IL to silica support. The same successful functionalization can be observed with [Si][N3114]Cl, once the reasons of all three elements was also very close to 1. However, analysing the results for [Si][N3222]Cl, [Si][N3444]Cl and [Si][N3888]Cl regarding to nitrogen content (0.48, 0.29 and 0.37 respectively), we can conclude that this is a residual presence of this element. This might be indicative of less effective functionalization of the support with these ILs, which might be due to stereochemical impediment of the alkyl chains from the cation that may difficult the binding process. Functionalization with these types of ILs might be enhanced with the increasing of

ligand quantity used in synthesis reactions. Nevertheless, despite these three supports might not present such an effective functionalization, it was possible to analyse their behaviour in screening assays realized with nucleic acids.

Table 6: Elemental analysis results of carbon, nitrogen and hydrogen for each synthesized support. Comparison between real sample and theoretical percentages.

Support	Element	Real Mass (%)	Real Sample % (RS%)	Molar Mass (g/mol)	Theoretical Sample % (TS%)	$\frac{RS\%}{TS\%}$
[Si][C3C1Im]Cl	Carbon	6.86	63.69	84.08	67.70	0.94
	Hydrogen	1.55	14.35	12.10	9.74	1.47
	Nitrogen	2.37	21.97	28.01	22.56	0.97
	Total	10.77	100	124.19	100	-
[Si][N3222]Cl	Carbon	4.83	76.70	108.10	75.45	1.02
	Hydrogen	1.17	18.60	21.17	14.77	1.26
	Nitrogen	0.30	4.70	14.01	9.78	0.48
	Total	6.30	100	143.28	100	-
[Si][N3444]Cl	Carbon	4.48	78.15	180.17	79.22	0.99
	Hydrogen	1.15	20.05	33.26	14.63	1.37
	Nitrogen	0.10	1.80	14.01	6.16	0.29
	Total	5.74	100	227.43	100	-
[Si][N3888]Cl	Carbon	4.46	80.53	324.30	81.94	0.98
	Hydrogen	1.01	18.15	57.46	14.52	1.25
	Nitrogen	0.07	1.32	14.01	3.54	0.37
	Total	5.54	100	395.76	100	-
[Si][N3114]Cl	Carbon	5.64	73.83	108.10	75.45	0.98
	Hydrogen	1.29	16.83	21.17	14.77	1.14
	Nitrogen	0.71	9.35	14.01	9.78	0.96
	Total	7.64	100	143.27	100	-

4.1.2. Nuclear Magnetic Resonance

NMR is a characterization technique that can be used to study samples of nucleus that present spin numbers, allowing the evaluation of different chemical environments of the nucleus. ^{13}C NMR provides information about carbon nucleus in the structure. In Figure 9 are represented the NMR spectra for each synthesized material.

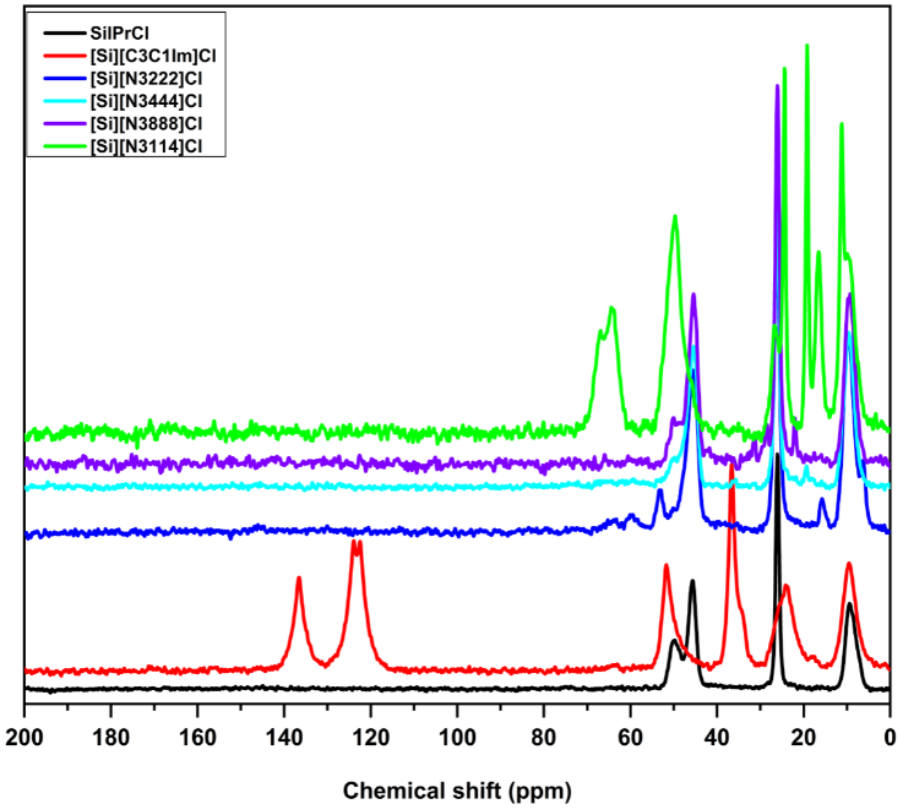


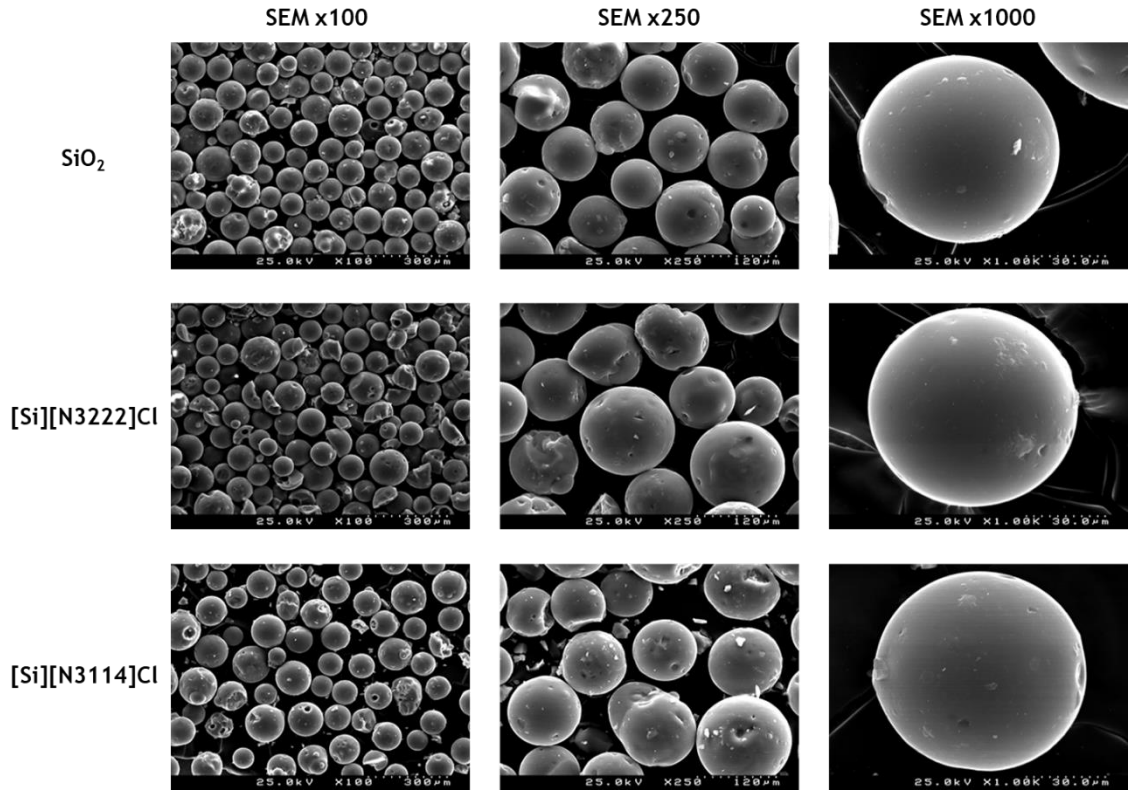
Figure 9: Results of NMR ^{13}C analysis of functionalized silica supports in the solid state with the different ILs.

Comparing de spectra obtained by NMR, we can see significant differences between the different supports. We can verify that the support [Si][C3C1Im]Cl (red) is well functionalized since there are present two of the characteristic signals for the carbons of the imidazole ring between 120 and 140 ppm. Relatively to the other supports, [Si][N3222]Cl (dark blue), [Si][N3444]Cl (light blue) and [Si][N3888]Cl (purple) it is not possible, through NMR spectra, to affirm if the functionalization occurred correctly, since the chemical deviations of the cations' carbons are similar among them (alkyl chains) and also similar to the chemical deviations of the silane carbons, which was used as compatibilizer. Regarding [Si][N3114]Cl (green) the signal observed approximately at 51 ppm corresponds to the methyl groups bound to the nitrogen, giving an indication of effective functionalization of the support.

4.1.3. Scanning Electron Microscopy

Since the synthesis conditions may sometimes be aggressive to the supports, due to the use of high temperature and high agitation rates, it is necessary to confirm if there is not an alteration in the morphology of the functionalized silica supports that may compromise their application as chromatographic supports. For this, in Figure 10 we have some SEM representative images of SiO_2, [Si][N3222]Cl and [Si][N3114]Cl with different magnifications.

Figure 10: SEM analysis of activated and functionalized silica surface. Observations with x100, x250 and x1000 of magnification.



By analysing and comparing the SEM images of activated silica with the two representative IL-functionalized materials, we can observe that the IL binding to the silica surface, despite de aggressive conditions used during the synthesis process, did not compromised the beads morphology since their round shape is maintained intact. Although only SEM images of two SILs are shown, one with a symmetrical amine and other with an asymmetrical amine, it is expected that the other materials show similar morphologies.

4.2. Screening of binding/elution conditions for RNA

As mentioned, the ability of these new ligands to interact with RNA must be tested and analysed, and for that different experiments were performed adapting experimental conditions to mainly favour ionic or hydrophobic interactions, establishing binding and elution profiles for each one of these conditions. The regeneration protocol with NaOH and HCl was performed after 3 assays, since the binding capacity was decreased in consecutive assays, thus allowing the normal conditions of the matrix to be restored between experiments.

4.2.1. Electrostatic interactions

For evaluating the possible interactions occurring between RNA and the different ligands in conditions that mainly favour electrostatic interactions, the binding step was established with 10 mM Tris-HCl, while the elution step was accomplished with 1.5M NaCl in 10 mM Tris-HCl pH 8.0 in order to increase the ionic strength of the buffer. The results of RNA behaviour regarding the binding and elution were analysed by absorbance measurement at 260 nm, calculating then the elution percentage in each step of the chromatographic assay.

When analysing essentially electrostatic interactions, the main contribution is from the charged group in the cation, the charged tetrasubstituted amine. Regarding the activated silica (SiO_2) and the intermediate support with the spacer arm (SilPrCl) (Figure 11, A and B) we can observe that there is no interaction with the sample, since there are no charged groups in these two supports. On the other hand, with [Si][C3C1Im]Cl and [Si][N3114]Cl (Figure 11, C and G) occurred total binding of the injected RNA, thus showing an excellent retention ability of the supports for RNA, when using these conditions.

As mentioned above, with the increase of the alkyl chains in the cation, it was observed an increase of the hydrophobicity evaluated by the low dispersion of the supports in water, and this was also confirmed in the chromatographic assays. With [Si][N3222]Cl occurred a good binding of RNA as well as total recovery with the increase of the ionic strength of the buffer. Meanwhile, [Si][N3444]Cl showed less ability to bind RNA than [Si][N3222]Cl (Figure 11, E and D) probably because the alkyl chains are longer, and the charged group is less exposed for the interaction to occur. Hereupon, there can exist a higher stereochemical impediment for the access of RNA to the charged region, and the more hydrophobic characteristics of the ligand become more evidenced. So, the binding of RNA in conditions that favour electrostatic interactions is less effective. Similarly, with [Si][N3888]Cl, RNA binding was even less effective since the alkyl chains are even longer, as well as the stereochemical impediment, thus conferring very high hydrophobic properties to this support (Figure 11, F).

In general, the purification of biomolecules using electrostatic interactions are easier and preferable to perform since, comparatively to the salts used in hydrophobic interaction (ammonium sulphate), sodium chloride is easier to remove from purified samples and is less hazardous to the environment. Considering this, it is reasonable to consider [Si][N3114]Cl and [Si][N3222]Cl as promising supports to exploit other applications in downstream processing.

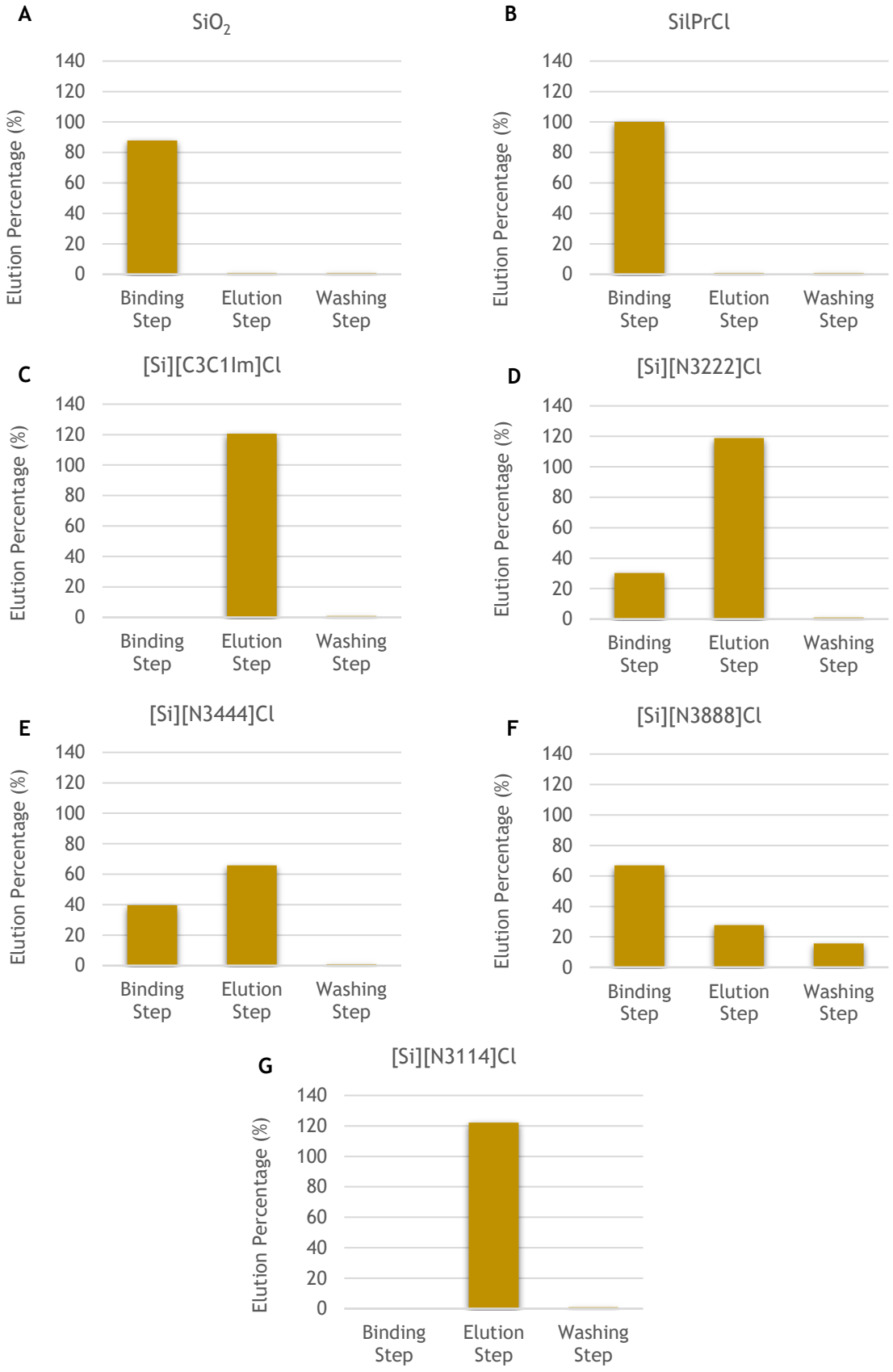


Figure 11: Elution behaviour of RNA at each step of the chromatographic process in conditions that favour electrostatic interactions. A - SiO_2; B - SilPrCl; C - [Si][C3C1Im]Cl; D - [Si][N3222]Cl; E - [Si][N3444]Cl; F - [Si][N3888]Cl; G - [Si][N3114]Cl.

4.2.2. Hydrophobic interactions

For evaluating the binding of RNA to the different ligands in conditions that favour mainly hydrophobic interactions, the binding step was defined as 2M $(NH_4)_2SO_4$ in 10 mM Tris-HCl while the elution step was accomplished with 10 mM Tris-HCl in order to decrease the ionic strength of the buffer. The results of RNA behaviour regarding to binding and elution were analysed by absorbance measurement at 260 nm, calculating the elution percentage in each step of the chromatographic assay.

When analysing essentially hydrophobic interactions, the main contribution is from the alkyl chains. Regarding activated silica and the intermediate support with the spacer arm (Figure 12, A and B) there was a significant interaction with RNA sample. Since the spacer arm is essentially an alkyl chain, and can demonstrate some hydrophobic behaviour, thus resulting in significant interaction when these conditions are favoured. However, this result does not compromise further analysis with functionalized supports. For the support [Si][C3C1Im]Cl it was observed a good retention in the binding step (Figure 12, C), proving the multimodal capacity of this ligand since it has shown also good binding properties when electrostatic interactions were favoured. The same was also observed with [Si][N3114]Cl (Figure 12, G). Concerning the other supports, [Si][N3444]Cl and [Si][N3888]Cl (Figure 12, E and F), have shown excellent binding ability of RNA sample, being the main issue the elution step, as it was verified a difficulty for the effective recovery of bound RNA. For [Si][N3222]Cl (Figure 12, D), it was observed partial elution of the sample, which may be interesting since it can already show some selectivity of this ligand. Meanwhile, as [Si][N3444]Cl is more hydrophobic than [Si][N3222]Cl, the interactions that occur with RNA are so strong that elution is significantly decreased (Figure 12, E). In the same way, with [Si][N3888]Cl, which is more hydrophobic, elution was even more difficult, being only possible to elute RNA sample with a second elution step with 1.5M NaCl in 10 mM Tris-HCl (Figure 12, F). This is because, when loading Tris-HCl 10 mM in the column, electrostatic interactions are also being favoured, thus not being enough for the recovery of all sample loaded. When NaCl flows through the column, electrostatic interactions are not favoured, leading to total elution of RNA sample.

Besides the binding and elution profiles, it was also evaluated the packing characteristics of the materials. As mentioned above, the more hydrophobic the material, more difficult it was to disperse it in water and therefore, more difficult turned out the packing of the columns. [Si][N3444]Cl and [Si][N3888]Cl when mixed with water formed aggregates of material which compromised the packing, and therefore, was not a good choice to explore in Akta equipment. [Si][C3C1Im]Cl, [Si][N3222]Cl and [Si][N3114]Cl were easy to pack since they had a good dispersion in water, not forming any type of aggregates. Since [Si][C3C1Im]Cl has already been tested by the group and proven to show good selectivity between gDNA and RNA, [Si][N3222]Cl and [Si][N3114]Cl seemed to be the most promising supports for further purification assays, due to their good binding ability at both ionic and hydrophobic conditions, and their good packing characteristics.

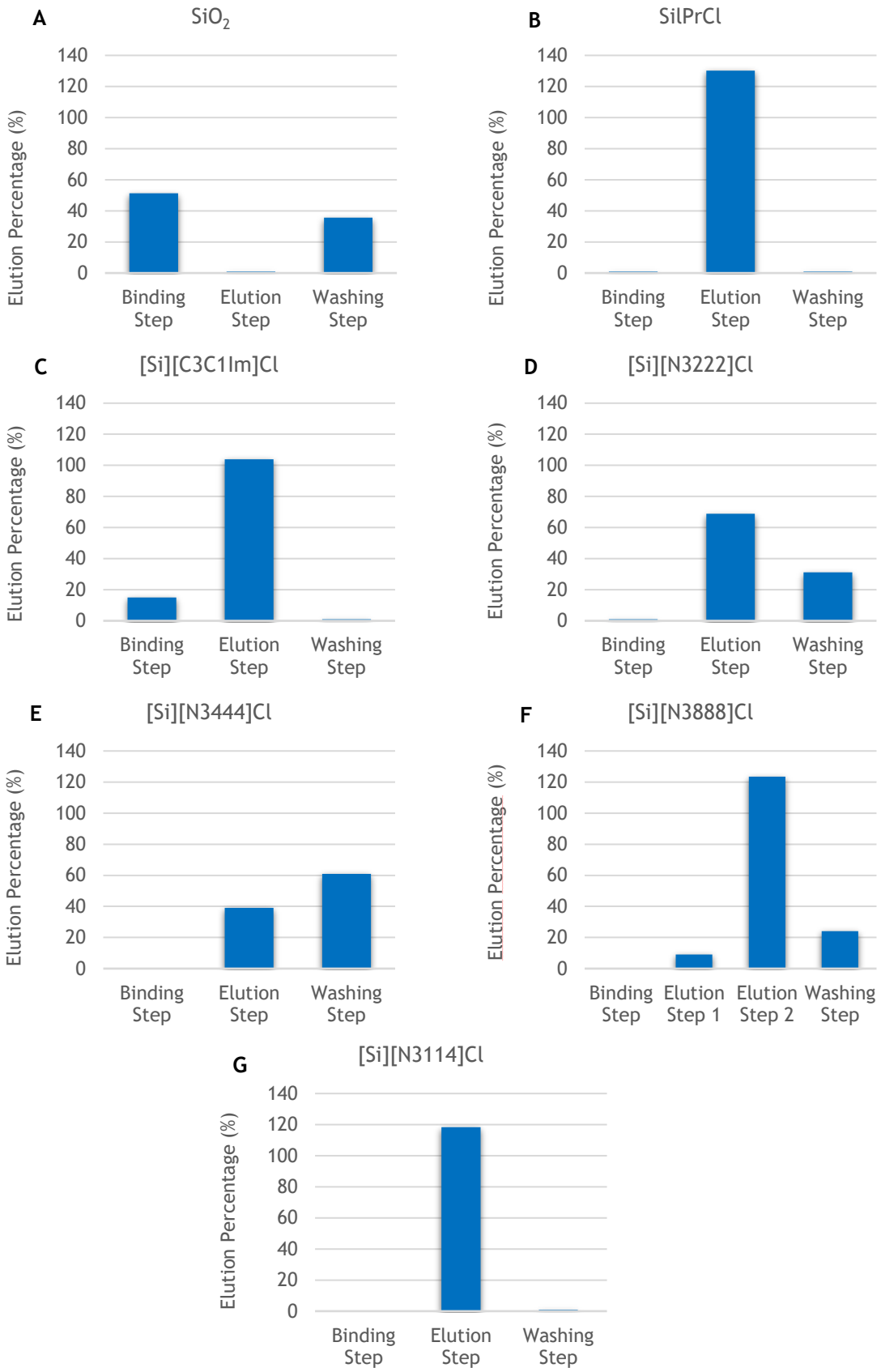


Figure 12: Elution behaviour of RNA at each step of the chromatographic process in conditions that favour hydrophobic interactions. A - SiO_2; B - SilPrCl; C - [Si][C3C1Im]Cl; D - [Si][N3222]Cl; E - [Si][N3444]Cl; F - [Si][N3888]Cl; G - [Si][N3114]Cl.

4.3. Separation between gDNA and RNA

Once completed the screening of all synthesised supports, was time to try to use of this spherical silica in the AKTA Avant equipment to, firstly study the effect of pH on the retention of RNA in each support. Silica is an interesting choice for the screening of new ligands since it is cheap and synthesis protocols with ILs are well stablished with this material. Although the supports had shown good binding and elution capacities when exploring hydrophobic interactions, the use of high salt concentrations imply more time to remove the salt from recovered peaks and has more environmental impact than the salts used when establishing electrostatic interactions. By this, the conditions able to mainly promote electrostatic interactions were chosen to test the supports for the purification of RNA. The assays performed with [Si][N3222]Cl are represented in Figure 13, A. For the assay with a pH 8 in the binding step, it was observed that the support had almost no binding ability for the RNA sample. However, when a pH 7 was tested in the binding step, de retention of RNA significantly increased comparatively to the previous assay, but binding was not complete since in the first peak we can observe some elution of the RNA (Figure 13, B). In the linear gradient it was possible to verify that with pH 7, there was a need of a higher salt concentration for the total elution of the sample comparatively to the assay at pH 8. In the same way, when using a pH 6 it was also observed a better retention of the sample in the binding step, and even a higher salt concentration was needed for the total elution of RNA, in comparison to the ionic strength required to elute RNA in the assay at pH 7. These results indicate that, with the decrease of the pH, there is a higher retention of RNA onto the support, favouring this interaction.

The experiments performed with [Si][N3114]Cl are represented in Figure 13, C, in which the RNA binding was even more pronounced than with [Si][N3222]Cl. In all the pH range under study, the binding was stronger, however, retention times were different from the observed in the other support. With pH 8 the retention was higher, since the total elution of RNA occurred later, this is, with a higher salt concentration. The behaviour of the support in the assay at pH 7 and pH 6 was very similar in terms of retention time of the samples, eluting almost at the same salt concentration. However, was expected that RNA retention would increase with de decreasing of the pH buffers, but at pH 8 RNA was more retained, this result can be influenced by the fact that this was the first assay performed in the matrix, thus being in the best binding conditions to perform an assay since it had not been submitted to any previous assay nor regeneration process, which means that the binding capacity was not compromised. By the agarose gel electrophoresis of the assays at pH 8, 7 and 6 in [Si][N3114]Cl (Figure 13, D), and comparing to the agarose gel electrophoresis of the assays at pH 8, 7 and 6 in [Si][N3222]Cl (Figure 13, B), we can see that the retention of RNA onto the [Si][N3114]Cl is much higher than the observed with [Si][N3222]Cl. This may indicate some contribution of the ligand symmetry, this is, since [Si][N3114]Cl is an asymmetrical cation it can establish interaction with different conformations of different types of RNAs that constitute the sample. It should be noted that, through supports characterization, [Si][N3114]Cl was more effectively functionalized than [Si][N3222]Cl, which can also have influence in the analysis of binding ability.

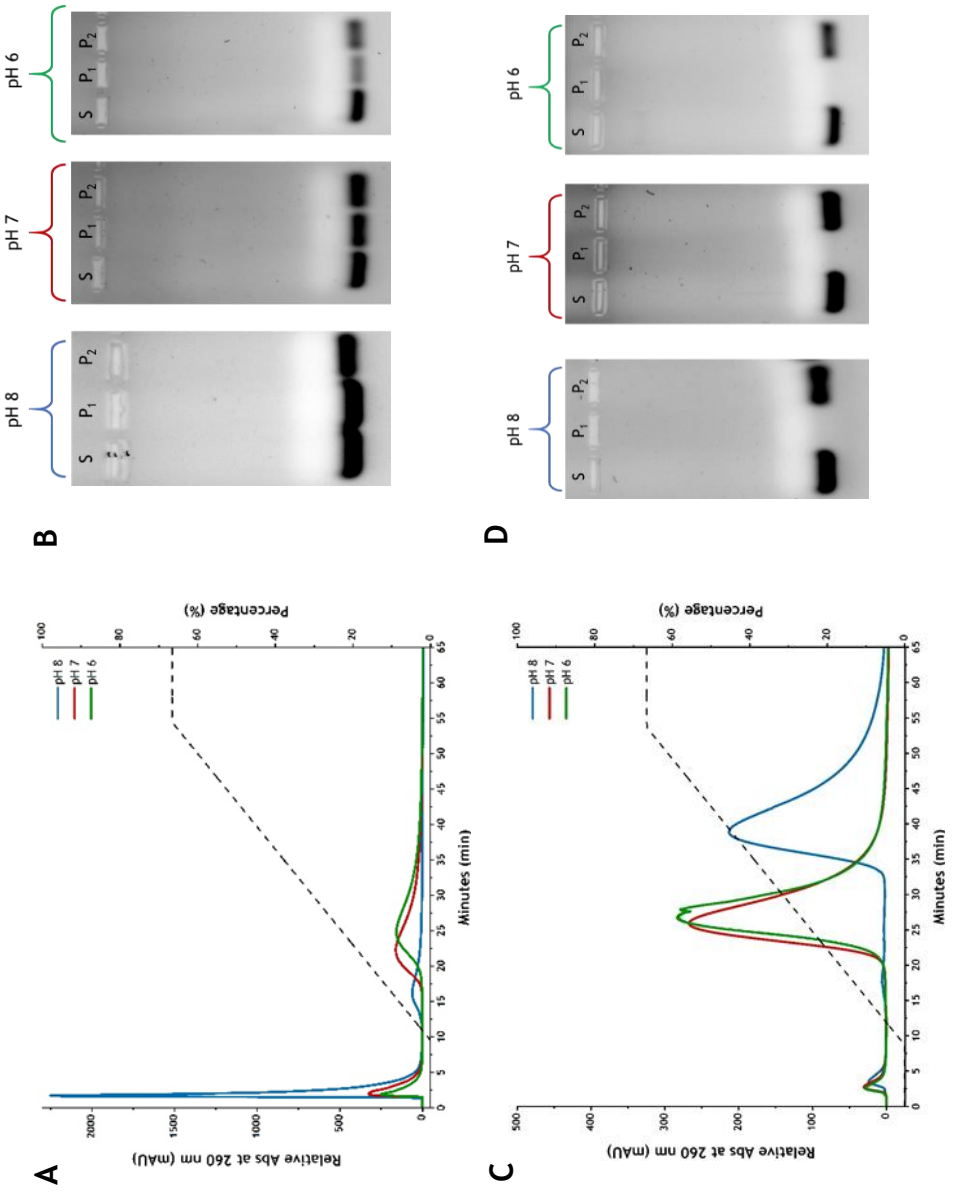


Figure 13: Representative chromatogram of the assays performed with RNA in [Si][N3222]Cl (A) and respective agarose gel electrophoresis of pH 8, 7 and 6 assays (B). In (C) is the chromatogram of the assays performed in [Si][N3114]Cl and respective agarose gel electrophoresis of pH 8, 7 and 6 assays (D). The assays were performed in the same conditions in which binding was favoured with low salt concentration following a linear gradient and finishing with a washing step. (S – Injected sample; P_1 – First Peak; P_2 – Second Peak).

After the assays with RNA, it was important to test if these supports had some selectivity between different types of biomolecules. For this, it was used a more complex mixture of genomic DNA (gDNA) and RNA, and the chromatographic assays were performed in the same conditions used in the previous assays.

In the assay performed with [Si][N3222]Cl (Figure 14, A) at pH 8 it was observed that the binding step did not allow the retention of neither RNA or gDNA. However, in the same way as it was previously observed with RNA, a stronger binding was achieved with the decrease of the pH, this is, at pH 7 and pH 6 RNA bound totally to the column (Figure 14, B). In the electrophoresis of the assay at pH 6 it was possible to observe that in the first peak (binding step), gDNA did not bound to column, eluting at lower salt concentrations. On the other hand, when linearly increasing salt concentration, it was possible to conclude that the second peak in the chromatogram corresponded to RNA, although being possible to see a little gDNA contamination. The same was verified in the assay performed at pH 7. In this way, it was possible to prove that [Si][N3222]Cl has selectivity and is able to distinguish between different nucleic acids, being gDNA eluted in the binding step, and since RNA is more retained, it only elutes with the increasing of salt concentration.

Ultimately, when tested the mixture of gDNA and RNA in [Si][N3114]Cl it was possible to conclude that this support had better binding ability than [Si][N3222]Cl, since RNA was retained in the column at all tested pH values (Figure 14, C) and the same was observed in the previous assays with the sample containing only RNA. Regarding the retention time, it was possible to conclude that in the assay performed at pH 6, RNA was more retained in the column since it was needed a higher salt concentration in order to elute all bound RNA. For the assay at pH 8, previously it was observed that RNA was more retained in this support, however since it has been submitted to other assays and regenerations this behaviour changed, thus, in these case with a more complex sample RNA did not bound so efficiently. In the electrophoresis of the assay at pH 8 (Figure 14, D) we can verify that the first peak corresponds to gDNA, and the second peak corresponds to RNA. gDNA may elute in the first place, since its structure is double-stranded and bigger, and nucleotide bases are not so exposed as RNA, thus being less accessible for establishing interactions with the ligands. On the other hand, RNA for being smaller and having bases exposed, can easily interact with the ligands, being more retained (Diogo *et al.*, 2002).

In all the assays, sometimes it was difficult to visualize the gDNA band in the electrophoresis gel, since this is a more fragmented sample and more subjected to denaturation. Therefore, it was necessary to increase de gDNA injected into the column, thus allowing the recovery of more gDNA quantity and after concentration it was possible to observe, although faint, a band of gDNA in the first peak of elution. In general, [Si][N3114]Cl showed better selectivity capacity than [Si][N3222]Cl, since in electrophoresis with this last support it is possible to see some contamination of RNA recovered fraction with gDNA. In this way, the asymmetry of the ligand seems to contribute to better selectivity in the separation of these two species of biomolecules.

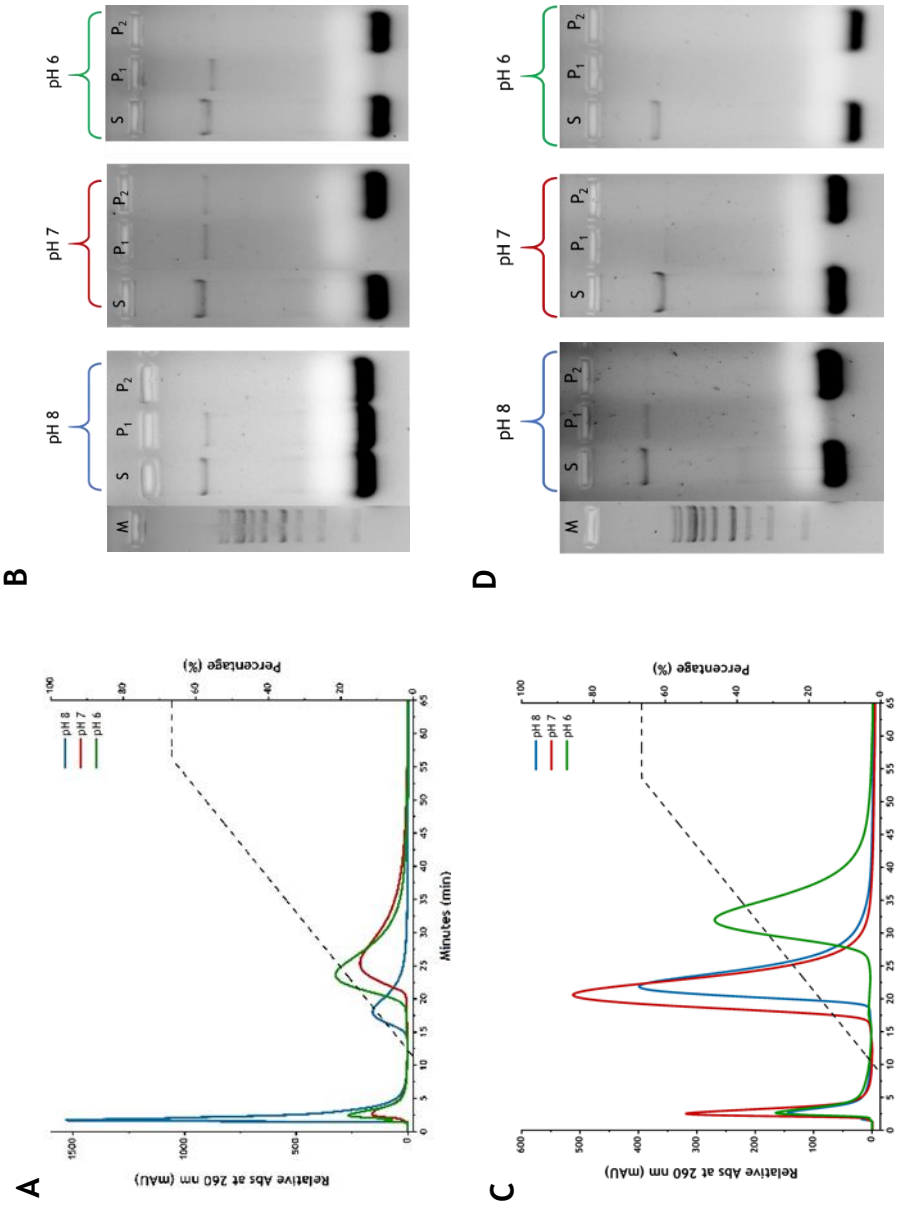


Figure 14: Representative chromatogram of the assays performed with more complex samples of gDNA and RNA in [Si][N3222]Cl (A) and respective agarose gel electrophoresis of of pH 8, 7 and 6 assays (B). In (C) is the chromatogram of the assays performed in [Si][N3114]Cl and respective agarose gel electrophoresis of pH 8, 7 and 6 assays (D). The assays were performed in the same conditions in which binding was favoured with low salt concentration following a linear gradient and finishing with a washing step. (M – Molecular weight marker; S – Injected sample; P_1 – First Peak; P_2 – Second Peak).

4.4. Circular Dichroism

In order to test the integrity and stability of RNA samples after the purification assays, CD spectra were made for either RNA purified with [Si][N3222]Cl and with [Si][N3114]Cl. Typical RNA CD spectra is characterized by having two main ellipticity peaks: a maximum at approximately 265 nm (positive band) and a minimum at approximately 210 nm (negative band) (Yao *et al.*, 2018). In Figure 15, RNA spectrum is represented in purple line, and it is possible to verify the two characteristic bands at 265 and 210 nm. RNA spectra from a purified fraction obtained with [Si][N3222]Cl is represented in pink line, being possible to observe a decrease of the ellipticity in the positive band, thus demonstrating some destabilization of RNA sample, although is not very significant. In the other hand, in RNA spectra from a purified fraction of the purification assay with [Si][N3114]Cl, represented in green line, the ellipticity in the positive band was maintained similar comparatively to the initial RNA sample, indicating that after purification, RNA structure is maintained. These results show that purification process might be better with [Si][N3114]Cl than with [Si][N3222]Cl, since the first did not compromise RNA integrity after the experiment.

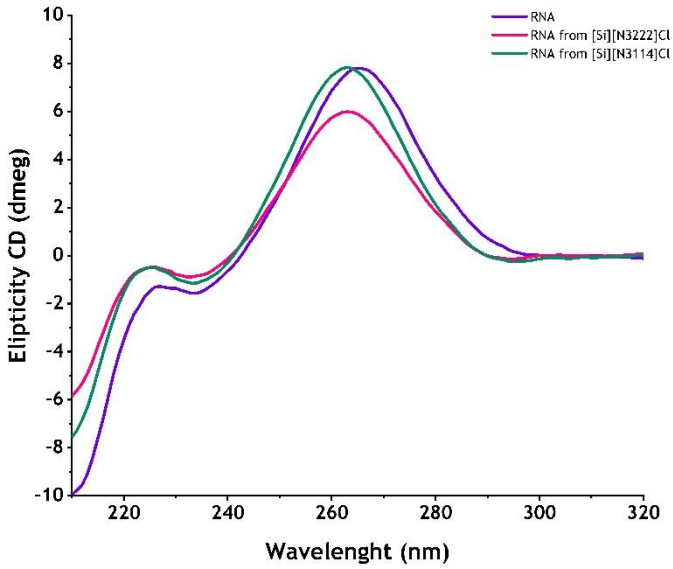


Figure 15: CD spectra (210 – 320 nm) of RNA collected from the purification assays with [Si][N3222]Cl and [Si][N3114]Cl. The purple line represents RNA, the pink line corresponds to RNA purified with [Si][N3222]Cl and the green line to RNA purified with [Si][N3114]Cl.

In the same way, CD spectra were measured for RNA with different types of ILs namely tetramethylammonium chloride ([N1111]Cl), tetraethylammonium chloride ([N2222]Cl), tetrapropylammonium chloride ([N3333]Cl) and tetrabutylammonium chloride ([N4444]Cl) (Figure 16). These specific ILs, analogues to the ligands used in the supports, were chosen because they are constituted by a tetraalkylammonium-based cation and the counter-ion chloride. Despite all the ILs being constituted by symmetrical amines, it was possible to evaluate the impact they have in the stabilization of RNA and thereby correlate the results with the alkyl chain length since we had a range from shorter to longer alkyl chains.

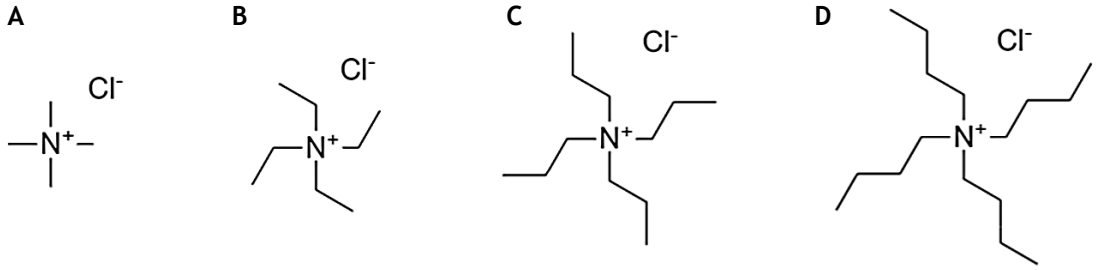


Figure 16: Chemical structure of the four different ILs used for RNA stabilization. A - tetramethylammonium chloride ([N1111]Cl); B - tetraethylammonium chloride ([N2222]Cl); C - tetrapropylammonium chloride ([N3333]Cl); D - tetrabutylammonium chloride ([N4444]Cl).

Therefore, in order to ascertain this, samples of RNA were prepared with 20% (w/w) of each IL and were incubated for 1 hour at 4 ºC before the spectra acquisition. In Figure 17, CD spectra of RNA in presence and absence of ILs are presented, as well as the spectra for each IL. RNA spectra without any IL is represented in dark grey (control), being verified the presence of the two characteristic bands at 265 and 210 nm. Analysing spectra obtained with RNA in 20% (w/w) [N1111]Cl (red line) we could see that ellipticity of the positive band was almost maintained comparatively to the control, as well as the negative band, thus indicating no destabilization of the RNA sample. The same analysis cannot be applied to the other three ILs. Regarding the sample of RNA in 20% (w/w) [N2222]Cl (dark blue) it was possible to see a significant ellipticity decrease in the positive band indicating some destabilization of the sample. With RNA in 20% (w/w) [N3333]Cl (green line) ellipticity decrease was less accentuated, however when observing this IL spectra (brown) it is clear a great interference with CD signal below 240 nm, compromising spectra analysis below this wavelength. Lastly, the analysis of RNA in 20% (w/w) [N4444]Cl showed a decrease on the ellipticity at 265 nm, which may also indicate some destabilization of RNA sample.

At first analysis, ILs with longer alkyl chains seem to compromise more RNA integrity since the main changes on the spectra occur with these ILs. A way to improve the stabilization of RNA would be adjusting ILs pH, since when we have measured the corresponding pH of each IL solution an acidic character prevailed. Pedro and co-workers verified that non-buffered [Ch][DHP] highly impaired RNA stability, whereas GB-ILs with more alkaline character beneficially maintained RNA integrity (Pedro *et al.*, 2019).

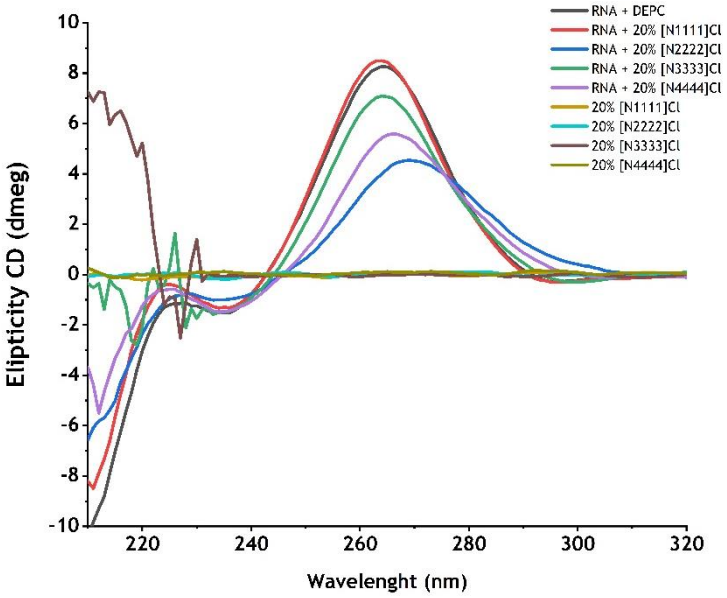


Figure 17: CD spectra (210 – 320 nm) of RNA in the absence and presence of ILs. The dark grey line represents RNA, the red line corresponds to RNA in 20% (w/w) [N1111]Cl, the dark blue line to RNA in 20% (w/w) [N2222]Cl, the green line to RNA in 20% (w/w) [N3333]Cl and the light purple line to RNA in 20% (w/w) [N4444]Cl. ILs spectra, without RNA, are also represented. RNA was incubated for 1 hour at 4 ºC with each ILs before measurements.

As an additional analysis, thermal denaturation experiments were performed with the view of studying RNAs secondary structure stability in presence and absence of the four ILs. Melting temperature (T_m) is assessed by following changes in the spectrum at 265 nm with increasing temperature, from 20 up to 110 ºC (Ranjbar & Gill, 2009). The T_m determined at 265 nm for RNA dissolved in DEPC water was of 49.1 ºC (Figure 18 and Table 7). Analysing all melting temperatures obtained with the presence of ILs, only [N1111]Cl showed a great improvement in the stabilization of RNA sample, by increasing T_m aproximatly 14 ºC. On the contrary, [N2222]Cl, [N3333]Cl and [N4444]Cl all shown to decrease RNA stability by decresing its T_m aproximatly 7, 11, and 19 ºC, respectively. This can be justified by the high stereo chemical impediment of these 3 ILs since longer alkyl chains limit interation of RNA with charged amine of the cation, what could limit some stabilization effect. An interesting test to perform would be to ensure if [N1111]Cl at a higher concentration than 20% would be capable of incresing even more RNAs T_m.

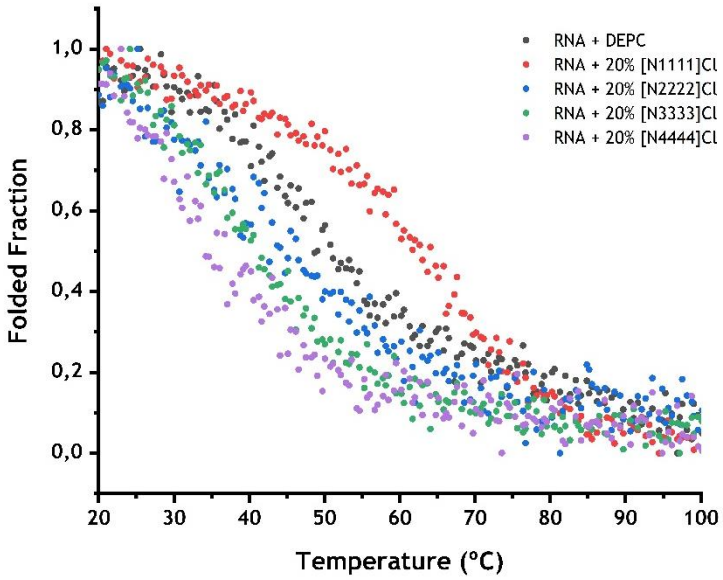


Figure 18: CD melting curves (265 nm) of RNA fractions incubated for 1 hour at 4 ºC with different ILs. The curves were obtained in a temperature range from 20 ºC to 100 ºC. The dark grey spots represent RNA, the red spots correspond to RNA in 20% (w/w) [N1111]Cl, the blue spots to RNA in 20% (w/w) [N2222]Cl, the green spots to RNA in 20% (w/w) [N3333]Cl and the light purple spots to RNA in 20% (w/w) [N4444]Cl.

Table 7: Melting temperatures (T_m) obtained from CD melting curves for each sample of RNA with the different ILs.

Sample	Melting Temperature (T_m) ˚C
RNA + DEPC	49.10 ± 0.59
RNA + 20% [N1111]Cl	63.22 ± 0.28
RNA + 20% [N2222]Cl	42.21 ± 1.14
RNA + 20% [N3333]Cl	37.82 ± 0.64
RNA + 20% [N4444]Cl	30.28 ± 1.80

4.5. Cytotoxicity effect of ILs

Since in this work, we are testing new ligands for the purification of biomolecules with the aim to be used as biopharmaceuticals, it is of great importance to test any cytotoxic effect that these ligands might present, once, although not expected, sometimes ligand leaching might occur. In this sense, the colorimetric assay MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used for the assessment of human fibroblasts (hFIB) viability in the presence of [Si][N3114]Cl and [Si][N3222]Cl in three different quantities, 25, 50 and 100 µg of support (Figure 19).

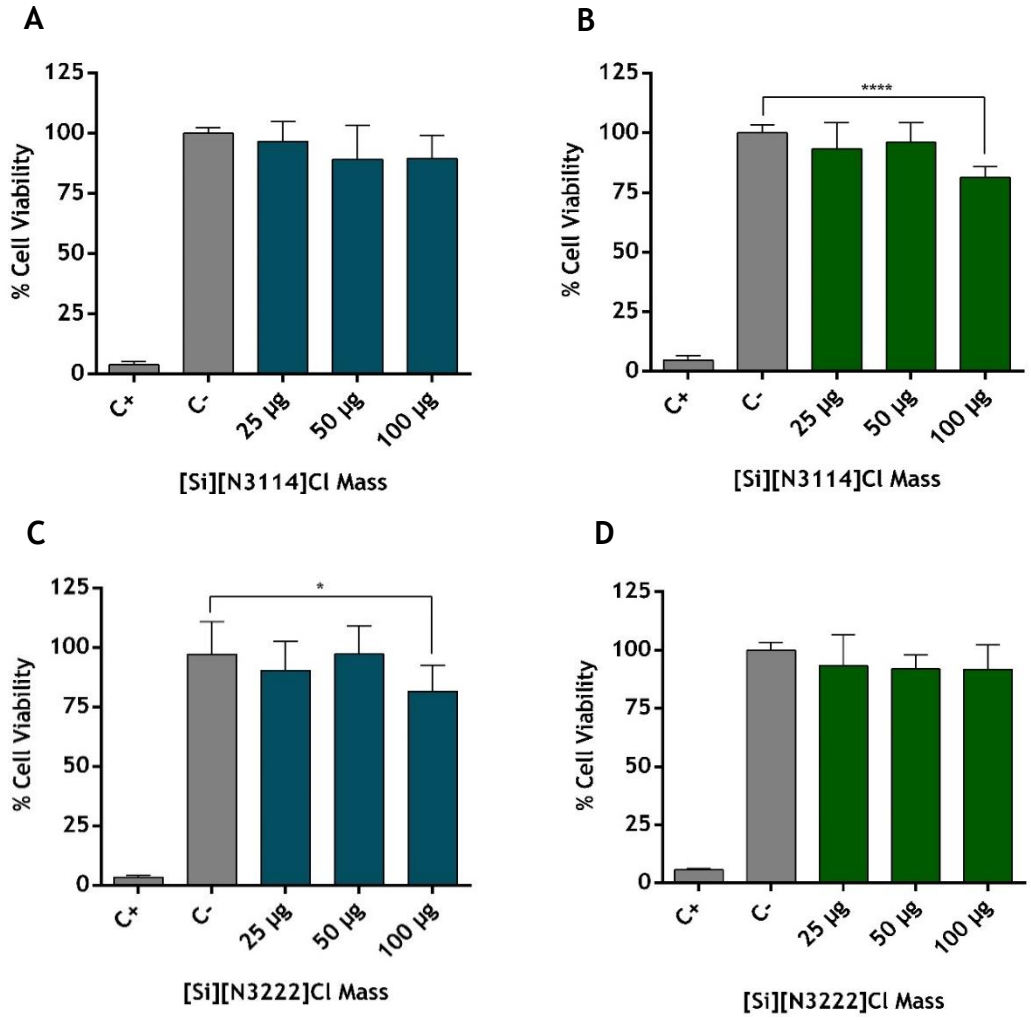


Figure 19: Cellular viability (MTT assay) of human fibroblasts after 24 (A) and 48 hours (B) incubation with [Si][N3114]Cl support, and 24 (C) and 48 hours (D) incubation with [Si][N3222]Cl support. Untreated cells (C-) and ethanol treated cells (C+) were used as negative and positive control, respectively. Viability percentage is expressed relatively to the control cells. Values were calculated with the data obtained from three independent measurements (mean ± SD, n = 3). Statistical analysis was performed using "One-way ANOVA". (ns = $p > 0.05$; **** $p \leq 0.0001$; *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$)

Through analysis of Figure 19 at 24h and 48h (A and B) in which hFIB were maintained in culture with [Si][N3114]Cl support, it was possible to assure that this ligand did not induce any cytotoxic effect on these cells. Despite of at 48h, cell viability with 100 µg of support was significantly different from negative control, it was still above 80%, suggesting that even that quantity did not represent cytotoxicity. Regarding the [Si][N3222]Cl support at 24h and 48h (C and D), also no cytotoxicity was observed. Cell viabilities were inferior at 24h comparatively to 48h, and this might be due to errors in cell seeding or in materials mass. It is important to emphasize that support masses used were much higher than the ligand leaching that can eventually occur, which also supports the safety of these materials. As additional results, cytotoxicity assay was also performed in hFIB with the ILs tested for stabilization of RNA, which are analogues to the ILs used in the functionalization of the chromatographic supports, in order to compare the behaviour between liquid solutions of ILs and immobilized ligands. For that, three different percentages of the four studied ILs were utilized in contact with hFIB, namely 0.5, 1 and 2% (w/w). Results at 24h and 48h are represented in Figure 20, A and B respectively.

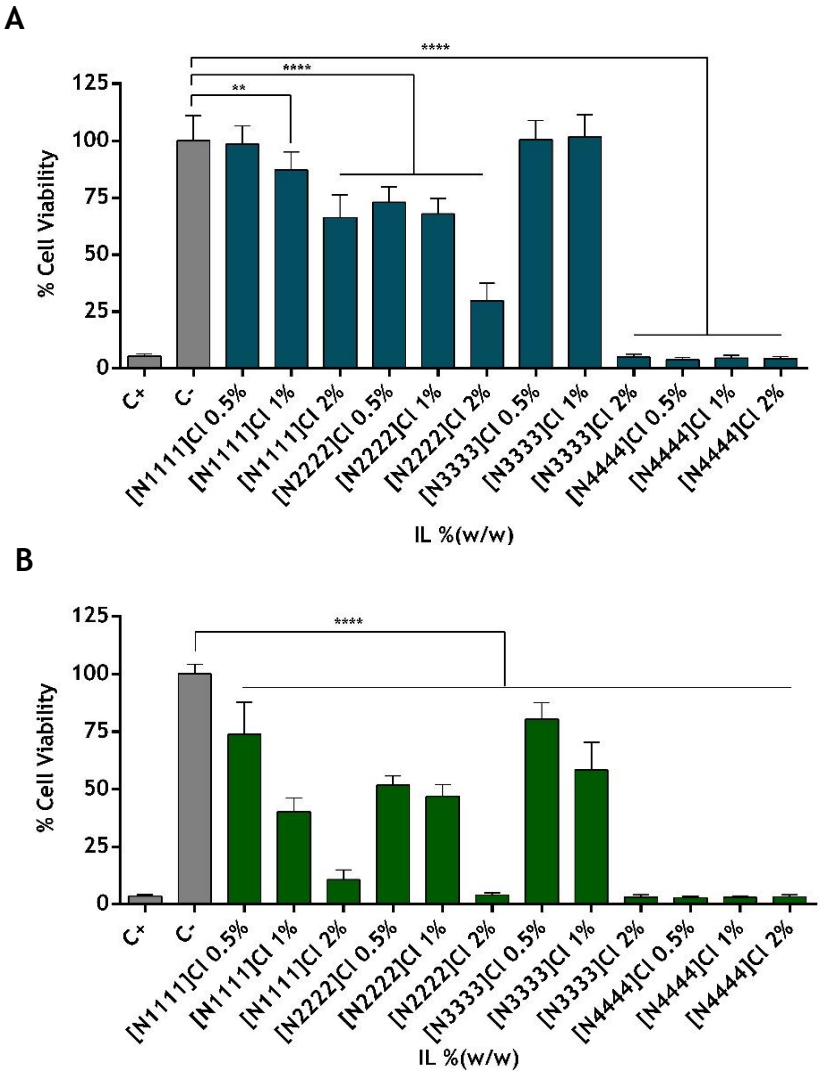


Figure 20: Cellular viability of human fibroblasts after 24 (A) and 48 hours (B) incubation with different ILs. Untreated cells (C-) and ethanol treated cells (C+) were used as negative and positive control, respectively. Viability percentage is expressed relatively to the control cells. Values were calculated with the data obtained from three independent measurements (mean ± SD, n = 3). Statistical analysis was performed using "One-way ANOVA". (ns = $p > 0.05$; **** $p \leq 0.0001$; *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$)

As presented in Figure 20, A, in general, cell viability is compromised when hFIB are incubated with the different ILs during 24h. Regarding experiments with [N1111]Cl at 0.5 and 1% (w/w) and [N3333]Cl at 0.5 and 1% (w/w), these were the only ILs and concentrations that did not induce toxicity in seeded cells, since cell viabilities were above 80%. On the contrary, [N2222]Cl and [N4444]Cl shown to be cytotoxic for all concentrations tested and also [N1111]Cl at 2% (w/w) showed cytotoxicity. It should be noted that for experiments performed with incubation time of 48h, all ILs at all tested concentrations shown to induce cytotoxicity (Figure 20, B). According to what is described in literature, it was expected that cytotoxicity would increase with the increase of the alkyl chains cation, however this did not occurred linearly, once [N2222]Cl showed to be more cytotoxic than [N3333]Cl (Mikkola *et al.*, 2015; Wang *et al.*, 2015).

In order to unravel these results, we thought that solutions pH could be causing these alterations, so pH of each solution was measured. As a matter of fact, [N2222]Cl presented a pH more close to 5 and [N3333]Cl close to pH 6, thus justifying [N2222]Cl to be more cytotoxic than [N3333]Cl, proving that not only the length of the alkyl chain influence cytotoxicity. However, when measuring [N4444]Cl pH, this was more close to 7, but since the alkyl chains are so long, this feature prevailed, being the most cytotoxic IL among all ILs tested. Similarly, to what was previously discussed in the RNA stabilization, pH control of ILs solution would maybe improve cell viability results by probably decreasing their toxic character.

In general, ILs with longer alkyl chains are more lipophilic than those with shorter versions. By this, it is presumed that they tend to be incorporated into the phospholipid bilayers of cells membranes. In this regard, increased toxicity of longer ILs can be accounted for enhanced membrane permeability, altering the physical properties of the lipid bilayer, thus conducting to decreasing cell viability (Pham *et al.*, 2010). Another important observation is the evident variation of toxicity with the increasing percentage of IL studied, with a significant cell viability decrease with increasing IL concentration. It was also reported by Feng and co-workers that molecular volume might affect the capacity of chemicals to cross the cell membrane, thereby leading to varied toxicity (Feng *et al.*, 2013).

It is important to emphasize that these liquid ILs are not exactly like the ligands immobilized onto the silica supports but can give an idea of the impact of alkyl chains length on stabilization and cytotoxicity. Comparing the results obtained in cytotoxicity assays between liquid ILs and immobilized supports, these shown to be much less toxic than the first ones.

CHAPTER 5 – Conclusion and Future Perspectives

Nucleic acids-based therapies have been arising as a new strategy for the treatment of numerous diseases namely those caused by alterations in gene expression. In this sense, it is extremely important to develop new purification strategies of these biomolecules in order to obtain them with total integrity, highly pure and with biological activity, in accordance to the guidelines of regulatory agencies for biopharmaceuticals. However, it is still difficult to have an ideal biotechnological process that allows biomolecules preparation in desired conditions, to be tested, in accordance to what is considered to be safe to be applied onto clinical trials.
Within the biotechnological processes, liquid chromatography is the method of choice for biomolecules purification, based on reversible interactions, to explore the selective separation and purification of biomolecules of interest. However, standard purification processes have some problems associated, namely the types of supports available and the use of solvents that can be environmentally hazardous.
This is where ionic liquids emerge as a new strategy for the purification of nucleic acids. Besides presenting excellent physicochemical characteristics, due to their characterization as "designer solvents", becomes possible to manipulate cation/anion combinations that present good selectivity capacity for purification of nucleic acids and non-cytotoxic character as well.
With this work, it was possible to prove the correct functionalization of silica supports with ILs, having the cation immobilized and anion as counter-ion, through covalent binding and nucleophilic substitutions. Besides, morphology support was not compromised after functionalization reactions turning possible its application for chromatographic experiments.
With the initial screening of binding and elution of RNA samples to each synthesized support, was possible to verify the multimodal capacity of all tested ligands, since they could establish interactions of either electrostatic or hydrophobic nature. After this screening tests, [Si][N3222]Cl and [Si][N3114]Cl were the chosen supports for further purification assays, as these showed to be the most promising ligands. Initially, were performed assays with only RNA and changing pH in the binding step from 6 to 8, in order to compare its influence on samples retention. In general, it was observed that with lower pH, RNA tends to be more retained in the column. Secondly, for analysing some selectivity of these ligands, a mixture of gDNA and RNA was loaded onto de columns, in the same conditions used for RNA experiments. With these chromatographic assays, both columns showed to have good selectivity between the two different nucleic acids species, with gDNA eluting in the beginning and RNA eluting with increasing salt concentration. After purification, CD spectra were obtained for RNA purified fractions and compared with initial sample of RNA, being possible to verify that these biomolecules could maintain their integrity. Therefore, this purification technique seems to be very promising for the purification of nucleic acids.
In the additional tests of stabilization with solutions of different ILs, it was possible to verify that with shorter alkyl chains in the cation, we could achieve an enhancement of RNAs melting temperature, proving its capacity of stabilizing RNA sample. Furthermore, in cytotoxicity assays, it was also observed an impact of the alkyl chains length on toxicity, since ILs with longer alkyl chains, such as [N4444]Cl, showed to be more harmful for cells. It is to be noted

that immobilized ligands showed to be much less harmful for cell than liquid solutions of ILs, proving a great advantage of using immobilized supports comparatively to liquid-liquid extractions, in which sample contamination with IL can be much higher.

As future perspectives, these ligands can be tested for purification of non-coding RNAs, once these represent a promising biomolecule to be used as biopharmaceuticals in the treatment of many diseases caused by gene defects. Besides, since they show to have multimodal characteristics, it would be interesting the use of these supports in continuous mode, thus achieving greater selectivity in the purification of different types of RNAs.

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