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Novel Excipient Candidates for RNA Stabilization

Sofia Pereira Oliveira

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Orientador: Profª. Doutora Fani Pereira de Sousa

Co-orientador: Profª. Doutora Mara Guadalupe Freire Martins

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Resumo Alargado

Até recentemente, o estudo de terapias usando a molécula de RNA como agente terapêutico era consideravelmente reduzido, quando comparado com terapias usando DNA e proteínas. A molécula de RNA era considerada como um mero intermediário na produção de proteínas. Mais recentemente, o estudo da molécula de RNA levou à identificação de outras funções, trazendo uma nova perspetiva para a potencial aplicação terapêutica desta molécula. A utilização de RNAs terapêuticos nomeadamente, os mRNAs e microRNAs tem vindo a aumentar, especialmente devido ao uso recente do mRNA na vacinação contra a COVID-19. Uma das principais limitações na distribuição global e utilização das vacinas de mRNA, que muito tem sido discutida, é a temperatura de armazenamento necessária à manutenção de estabilidade e eficácia da vacina. Isto revela a importância e urgência na identificação de compostos capazes de estabilizar a molécula de RNA de modo a facilitar, o seu armazenamento e distribuição, garantindo a sua atividade biológica. Considerando a crescente investigação na área dos RNAs terapêuticos, a identificação de estabilizadores pode ter um impacto transversal e alargado a outras moléculas de RNA, com diferentes aplicações.

No presente trabalho foram realizados ensaios de Dicroísmo Circular (CD) para avaliar a estabilidade da estrutura do RNA, após a incubação com diferentes categorias de excipientes, nomeadamente aminoácidos (arginina e glicina), líquidos iónicos (*N-acetyl amino acid N-alkyl cholinium-based*), açúcares (glucose, sacarose e trealose) e *Deep Eutectic Solvents* (DES) (*glucose-based DES* e *trehalose-based DES*). Os estudos de estabilidade envolveram também a avaliação da estabilidade de um RNA modelo ao longo do tempo (0, 7, 15, 30 e 60 dias) e também quando armazenado a diferentes temperaturas (4°C e temperatura ambiente). De uma maneira geral os resultados obtidos demonstraram a possibilidade de estabilizar o RNA na presença de aminoácidos, durante os 60 dias de armazenamento, quer a 4°C quer à temperatura ambiente. As novas classes de estabilizadores também demonstraram grande potencial, tendo em conta que as amostras estabilizadas por *DES* composto por trehalose e colina mantiveram a sua estabilidade após os 60 dias de armazenamento, a ambas as temperaturas, com um aumento da temperatura de *melting* de cerca de 18 °C. Os resultados obtidos por *docking* molecular permitiram um melhor entendimento das interações estabelecidas entre os *DES* e a molécula de RNA, permitindo estimar qual dos componentes do *DES* é que apresenta uma maior contribuição para a estabilização global da molécula de RNA. Outra das novas classes estudadas foram os líquidos iónicos com aminoácidos acetilados que também demonstraram resultados encorajadores. Neste caso, grande parte das amostras de RNA manteve a estabilidade ao longo do tempo, para ambas as temperaturas,

demonstrando assim uma melhoria relativamente à amostra de RNA sem estabilizadores.

Considerando os resultados obtidos, foi possível confirmar a possibilidade de estabilizar RNA usando alguns excipientes já estabelecidos e verificar o potencial de novas classes na indução da estabilidade desta molécula por um tempo médio de armazenamento à temperatura ambiente ou em refrigeração a 4 °C. Com este trabalho, criam-se novas perspetivas e novas possibilidades de formulação de produtos terapêuticos de base génica.

Palavras-chave

DES, Dicroísmo Circular, Estabilidade, IL, RNA

Abstract

Until recently, the study of therapies using the RNA molecule as a therapeutic agent was considerably low when compared to therapies using DNA and proteins. For a long time, the RNA molecule was considered as a mere intermediary in the production of proteins. More recently, the study of the RNA molecule has led to a better understanding of its function, thus bringing a new perspective to potential therapeutic applications of this molecule. The use of therapeutic RNAs namely, mRNAs and microRNAs has been increasing, especially due to the recent use of mRNA in vaccination against COVID-19. One of the limitations in the use of mRNA vaccines, which has seen a lot of discussion, is the temperature at which they have to be stored, in order to maintain vaccine stability and effectiveness. This reveals the importance and urgency in identifying compounds capable of stabilizing the RNA molecule in order to facilitate, the storage and handling, while maintaining its biological activity. Taking into account the growing research in the therapeutic RNAs field, identifying stabilizers might have an impact when working with other RNA molecules with different applications.

In the present work, Circular Dichroism (CD) assays were performed to assess the stability of the RNA structure, after incubation with different categories of excipients, namely amino acids (arginine and glycine), ionic liquids (N-acetyl amino acid N-alkyl cholinium-based ILs), sugars (glucose, sucrose and trehalose) and Deep Eutectic Solvents (DES) (glucose-based DES and trehalose-based DES). These studies also involved the assessment of stability over time (0, 7, 15, 30 and 60 days) at different storage temperatures (4 ° C and room temperature). In general, the results obtained demonstrate the potential of amino acids to stabilize RNA, for 60 days of storage at both temperatures. Moreover, the samples stabilized by the new excipient candidates also demonstrated great potential, taking into account that the samples stabilized by trehalose-based DES not only maintained their stability after 60 days at both temperatures, but also increased the melting temperature by around 18 °C. Data gathered by molecular docking simulations allowed for a better understanding of the interactions that happen between DES and RNA and which of the compounds might have a higher contribution for the RNA stabilization. Results using amino acid-based ionic liquids as stabilizers were also very encouraging, as most of the samples tested maintained stability over the testing period at both temperatures.

Considering the results obtained, these confirm the stabilizing capacity of the classes already used as excipients and the potential of new classes as RNA stabilizers. Thus opening the door to new possibilities for formulating therapeutic products.

Keywords

Circular Dichroism, DES, IL, RNA, Stability

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List of Acronyms

AcAla	N-Acetyl-L-alanine
AcCys	N-acetylcysteine
AcGly	N-acetylglycene
AcMet	N-Acetyl-L-methionine
AcPhe	N-Acetyl-L-phenylalanine
aFGF	Acidic fibroblast growth factor
ALS	Lateral sclerosis
API	Active pharmaceutical ingredient
ASOs	Antisense oligonucleotides
C_2Ch	Choline 2-C
C_5Ch	Choline 5-C
CD	Circular Dichroism
DEPC	Diethylpyrocarbonate
DES	Deep eutectic solvents
DNA	Deoxyribonucleic acid
EMA	European Medicines Agency
dNTPs	Deoxyribonucleotide triphosphate
dsRNA	Double stranded RNA
E. coli	*Escherichia coli*
FDA	Food and Drug Administration
FTIR	Fourier transform infrared
FW-primers	Forward primers
Gly	Glycine
His	Histidine
HPLC	high performance liquid chromatography
Ils	Ionic Liquids
K_2HPO_4	Potassium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
LB agar	Luria Broth agar
miRNA	MicroRNA
mRNA	Messenger RNA
NADES	Natural deep eutectic solvents
PCR	Polymerase chain reactions
PEG	Polyethylene Glycol
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
RNA	Ribonucleic acid
RNAP	RNA polymerase
rRNA	Ribosomal RNA
RV-primers	Reverse primers
SEC	Size exclusion chromatography
siRNA	Small interfering RNA
sncRNA	Small non-coding RNA
Tm	Melting temperature

TBDMS	T-butyldimethylsilyl
TOM	Tri-iso-propylsilyloxymethyl
tRNA	Transfer RNA
UV	Ultraviolet

Chapter 1

1. Introduction

1.1 Nucleic acids

Nucleic acids are at the center of cell function, as they contain the genetic information required for the production of all effector biomolecules. Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) are two different types of nucleic acids (figure 1), both carrying genetic information, and involved in distinct but related biological mechanisms. Both are composed by linear chains of nucleotides, being the DNA constituted by two complementary nucleotide chains, whereas RNA is composed by a single chain [1]. Nucleic acids chains are obtained by the binding of successive nucleotides, which are the basic units for the construction of DNA and RNA macromolecules, composed of a sugar, a phosphate group and a nitrogenous base. The connection between nucleotides occurs by covalent binding between the sugar and the phosphate of two consecutive nucleotides, which creates a backbone that provides structure for the rest of the molecule. The slight differences between DNA and RNA affect both structure and function. DNA is double stranded and forms a double helix, while RNA is made up of a single strand. The nitrogenous bases that are present in DNA are adenine, thymine, guanine and cytosine however in RNA thymine is replaced by uracil [1]. Also, the sugar differs between the nucleic acids, being the ribose present in RNA molecules, while DNA contains the deoxyribose.

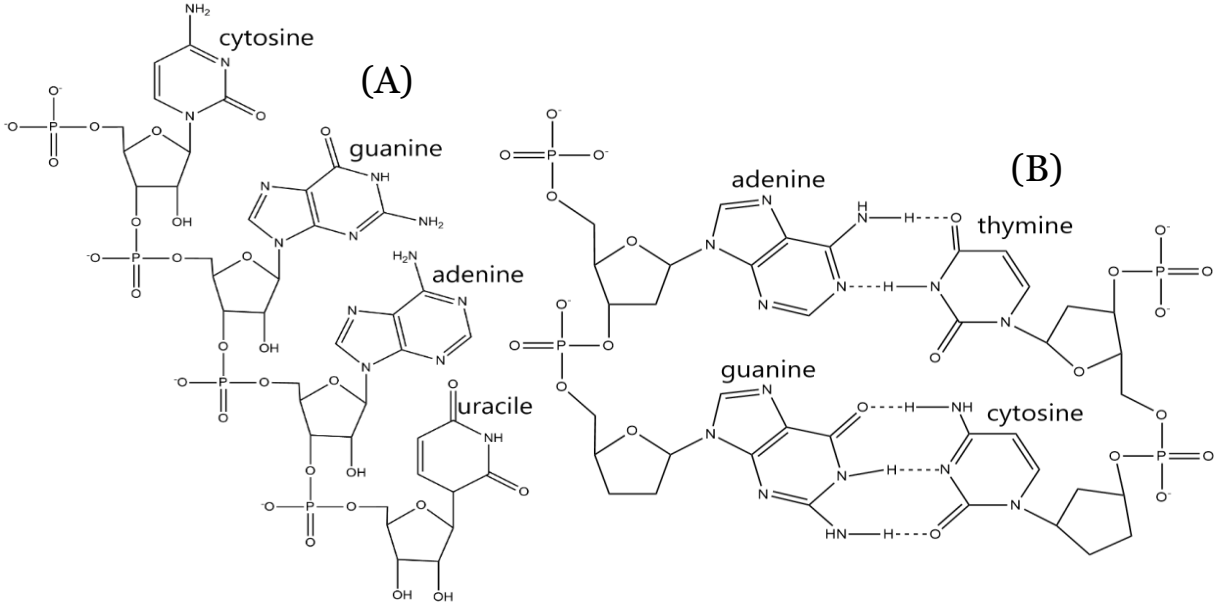


Figure 8 – Representation of RNA(A) and DNA(B) chemical structure.

1.2 Ribonucleic acid

When RNA was first discovered it was thought that it was an exclusive intermediary to protein production [2], however, more recently, various new functions have been attributed to RNA. In general, we can distinguish between two types of RNA, the coding and non-coding RNAs. Messenger RNA (mRNA), which is a coding RNA, is responsible for the transportation of the genetic information from the nucleus to ribosomes so that proteins can be produced. The rest are included in the group of non-coding RNAs, such as the transfer RNA (tRNA) which is a facilitator in the production of proteins by transporting the necessary amino acids to the ribosome, the ribosomal RNA (rRNA), forming the basic structure of the ribosome and catalyzing protein synthesis [1, 3], and small interfering RNA (siRNA) and microRNA (miRNA) which have a more regulatory role.

RNA has an extra hydroxyl group in the ribose that is associated to a higher susceptibility of RNA to hydrolysis [4, 5], and thus, maintaining the structural integrity of RNA might be more complex when compared to DNA. RNA has tendency to form secondary structures which facilitate RNA interactions, by covering some sequences or exposing others for recognition by other molecules. This recognition phenomenon can also be enhanced by the tertiary structures that RNA can adopt, which allow for specific sequences to be more accessible for interaction and create internal environments for metal ion binding sites [5]. The most common secondary structure assumed by RNA is hairpin (figure 2) which consists of an intramolecular double stranded RNA (dsRNA) chain with unpaired sequences at one end and a terminal loop. This RNA structure can be associated with many functions depending on the RNA where it occurs. Some important roles include protecting mRNA from degradation, promoting enzymatic reactions by providing a recognition motif for proteins to bind to RNA, guide RNA folding and determine interactions in a ribozyme [6].

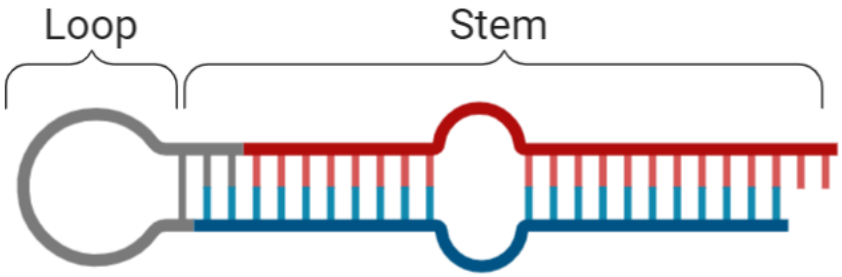


Figure 9 - Hairpin RNA structure.

1.2.1 RNAs with therapeutic value

In recent years, the RNA molecule has received increasing interest in its therapeutic capabilities. A large number of RNA-based therapeutics are being investigated and are in various stages of research [15]. The first RNA-based drug approved by the U.S. Food and Drug Administration (FDA) was Macugen, and since then many RNA-based drugs have entered clinical trials for various diseases, from genetic diseases to virus infection and malignant cancers, with very satisfying results [16]. There are various types of RNAs that can be used in a therapeutic way, such as, microRNA (miRNA), small interfering RNA (siRNA), antisense oligonucleotides (ASOs) and mRNA. Actually, due to the recent use of mRNA for vaccination, creating an opportunity for the control of COVID-19 pandemic, the study of innovative therapies based on nucleic acids has dramatically increased. mRNA-based therapies bring forth various advantages, comparatively to DNA, such as the location where the molecules exert their functions, since mRNA is translated after reaching the cytoplasm whereas, the DNA molecule needs to enter the nucleus. This facilitates transport and avoids the risk of insertional mutagenesis. Moreover, after a few days the RNA molecule is metabolically degraded, avoiding accumulation and minimizing toxicity [17]. Therefore, mRNA presents itself as a safer alternative to gene therapy comparatively to DNA and also has a transfection process which is considerably easier and more efficient [18]. One of the most popular uses of mRNA vaccines is the immunization of cancerous patients with autologous dendritic cells loaded with mRNAs encoding tumor-associated antigens, since dendritic cells play a main role in the immune system to initiate the adaptive immune response [16]. siRNAs are agents of RNA interference which are a class of double stranded RNAs that can serve as mRNA inhibitors, to silence gene expression [19, 20]. Thus, siRNAs has the potential to help treating numerous types of diseases, namely, genetic diseases, viral infections and cancer. For example, the application of siRNA to selectively degrade a mutate allele which encoded for the SOD1 gene has been achieved, therefore demonstrating the potential therapeutic value of siRNA when treating amyotrophic lateral sclerosis (ALS) [21]. Antisense oligonucleotides inhibit gene expression by modifying mRNA splicing, hindering mRNA translation, and targeting mRNA for degradation. While most antisense therapeutics target mRNA, one clinical drug was developed to target an endogenous miRNA, considering that the dysregulation of endogenous miRNAs has been linked to various diseases [19]. Thus, antisense oligonucleotides might prove to have much more therapeutic potential than initially expected. Research on the various RNAs with therapeutic potential has never been more dominant, and considering these RNAs have different mechanisms of action and

different structures, finding stabilizers that will help maintain RNA structure and consequently their function has never been more important.

1.2.1.1 MicroRNA

MicroRNAs (miRNAs) are small non-coding RNA (sncRNA) molecules which have regulatory functions and can affect mRNA expression [7]. When miRNA is first transcribed, the pri-miRNA is formed which typically has a poly(A) tail at the 3' end. Then, to have a functional miRNA, several post-transcriptional steps are required. First, pri-miRNA is processed by Drosha enzyme into a double-stranded hairpin structure which is called precursor miRNA (pre-miRNA). Pre-miRNA then needs to be exported from the nucleus to the cytoplasm by Exportin-5 and Ran proteins. In the cytoplasm, pre-miRNA, is then cleaved by the Dicer RNaseIII enzyme into double-stranded RNA (dsRNA). For the recognition of its target mRNA one of the strands must degraded and the other forms the miRISC complex which will regulate mRNA expression (figure 3)[10, 11]. Complementarity of sequences will determine what happens to the target mRNA, as the perfect complementarity of mRNA-miRNA will lead to the cleavage of the mRNA strand by proteins of the Argonaut family, while partial complementarity, with central mismatches, will promote the repression of mRNA translation [2].

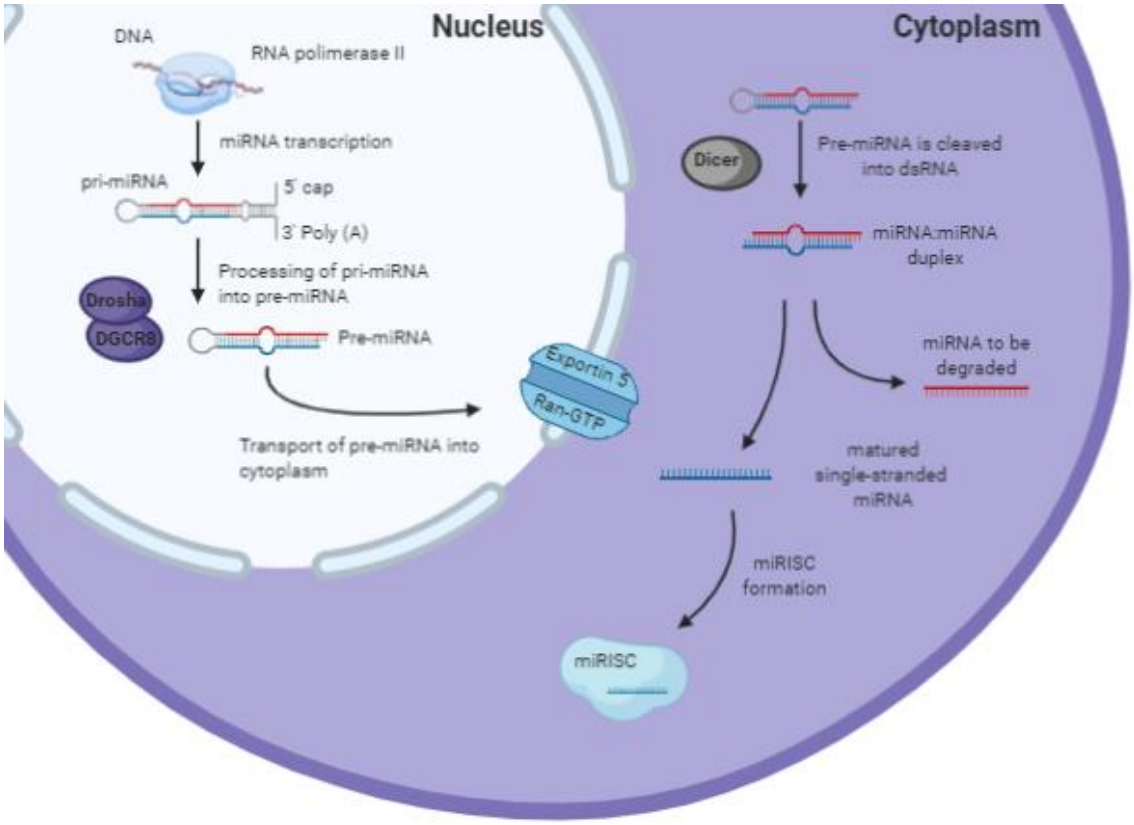


Figure 10 – Schematic representation of miRNA biogenesis.

MicroRNA has been widely studied has a potential biomarker. Recent studies have found that the deregulation of miRNAs can help diagnose patients or even determine patient survival chances [2, 10]. A study was conducted where different tissue samples of pancreatic cancer, benign pancreatic tissue, chronic pancreatitis and normal pancreas were tested for abnormal miRNA presence. The microarray noted that there was an up-regulation of 30 different miRNAs in pancreatic cancers when compared with normal pancreatic tissue. There were also differences between the tissues, with different levels of miRNAs, found in pancreatic cancer and chronic pancreatitis, where 15 of those 30 miRNAs were overexpressed and 8 were under expressed in cancers. Thus, searching for the differential expression of miRNAs can help determine which of the diseases is affecting the patient [10]. Another study described that some miRNAs, like miR-10a, can induce rather than inhibit protein expression [2, 13]. If the miRNAs in question induce the expression of a protein which helps the cell fight the disease, miRNAs may have an unexplored use as a therapeutic product. Ongoing clinical trials aim to study the effect of miRNA on target cells and the organism as a whole. MiRNAs have also shown potential in altering drug sensitivity which would overcome drug resistance, one of the most common issues in clinical medicine. Clinical trials have been ongoing in various areas of medicine such as treatment of, Hepatitis C virus infection, lung cancer, T-cell lymphoma, heart failure, among others [12]. Little is known regarding miRNA stability, however a study has shown that some miRNAs are unstable in the cell, what may be dependent on the presence of specific sequences. Other miRNAs may also have intrinsic elements that modulate their stability, but this is an unexplored field and more research is required on the subject to fully understand it [13]. Pre-miRNA which precedes miRNA is double stranded and therefore might have improved stability and easier handling. In recent times pre-miRNA has been gaining attention has a potential biomarker and therapeutic product [14].

1.2.2 RNA production

RNA Manufacturing for pharmacological use has to meet various standards for quality, stability and bioactivity in order to be approved by authorities such as the Food and Drug Administration (FDA) and European Medicines Agency (EMA). Three different methods for preparation of RNA are currently used, chemical and enzymatic synthesis, and recombinant production (figure 4). Chemical synthesis is the most commonly used method and it is based on phosphoramidite chemistry which has already been automated for oligonucleotide production. Chemical synthesis of RNA requires additional steps when compared to DNA synthesis, namely the protection of the 2'-hydroxy group using t-butyldimethylsilyl (TBDMS) or tri-iso-propylsilyloxymethyl

(TOM), which can be removed afterwards using a fluoride treatment. The success of the synthesis depends on the sequential deprotection-coupling-oxidation reactions. When the synthesis is completed, most target RNAs are purified by high performance liquid chromatography (HPLC) in order to meet industry standards. The chemical synthesis method allows the inclusion of modifications in order to improve RNA stability or to give some fluorescence properties. However, these alterations might lead to changes in structure, stability, biological activity and safety, and this must be evaluated. Production of lengthier RNA sequences may prove to be an issue, with higher costs, making RNA chemical synthesis a better option for sRNA agents and lower quantities [22].

Enzymatic synthesis or *in vitro* transcription is a biochemical approach to produce single stranded RNA. It is based on the capability of RNA polymerase (RNAP) to construct specific RNA molecules using DNA sequences as a template, under a particular promoter. The DNA template is required and the efficiency is dependent on the appropriate promoter and fidelity of purified RNAP to transcribe the template *in vitro*. The most common templates are linearized plasmids which contain the sequence of interest, PCR products or synthetic oligonucleotides. This method can be applied to produce RNA molecules of various lengths and can also be used for other purposes such as, detection assays, structural and functionality studies. Even though many kits are now readily available and well established, some RNA products display heterogeneity at 3' and 5' ends, and the reliability of t7 RNA polymerase decreases as the transcript length increases [16, 17].

Recombinant production has seen a growing interest in the last years. This novel method involves a fermentation-based strategy in order to cost-effectively produce RNA agents on a much larger scale. Since the resulting RNAs are produced by the cell all the necessary posttranscriptional modifications which are critical for secondary structure, folding and stability, should be assured. The general process is based on the use of a recombinant plasmid encoding the target RNA construct with expression signals, which is introduced into the cell. Host transcription mechanisms will be responsible for the production of the desired RNA, which depending on host will either accumulate in the cytosol or be secreted to the growth medium. After the culture comes to an end the cells or medium are recovered by centrifugation, lysed in case of the cells, and the RNA is then purified by chromatographic techniques. The process seems to be simple, but two major issues must be addressed, the RNA susceptibility to nucleases and its heterogeneity [16, 18].

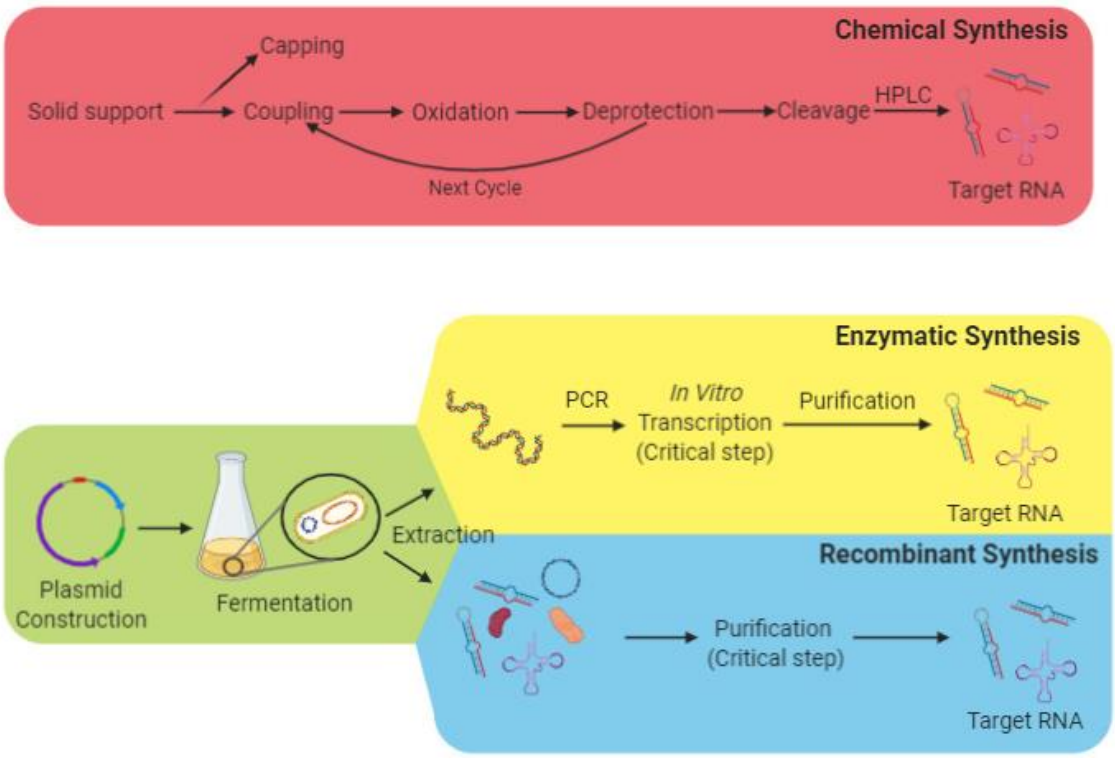


Figure 11 - Schematic representation of the RNA synthesis methods: Chemical, Enzymatic and Recombinat production.

1.3 Importance of stability and bioactivity

Pharmaceuticals are required to have a long shelf life to maintain their stability and bioactivity from the production until it gets to the end-user. Many excipients are used in the formulation of common chemical pharmaceuticals in order to improve stability. However, the stability of biologics is a far more unknown field. In biologics, it can be more challenging to maintain their stability since instead of relatively stable and simple chemicals we have proteins or nucleic acids with a complex secondary and tertiary structure, which directly influences their activity. Moreover, biologics or biopharmaceuticals are usually larger than chemicals and more susceptible to enzymatic cleavage. Proteins are structurally unstable in solution and conformational changes may happen due to induced stress during purification, processing and storage [25] and the same can happen to nucleic acids. If this structure is compromised, most likely that the function is affected, and most biomolecules cannot recover from this destabilization. So, the biopharmaceuticals stability must be guaranteed during the preparation, storage, and delivery. Formulation of these biopharmaceuticals with other substances may help to overcome these issues. When it comes to storage, pharmaceuticals can be kept in solution, however, as this type of molecules has a tendency be to destabilized in solution,

freeze drying has been an effective alternative to improve long term stability. In order to maintain the biomolecules structure and subsequent function, the use of cryoprotectants and other molecules that promote stabilization of the biopharmaceutical has been very common during the process of freeze-drying [26]. Taking into account the expansion of biopharmaceuticals market and the industrial and biomedical interests, new methods and excipients have to be established to ensure biologics stability and respond to this challenge with higher efficacy.

1.3.1 Chemical and physical mechanisms of instability

When developing biopharmaceuticals for therapeutic purposes the mechanisms of degradation of the biomolecule must be taken into account, otherwise biomolecule function might be lost. Macromolecule degradation is associated with instability phenomena that can be divided into chemical mechanisms and physical mechanisms [27].

Chemical degradation in proteins can happen due to alteration in the amino acids which compose the biomolecule, for example disulphide bond interchange can occur at cysteine-cysteine links which might lead to aggregation, but this can be avoided by the addition of surfactants. In nucleic acids depurination and β-elimination can lead to cleavage of phosphodiester backbone and therefore induce the destabilization of the nucleic acid structure. To have a better understanding of what is happening to the biomolecule, a range of complimentary tests should be done to ensure full understanding of the degradation process, namely, gel electrophoresis, size exclusion chromatography (SEC) and high pressure liquid chromatography (HPLC) [27].

Physical degradation of proteins is mainly related with alterations in their secondary, tertiary or quaternary structure, for example aggregation, precipitation, adsorption and denaturation. Chemical alterations might lead to aggregation and in its turn aggregation might lead to precipitation. SEC-HPLC is a technique commonly used to determine soluble covalent protein aggregates. Denaturation can be caused by heating, freezing, pH and shear. In order to determine if the molecule has been denatured or not, Circular Dichroism can be used to follow alterations in the secondary or tertiary structure of the molecule [27]. Overall, understanding why the biomolecules have lost their stability will help determine what needs to be included in the formulation to avoid these mechanisms of instability and extend the biomolecules shelf life.

1.4 Formulation

A therapeutic drug is not only composed of the active pharmaceutical ingredient (API) but also of other compounds which will help overcome certain shortcomings, such as poor solubility, insufficient bioavailability, limited chemical and physical stability, or reduced rate of drug delivery [28]. Both pharmaceuticals and biopharmaceuticals require a formulation to address these issues. For example chemicals are usually smaller molecules, so while they also have issues when it comes to structure and delivery, these are easier to overcome when compared to biopharmaceuticals. In order to bypass these issues, excipients must be added to the API. Recent studies have focused in overcoming these problems by using a range of different molecules which have a stabilizing effect on biomolecules [29].

Usually RNA formulations require the involvement of delivery systems to encapsulate the molecule of interest, facilitating the administration, delivery to the target but also protecting RNA from nucleases [29]. However, the administration of nanoparticles still faces some challenges since these nanoparticles tend to be routed to the liver, which could lead to the metabolization of the biopharmaceutical. Thus, directing the molecule to target cells or tissues is another challenge faced in formulation. Formulation of therapeutic drugs plays an essential role in the efficiency of the API, therefore it must be carefully thought out in order to obtain the best possible results.

1.4.1 Excipients

Excipients are inert ingredients that may improve solubility, stability, avoid adsorption, minimize precipitation, add antioxidant effect, buffering and control the delivery of the API [28]. To select which excipients should be added to a formulation many factors must be taken into account, like compatibility, function, and cost [30]. Excipients can have various roles as previously mentioned and to reach those goals different categories of excipients must be used like salts, sugars, polyols, amino acids, polymers and surfactants (table 1) [28].

Table 1 - Excipient type and function [27].

Excipient categories	Examples	Main function
Amino acids	Glycine, arginine, cysteine, methionine, alanine	Reduce aggregation; Increase thermal stability;
Sugars	Glucose, sucrose, trehalose, lactose, fructose	Protect macromolecules from denaturation; Cryoprotectants;
Salts	Sodium phosphate, sodium acetate, potassium phosphate	Increase thermal stability; Increase solubility
Surfactants	Poloxamer 407, polysorbate 80, polysorbate 20	Prevent adsorption; Reduce aggregation induced by agitation;
Polyols	Glycerol, xylitol, sorbitol, mannitol	Stabilize proteins through selective solvation of the protein; Prevent aggregation;
Polymers	Polyethylene glycol (PEG), dextran, polyvinylpyrrolidone	Cryostabilizer; Inhibit antibody aggregation;

1.4.1.1 Amino acids

Amino acids have plenty of biotechnological applications one of which is their use as excipients in pharmaceutical drugs to improve water-solubility, also facilitating the delivery to cells, with the advantage of presenting low toxicity. All the aforementioned characteristics make them great candidates to improve drug solubility and delivery [31]. Some amino acids like aspartic and glutamic acid have been used to reduce aggregation, while glycine, lysine and arginine have been reported to prevent aggregation altogether [27]. An example is the reduction in 50% of heat induced aggregation of acidic fibroblast growth factor (aFGF) using 1.6 mM of histidine and 180 mM of glycine [32]. A recent study using DNA hairpin to investigate the effect of amino acids on the secondary structure of nucleic acids has shown that positively charged amino acids, such as arginine, at a concentration of 100 mM can improve the DNA secondary structure stability [33]. In addition, in a different study, it was reported that amino acids might

reduce chemical degradation of proteins. Methionine is mainly used due to its effective antioxidant feature, but in particular situations, histidine also showed the ability to protect papain from oxidation-induced inactivation [32]. Taking into account the stabilizing capabilities of amino acids, they are considered perfect candidates to help stabilize the RNA molecule.

1.4.1.2 Sugars

Extensive studies have been done with sugars as excipients, due to their benefits in cryopreservation and freeze-drying of pharmaceuticals [34]. The process of lyophilization is associated with freezing, dehydration and solid-liquid interfacial stresses. Studies have shown that sugars can help protect proteins, and particularly their structure, when exposed to these stress conditions. In the drying process, the stabilization of the proteins depends on the sugar used, as the interactions it creates with the protein will help to reduce the local and global mobility. The drying process can involve an increase in temperature, and as it is known, degradation usually increases with the increase of temperature. Sugars can help stabilize against thermal stress by forming a matrix, which immobilizes the protein, reducing the mobility and interaction, therefore maintaining the protein structure intact[35]. A study showed that by using sucrose, trehalose, glucose and fructose at concentrations of 10 and 20 %, the melting temperature (T_m) of myoglobin would increase by around 4-5 ºC [36]. In addition, chemical degradation is reduced by sugars through a decrease in solubility and reduction of diffusion of reactive species present [37]. In order to maximize protein stability in freeze-drying a lot of thought must go into the selection of the appropriate sugar, as not all present the same stabilizing capabilities, and also the conditions of drying must be taken into account [35].

Sugars such as glucose, trehalose and sucrose, all have promising results is this area, however when it comes to the stabilizing effect in samples which are not freeze-dried, very few studies have been done. The most common uses of these sugars in formulations are as flavor enhancers, freeze-drying agents, sweetening agents and as thickening agents [38], and thus further studies must be conducted to exploit the advantages of sugars on biomolecules stabilization and formulation.

1.4.1.3 Salts

A salt is a compound composed of an anion and a cation, that can be organic or inorganic. Salts have had extensive studies done on their effects regarding thermal stability of proteins, with positive results [39]. Some salts such as NaCl and KCl have shown to be able to stabilize RNase H2 and increase the proteins activity when heated using concentrations of 80 mM and 60 mM, respectively [40]. Also, a study using arginine salts described the improvement of refolding of the unfolded domain of IGg1 monoclonal antibody using 50mM of Arg_2SO_4 [41]. Another study has found that chaperonin GroEL in the presence of 100 mM of $(NH_4)_2SO_4$ and 10 mM of $MgCl_2$ has improved stabilization against thermal stress [42]. Moreover, salt concentrations of up to 0.3 M have shown to increase melting temperature (T_m) of DNA molecules, however higher concentrations lead to a decrease in T_m, which is due to the fact that at high concentrations anions can act as hydrophobic bond-breaking agents [43]. In general, salts have demonstrated potential as stabilizers of biomolecules, however at high concentrations the opposite effect tends to happen, therefore careful consideration of the salts concentration must be taken in order to obtain the best stabilizing effect.

1.5 Novel candidates for nucleic acids stabilization

1.5.1 Ionic liquids

Ionic liquids (ILs) are usually defined as compounds made up of ions, with melting points lower than 100°C [33, 34]. These compounds have been widely studied due to their unique characteristics, such as negligible vapor pressure, good thermal stability, tunable viscosity, and miscibility with water and organic solvents [46]. These characteristics have also led to the use of ILs in various areas of research like analytical chemistry, chromatography, extraction processes, and more recently in drug synthesis and drug delivery systems [33, 35]. Actually, ILs have been shown to be stable in various physicochemical conditions such as high temperature, irradiation, electrical current and oxidation agents [45]. Moreover, ILs are considered as designer solvents since they are usually composed by two asymmetrical ions, an inorganic or organic anion and an organic cation, and these can be combined to give the IL different properties and be exploited in different ways [20]. Due to this versatility, it is estimated that over a million different combinations of cations and anions can be made to form an IL [36, 37]. Four main types of interactions and mechanisms, including the hydrogen bonding, base stacking, conformational entropy and cation binding determine the structure of nucleic acids. Taking into account the versatility of ILs, these can be designed and selected to

interact with specific parts of the RNA molecule, to provide an even stronger stabilizing effect. Therefore, it is expected that ILs will affect the structure of nucleic acids. As an example, it can be described the use of amino acid-based ILs, namely, [Ch] [Lys], [Ch] [Arg], [Ch] [Glu] and [Ch] [Asp] which showed the ability to maintain RNA stability during extraction, leading to an improved T_m of around +15 °C after one hour in the presence of [Ch] [Glu] and [Ch] [Asp], and +10 °C after 15 days in the presence of the same ILs [49]. Another study described that DNA in choline dihydrogen phosphate (choline DHP) showed only a slight decrease in the intensity in the CD spectra after 6 months of storage at room temperature, suggesting a stabilization effect induced by the IL, in comparison to the control consisting on DNA in water [47]. Another advantageous characteristic of ILs is the biocompatibility, as various types of ILs, such as cholinium-based ILs and amino acid-based ILs, have shown to have this attribute [48], [49]. These results indicate the great potential of ILs as stability enhancers, being possibly seen as novel excipient candidates in the near future.

1.5.2 Deep Eutectic Solvents

Deep eutectic solvents (DES) are eutectic mixtures in which the melting point is much lower than that of its individual components. The first deep eutectic phenomenon was described when a mixture of choline chloride and urea, which have melting points of 302°C and 133°C, respectively, resulted in a mixture with a melting point as low as 12°C. This mixture showed to be biocompatible and the enzyme dissolved in it, retained its activity [47]. DES have different chemical properties from ILs but retain the same beneficial physical properties. DES have some advantages over ILs such as easy availability due to the relatively inexpensive components, and easy preparation, just consisting in the mixing of the two components and heating until DES formation. Since their discovery, DES have shown a huge versatility, being applied in fields such as metal processing, metal electrodeposition, ionothermal synthesis, biotransformations and manufacturing of biodiesel [39, 40]. In the biological field, some studies have demonstrated that G-quadruplexes aquire ultrastability in DES when compared with water, and given its characteristics, like no vapour pressure and high chemical and thermal stability, it is also a better alternative to water at high temperatures [47]. Another study found that natural deep eutectic solvents (NADES) could be used as an inert medium for enzyme protection from denaturation or inhibition of proteins. It was also verified that enzymatic activity of dhurrin biosynthetic enzymes in glucose:tartrate NADES would also be maintained at room temperature for more than 30 days as long as a concentration of 25 % was not exceeded [52].

DES have appeared as a new and attractive alternative to ILs as they can be less expensive, more accessible, nontoxic, and biodegradable while sharing the characteristics that provide a favorable environment for chemical reactions and biomolecules preservation [53].

1.6 Evaluation of RNA structure

RNA structure can be assessed either empirically or theoretically, depending on the pretended result. Theoretical structure studies like molecular dynamics, RNA puzzels, and docking simulations allow for a prediction of RNA 3D structure and interaction with other molecules [54]. Practical studies using Circular Dichroism (CD) and Fourier transform infrared (FTIR) allow for an evaluation over time, and testing of different conditions. In order to obtain the best possible results different techniques must be conjugated as it will contribute to have more information about what is happening with the biomolecule.

1.6.1 Circular Dichroism (CD)

CD spectroscopy is a valuable biophysical technique for studying the secondary structure of biomolecules such as proteins, DNA and RNA. CD analyzes circularly polarized light and detects the differential absorption from the right and left, giving an ellipticity value which usually lies in a range of 10 mdeg [55]. Different parameters must be used for different biomolecules in order to obtain meaningful results. In regard to RNA the CD spectrum must be measured between 200 and 320 nm, since the characteristic RNA spectrum has a negative band around 215 nm and a positive band around 265 nm (figure 5). Any alteration to the typical spectrum may indicate conformational changes, therefore analysis must be made through comparison with the control RNA spectrum [56]. CD spectroscopy not only allows the analysis of the secondary structures of biomolecules but also enables the follow-up of the conformational changes that can occur by the interaction of the target proteins or nucleic acids with ligands or different agents in solution. CD has become an important asset for quick and cheap analysis of the secondary structure of biomolecules.

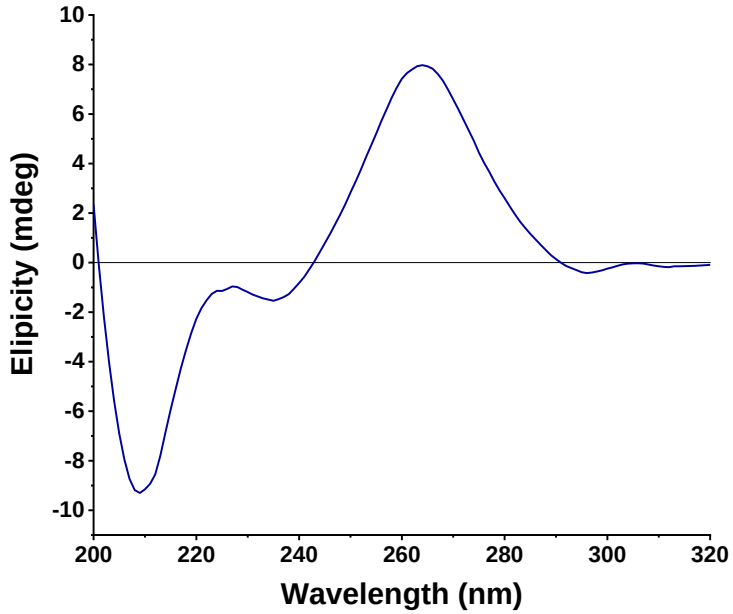


Figure 12 – Typical representation of a CD spectrum of RNA

CD spectroscopy also allows for temperature variation studies. Thermal stability is assessed in these studies by following the changes to the CD spectrum as the temperature rises. This method is commonly used to determine if additives like amino acids, salts or sugars alter the thermal stability of the molecule in study [57]. The resulting graph can be analyzed to determine the T_m of the molecule (figure 6). An increase in T_m indicates improved thermal stability [58]. Therefore, it is another indicator to determine molecule stability and along with the spectrum allows for a better understanding of how the molecule of interest is interacting with the other components in the sample.

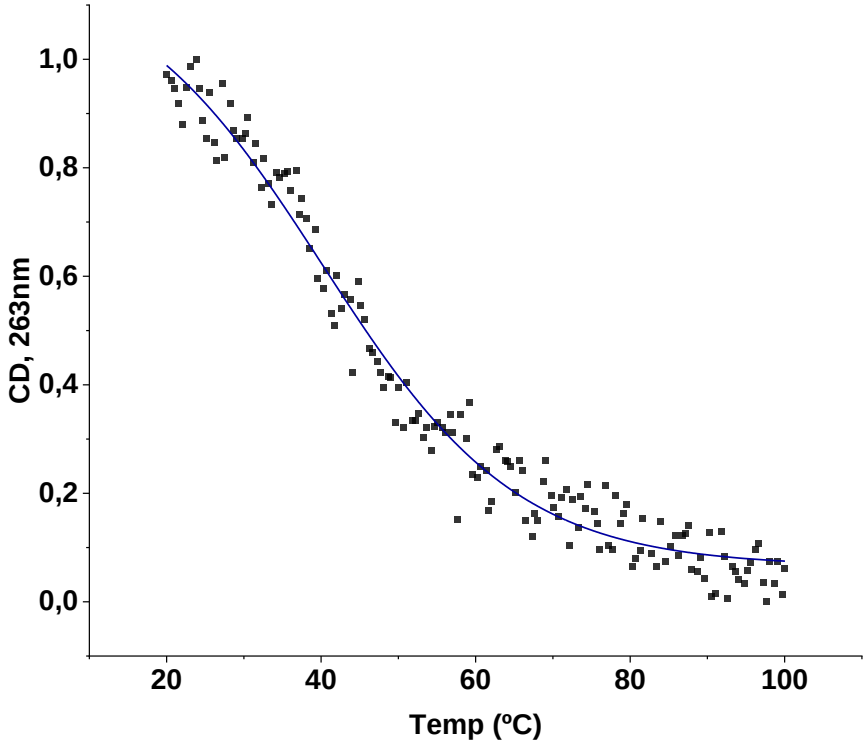


Figure 13 – Representation of a typical melting curve for RNA

1.6.2 Molecular docking

Molecular docking is a computational method that predicts the most favorable orientation of the binding of two molecules in order to form a stable complex. It can also help on the identification of the interactions between these two molecules and the area where these occur. This method allows for the prediction of binding affinity or strength of association between compounds. Molecular docking is commonly used to predict the binding orientation of drug candidates to the molecule of interest, allowing for an initial screening of candidates without any waste of laboratory resources. Docking also helps to understand what types of bonds are being created between two molecules that have already been determined to interact [48, 49]. Docking is a tool with versatility of application, as it can be used to screen potential candidates to interact with a particular molecule and can be used to study well known interactions to further improve our knowledge about the systems. In the case of this work molecular docking will help understanding the types of interactions predicted between the stabilizer and the RNA molecule, allowing for a better understanding of why the stability has improved or decreased and which excipients have better overall stabilizer potential.

Chapter 2

2. Aim of the work

RNA molecule has great therapeutic potential, however the structure of the molecule is highly susceptible to foreign factors that will lead to its denaturation or degradation. Therefore RNA structure must be maintained in order for the molecule to exert its function. With the objective of extending the long-term stability of RNA the effect of stabilizing compounds on the RNA structure must be analyzed.

The main objective of this work is to find new stabilizing candidates that will prolong the shelf life of RNA at more sustainable temperatures for future RNA formulations. The results of this work will lead to a better understanding of the RNA molecules behavior in aqueous solution, allowing the establishment of innovative and effective solutions for RNA stabilization.

Chapter 3

2. Materials and Methods

2.1 Materials

For *Escherichia coli* (*E. coli*) DH5α growth in a solid medium it was used the Luria-Bertani (LB) agar (Pronalab, Mérida, México), while growth in liquid medium was carried out using yeast extract and tryptone (Bioakar, Beauvais, France), glycerol (Himedia, Einhausen, Germany), potassium hydrogen phosphate (K_2HPO_4) (Panreac, Barcelona, Spain) and potassium dihydrogen phosphate (KH_2PO_4) (Sigma-Aldrich, St. Louis, Missouri, USA). Both solid and liquid culture media were supplemented with kanamycin antibiotic (Thermo Fischer Scientific Inc., Waltham, USA).

The reagents used for the extraction of RNA were guanidine tiocyanate, N-Lauroylsarcosine sodium salt, sodium citrate and isoamyl alcohol, from Sigma-Aldrich (St. Louis, Missouri, USA), isopropanol from Thermo Fischer Scientific Inc. (Waltham, USA), β-mercaptoethanol from Merck (Whitehouse Station, USA), and phenol and NaCl from VWR (Randor, Pennsylvania, USA). All solutions used in the extraction of RNA were previously prepared with 0.05% water treated with diethyl pyrocarbonate (DEPC) (Fluka, Sigma-Aldrich, St. Louis, Missouri, USA).

Polymerase chain reaction (PCR) reagents included DreamTaq DNA Polymerase and DreamTaq Green Buffer from Thermo Fisher Scientific Inc. (Waltham, USA), deoxyribonucleotides triphosphate (dNTPs) from NZYTech Genes and Enzymes (Lisbon, Portugal) and Forward (Fw) and Reverse (Rv) primers from STABvida (Lisbon, Portugal). The primers sequences are presented in Table 1. After the amplification of the PCR products, its purification was carried out with NucleoSpin Gel and PCR Clear-up Kit (Machery-Nagel, Düren, Germany).

Table 2 - Pre-miR-9 primers sequences used in the PCR reaction.

miRNA	Primers sequences	
pre-miR-9	Fw	5'-GTT TTT TTT AAT ACG ACT CAC TAT AGG CGG GGT TGG TTG TTA TCT TTG G-3'
	Rv	5'-TGG GGT TAT TTT TAC TTT CGG TTA TC -3'

In vitro transcriptions were performed with MEGAscript T7 Kit, and the transcripts were purified using MEGAclear T7 Kit from Thermo Fisher Scientific Inc. (Waltham, USA). To confirm the integrity and purity of the RNA samples, 1% agarose gel electrophoresis was used with GRS Agarose LE and Green-Safe (Grisp, Porto, Portugal).

For the RNA stability studies, the stabilizer candidates such as, glucose, sucrose and trehalose were acquired from Thermo Fisher Scientific Inc. (Waltham, USA). The ILs were synthesized and provided by project partners from the Chemistry Department of Universidade Nova de Lisboa and ChCl was provided by project partners from CICECO of Universidade de Aveiro. All solutions were prepared using Milli-Q water from Millipore (Billerica, MA, USA). Circular dichroism assays were performed using a rectangular quartz cell with a 1 mm optical path (Hellma analysis, Germany).

2.2 Methods

2.2.1 *E. coli* DH5α growth for RNA production

Total RNA was obtained through a culture of *E. coli DH5α* previously transformed with a plasmid containing the sequence for human pre-miR-9 (pBHSR1-RM-pre-miR-9). First, *E. coli* was cultured in a plate with Luria-Bertani Agar (LB Agar) medium which was supplemented with 50 μg/mL of kanamycin and left to grow overnight at 37 ºC. Colonies from the solid medium were transferred into an Erlenmeyer of 500 mL with 125 mL Terrific Broth (TB) medium with the following composition: 24 g/L of yeast extract, 12 g/L of tryptone, 0.072 M of K_2HPO_4, 0.017 M of KH_2PO_4, $5.5x10^{-5}$ M of glycerol and supplemented with 50 μg/mL of kanamycin. The pre-fermentation medium was incubated at 37 ºC in an orbital shaker at 250 rotations per minute (rpm). Optical Density (OD) was frequently measured at 600 nm to monitor the growth of the culture, using a spectrophotometer from Pharmacia Biotech Ultraspec 3000 UV/Visible (Cambridge, England). Once the culture reached an OD_{600} of 2.6. The volume of pre-fermentation required to start the fermentation at an OD_{600} of 0.2 was calculated using the following equation:

$$\boldsymbol{V_{pre-fermentation}} = \frac{(\boldsymbol{V_{fermentation}} \times \boldsymbol{OD_{fermentation}})}{(\boldsymbol{OD_{pre-fermentation}} - \boldsymbol{OD_{fermentation}})}$$

The *E. coli* growth was thus maintained in TB medium, at 37 ºC and 250 rpm for approximately 8 hours, up to a final OD around 6, corresponding to the final stage of the exponential phase. The bacterial cells were recovered through centrifugation at 3900 g for 10 min at 4 ºC, and the final pellets of 100 mL were stored at -20 ºC, until use.

2.2.2 Low molecular weight RNA extraction

RNA extraction was performed using the method of acid guanidinium thiocyanate-phenol-chloroform. The *E. coli* pellets which had been previously stored at -20 ºC were thawed and resuspended using 20 mL of 0.8 % NaCl, followed by a centrifugation at 6000 g for 10 minutes at 4 ºC. The supernatant was discarded and the remaining pellet was resuspended in 5 mL of D Solution (4 M guanidinium thiocyanate, 0.5 % sodium N-lauroylsarcosinate, 0.025 M sodium citrate pH 7 and 0.1 M β-mercaptoethanol) and incubated in ice for 10 min. Afterward, 0.5 mL of sodium acetate pH 4 was added, and the suspension carefully homogenized, followed by the addition of 5 mL of phenol and repeating the careful homogenization. A mix of chloroform/isoamyl alcohol (49:1) was prepared and 1 mL was added to the previous suspension and

vigorously mixed. The resulting mixture was then incubated in ice for 15 minutes. After the incubation period the mixture was centrifuged at 10000 g for 20 minutes at 4 ºC. Two aqueous phases are formed, where the upper phase is enriched in RNA and the bottom phase is enriched in DNA. Therefore, the upper phase must be carefully separated from the bottom phase and transferred onto new tubes, to avoid DNA contamination. 5 mL of isopropanol were added to the new tubes to precipitate the RNA and a centrifugation at 10000 g, for 20 minutes, at 4 ºC was carried out. The resulting supernatant was discarded and the pellet was resuspended in 1.5 mL of D solution, followed by the addition of 1.5 mL of isopropanol and proceeding to a new centrifugation at 10000 g, for 10 minutes, at 4 ºC. Supernatant was discarded, and the resultant pellets were resuspended in 2.5 mL of 75 % ethanol in DEPC water, followed by an incubation at room temperature, for 10-15 minutes. The mixture was centrifuged at 10000 g, for 5 minutes, at 4 ºC, and the resulting pellet was dried for 5-10 minutes at room temperature. Finally, the RNA pellets were dissolved in 1 mL of DEPC water and incubated at room temperature for 10-15 minutes. RNA concentration was measured using a Nano Photometer (IMPLEN, United Kingdom) and the integrity of the samples was verified by agarose gel electrophoresis. RNA samples were then stored at -80 ºC.

2.2.3 Plasmid DNA extraction from *E. coli DH5α*

When using RNA prepared by *in vitro* transcription, it was necessary to isolate plasmid DNA (pDNA) from *E. coli*. Thus, the bacterial cells were lysed using NZYMiniprep kit, according to the manufacturer's instructions. Briefly, the bacterial pellet was resuspended in 25 mL of 0.8 % NaCl, followed by the preparation of 2 mL fractions for centrifugation at 12300 g, for 1 min, at room temperature. After discarding the supernatant the pellet was resuspended in 250 µL of A1 buffer and vortexed. The process of lysis was started by adding 250 µL of A2 buffer and homogenizing. For the neutralization step, 3 min later, 300 µL of A3 buffer were added and then centrifuged for 9 min, at 12300 g, at room temperature. The supernatant was recovered to the NZYTech column and centrifuged for 1 min, at 11000 g, at RT. This step boosts DNA binding to the column, which is posteriorly washed by adding 700 µL of A4 buffer. The column was centrifuged for 1 min, at 11000 g, at RT. The pDNA was eluted by the addition of 50 µL of AE buffer, and recovered by centrifugation at 12300 g, for 1 min, at room temperature. The final sample of pDNA was quantified using a NanoPhotometer (IMPLEN, United Kingdom).

2.2.4 Agarose gel electrophoresis

Analysis of the extracted nucleic acids was performed by electrophoresis in 1 % agarose gel. In order to visualize the nucleic acids, the gel was prepared using 0.012 µL/mL of Green Safe (Porto, Portugal). The electrophoresis was performed at 120 V for 30 minutes in TAE buffer (40 mM Tris base, 20 mM acetic acid 1 mM EDTA, pH 8). The gel was revealed using ultraviolet (UV) light exposure in the Uvitec Cambridge Fire-Reader equipped with a UV chamber (UVITEC Cambridge, Cambridge, United Kingdom).

2.2.5 Conventional polymerase chain reaction

The pre-miRNAs sequences encoded in the pBHSR1-RM plasmid were amplified by polymerase chain reaction (PCR) in a T100 Thermal Cycler (BioRad). The program used for the amplification is described in table 3. In order to verify integrity and purity of the PCR products, each sample was analyzed by 1 % agarose gel electrophoresis.

Table 3 - Experimental conditions used in the PCR for pre-miRNA-encoding sequences amplification.

General PCR program			
Step	Temperature (°C)	Time	Cycle
Initiation of the reaction	95 °C	5 min	1x
Denaturation of DNA	95 °C	30 s	24 x
Hybridization of the primers to DNA	66 °C	30 s	
Extension	72 °C	30 s	
Final Elongation	72 °C	5 min	1 x
	12 °C	∞	

2.2.6 Purification of the PCR products

PCR products were purified using the "NucleoSpin® Gel and PCR Clear-up" kit (Machery-Nagel, Düren, Germany). All reactions were transferred to a tube of 1.5 mL and 20 µL of NTI buffer was added for each 10 µL of PCR products and homogenized. The mixture was then transferred to the purification column. The column was centrifuged for 30 s, at 11000 g, at RT. Then, 700 µL of N3 buffer were added and centrifuged for 1 min, at 11000 g, at RT until no buffer remains in the column. To recover the DNA, 30 µL of NE buffer were added to the column and another centrifugation was done with the same conditions. DNA was quantified using a NanoPhotometer and the integrity and purity was analyzed by 1 % agarose gel electrophoresis.

2.2.7 *In vitro* transcription

In vitro transcription was performed to obtain the pre-miR-9 and was achieved by using MEGAscript T7 Kit. The reaction mix was composed by: 2 µL of 10X T7 Reaction Buffer, 2 µL of each rNTP, 0.4 µg of purified PCR products previously prepared, 2 µL of T7 Enzyme Mix and the remaining volume was completed with nuclease-free water for a total of 20 µL. The reaction mix was incubated for 16 h at 37 ºC. Afterwards, 2 µL of DNase I was added to the mix and incubated for 15 min. Lastly, 2 µL of Stop solution (EDTA 0.5 M, pH 8) was added and the mixture incubated for another 15 min. In the end, samples were quantified using a NanoPhotometer, analyzed using a 1 % agarose gel electrophoresis to verify integrity and purity and finally stored at 80 ºC, until further use.

2.2.8 Purification and concentration of the RNA transcripts

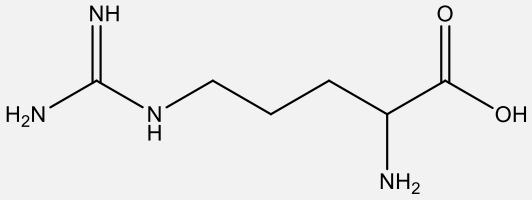
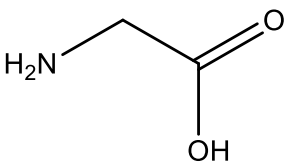
Samples obtained by *in vitro* transcription were purified using the MEGAclear T7 Kit, according to manufacturer's instructions. First, 80 µL of elution solution were added to 20 µL of RNA and homogenized. The mixture was then transferred to a new 1.5 mL tube and 350 µL of binding solution concentrate were added and homogenized. Afterwards, 205 µL of 100 % ethanol were added, the mixture was transferred to the purification column and centrifuged for 1 min, at 12300 g, at room temperature. The column was washed using 500 µL of wash solution and, centrifuged in the same conditions. Another centrifugation was performed to guaranty that there was no remaining buffer in the column. In order to recover the RNA, 50 µL of elution solution was added to the column and incubated at 70 ºC for 10 min in a heating block. A centrifugation was performed and the incubating step was repeated after adding another 50 µL of elution solution. After the purification, a concentration step was performed by

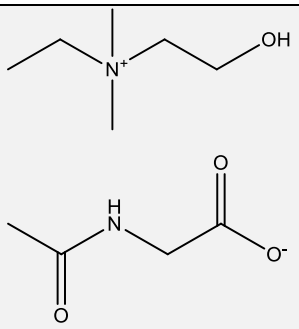
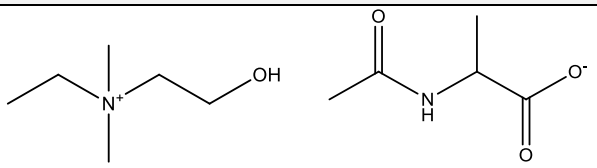
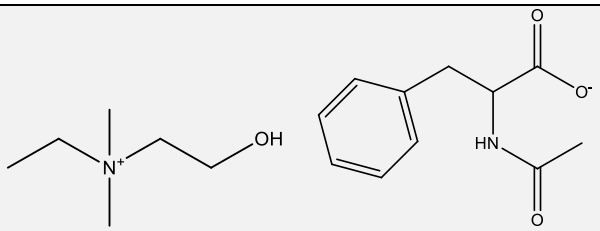
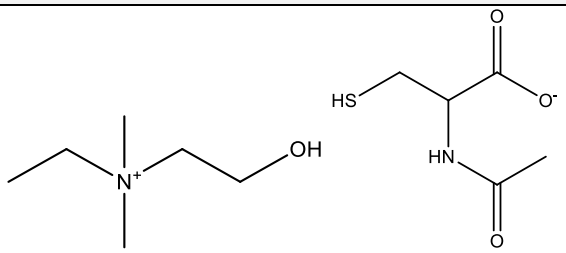
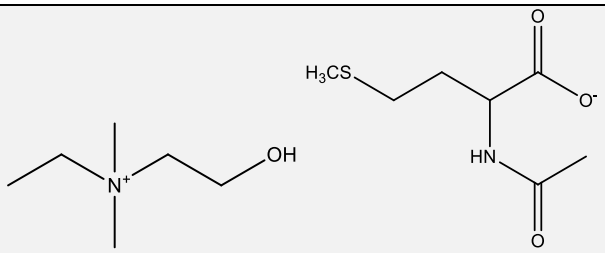
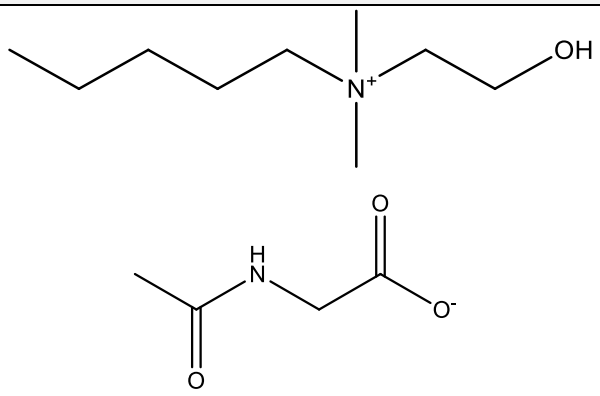
adding 10 µL of 5 M ammonium acetate, and 275 µL of 100 % ethanol and, incubating for 30 min at -20 ºC. Afterwards a centrifugation at 4 ºC, 15000, g for 15 min was carried out. Another centrifugation was done after adding 500 µL of cold 70 % ethanol. The resulting pellet was dried for 10 min at RT and resuspended in 30 µL of DEPC water. RNA concentrations were determined using a NanoPhotometer and integrity and purity were analyzed by 1 % agarose gel electrophoresis. The samples were then stored at -80 ºC.

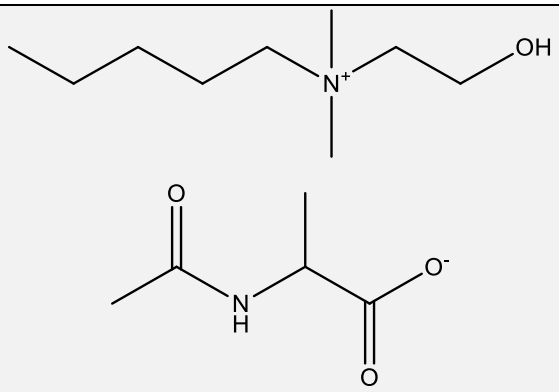
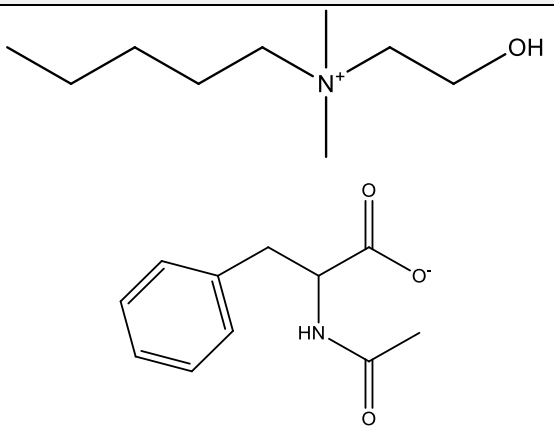
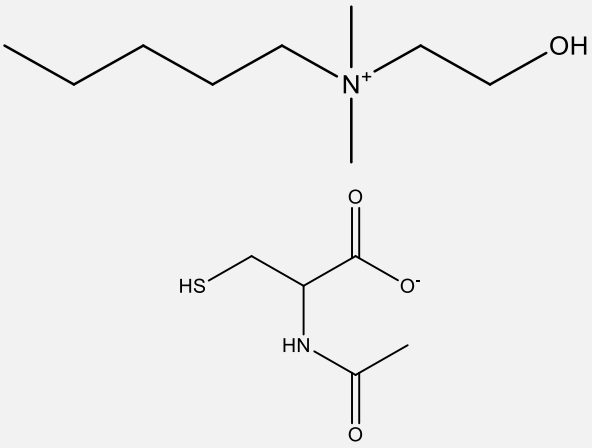
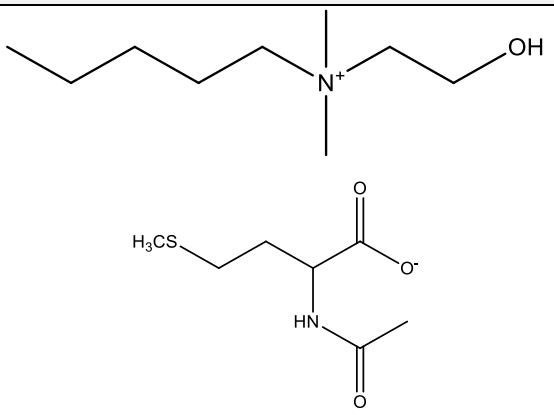
2.2.9 RNA samples preparation and conditions

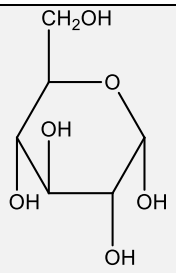
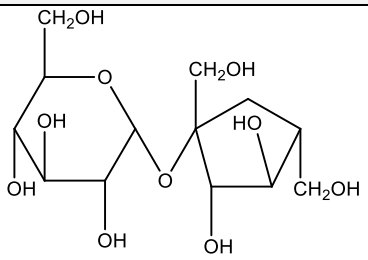
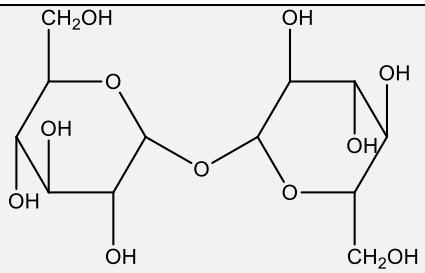
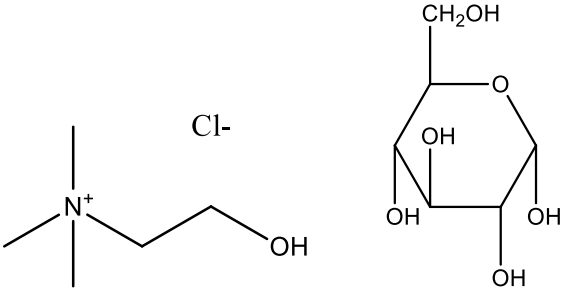
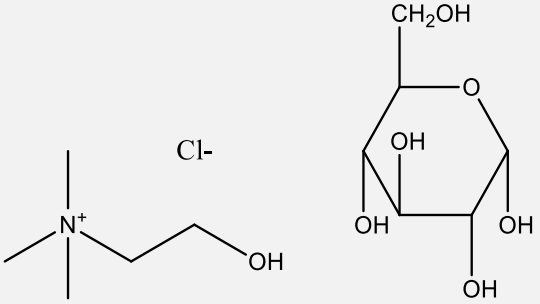
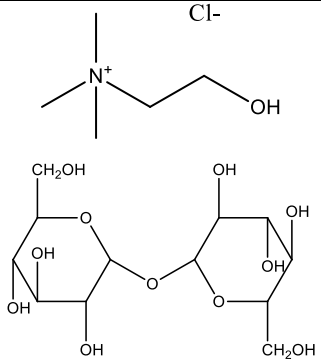
The samples were composed of RNA and the stabilizer agent solution under study. Initially, extracted RNA sample concentration was determined using Nanodrop, calculations were made in order to determine the volume needed of RNA for a concentration of 150 µg/mL in a volume of 1.5 mL, the remaining volume of the sample was then made up by the excipient solution up to the final concentration required for the excipient. After preparation of the samples they were stored at the temperature of testing (4 ºC or room temperature). The samples were tested at 5 different time points (0, 7, 15, 30 and 60 days). The different stabilizers tested and the different concentrations are displayed in table 4.

Table 4 - Stabilizers candidates, with the respective structure and studied concentration.

Category	Stabilizer agent	Concentration	Structure
Amino Acids	Arginine	1 and 4 mM	
	Glycine	1 and 4 mM	

Ionic Liquids	[C2Ch][AcGly]	1 and 4 mM	
	[C2Ch][AcAla]	1 and 4 mM	
	[C2Ch][AcPhe]	1 and 4 mM	
	[C2Ch][AcCys]	1 and 4 mM	
	[C2Ch][AcMet]	1 and 4 mM	
	[C5Ch][AcGly]	1 and 4 mM	

Ionic Liquids	[C5Ch][AcAla]	1 and 4 mM	
	[C5Ch][AcPhe]	1 and 4 mM	
	[C5Ch][AcCys]	1 and 4 mM	
	[C5Ch][AcMet]	1 and 4 mM	

Sugars	Glucose	10 and 20 %	
	Sucrose	10 and 20 %	
	Trehalose	10 and 20 %	
Deep Eutectic Solvent	DES choline-glucose (ratio 1:2)	10 and 20 %	
	DES choline-glucose (ratio 2:1)	10 and 20 %	
	DES choline-trehalose (ratio 2:1)	10 and 20 %	

2.2.10 Circular Dichroism spectroscopy

Circular Dichroism was used to evaluate the structural stability of the RNA molecule at various time points. CD was performed in a Jasco J-815 spectropolarimeter (Jasco, Easton, MD, USA), using a Peltier-type temperature control system. CD spectra were acquired at 20 ºC using a scanning speed of 100 nm/min, with a response time of 1 second over a wavelength range 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 of 1 mm (Hellma analytics, Germany). Three scans were measured per spectrum to improve the signal to-noise ratio. The spectra were posteriorly smoothed using the noise-reduction option in the operating software. CD melting experiments were performed from 20 to 100 ºC, with a heating rate of 1 ºC/min, by monitoring the ellipticity at the highest point from the corresponding spectrum, around 265 nm.

2.2.11 Molecular docking

Molecular docking analysis of RNA with DES were calculated using AutoDock Vina 1.1.2 program [61]. Ligand (hydrogen bond *donor* (HBD) and hydrogen bond *acceptor* (HBA)) 3D atomic coordinates were computed by Discovery Studio, v20 (Accelrys, San Diego, CA, USA) and ligands rigid root was generated using AutoDockTools (ADT) [62], setting all possible rotatable bonds defined as active by torsions. Additionally, Auto DockTools (ADT) was used to prepare the receptor (RNA - PDB: 1kxk) input file by merging non-polar hydrogen atoms, adding partial charges and atom types.

The grid center at the center of mass (x-, y-, and z-axes) of RNA was -25.587 Å× 51.471 Å × 141.78 Å (to cover the whole surface of RNA). The grid dimension used for RNA was 80 Å× 80 Å × 80 Å. The binding model was searched out from 10 different conformers (for each ligand (HBD and HBA)).

Chapter 3

3. Results and Discussion

3.1 Stability assays with RNA

Most biomolecules are structurally unstable in solution and therefore are susceptible to conformational changes which might lead to loss of function [63]. Circular Dichroism (CD) spectroscopy allows the detection of changes in the conformation of biomolecules, being a powerful technique to verify if there are significant structural modifications on the biomolecule when it is in contact with other compounds, over a period of time. CD can also be used to study melting temperature which indicates the sample stability in certain conditions. In this case, the ellipticity is measured in a specific wavelength as temperature rises and the point where denaturation occurs can be determined. By comparing different melting temperatures we can determine in what conditions the RNA molecule is more stable. This strategy also gives relevant information about macromolecule-ligand and macromolecule-macromolecule interactions [64].

The typical RNA spectrum has two characteristic bands, one negative at 215 nm and one positive at 265 nm. In the present study, RNA stability assays were performed under different conditions and studying, various classes of excipients (amino acids, sugars, ionic liquids and deep eutectic solvents). Moreover, it was studied the storage of RNA at different temperatures (4 ºC and room temperature) and for different times, to evaluate the RNA stability under these storage conditions. To address this issue, CD analysis were carried out at several time points, namely 0, 7, 15, 30 and 60 days.

Free RNA was used as a control sample, giving an idea of the behavior of RNA molecule in solution, when stored at different temperature conditions. The results indicated that at 4 °C the RNA sample maintained the stability throughout the timeframe in study, which was 60 days. However, at room temperature there is a significant decline in ellipticity between 30 and 60 days of storage, indicating a change on RNA structure and loss of stability (figure 7).

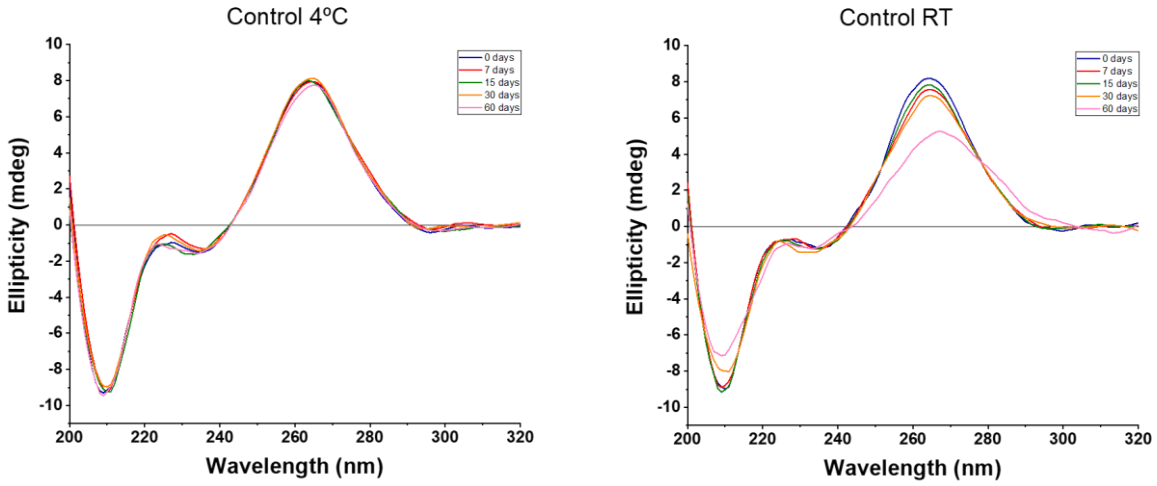


Figure 14 - RNA control sample behavior over time, after storage at 4 ºC and room temperature.

In order to evaluate if this behaviour would also be verified for the RNA molecules, adopting different secondary structures, a control stability study was conducted using pre-miRNA-9, which is a molecule presenting an hairpin structure. For this, a pre-miRNA-9 sample was stored at room temperature, over the same time period, for further comparison of these results with the previous ones obtained with an unspecific sample of RNA. The results revealed that the pre-miRNA sample maintained the characteristic CD spectrum over the 60 days, also maintaining the ellipticity values over this time of storage at room temperature (figure 8). Considering that the small RNAs sample had a significant decrease in ellipticity from 30 to 60 days it seems that the pre-miRNA is more stable on its own. This might happen due to the hairpin structure of the pre-miRNA which leaves the backbone more exposed and protects the nitrogenous bases, particularly on the sequence where it is found an intramolar complementarity. The results confirm that the secondary structure naturally enhances the stability of the RNA molecule. The improved stability was also noticeable in the melting temperature, since at 0 days pre-miRNA already presented a Tm 13 ºC higher than the small RNA sample, while a Tm higher in 8 ºC was achieved after 60 days of storage. In the future it would be interesting to test different types of RNAs to understand the influence of the structure of the molecule on their natural stability, and futher evaluate the effect of the addition of specific molecules on RNA formulation to potentiate stabilization and bioactivity on long-term storage.

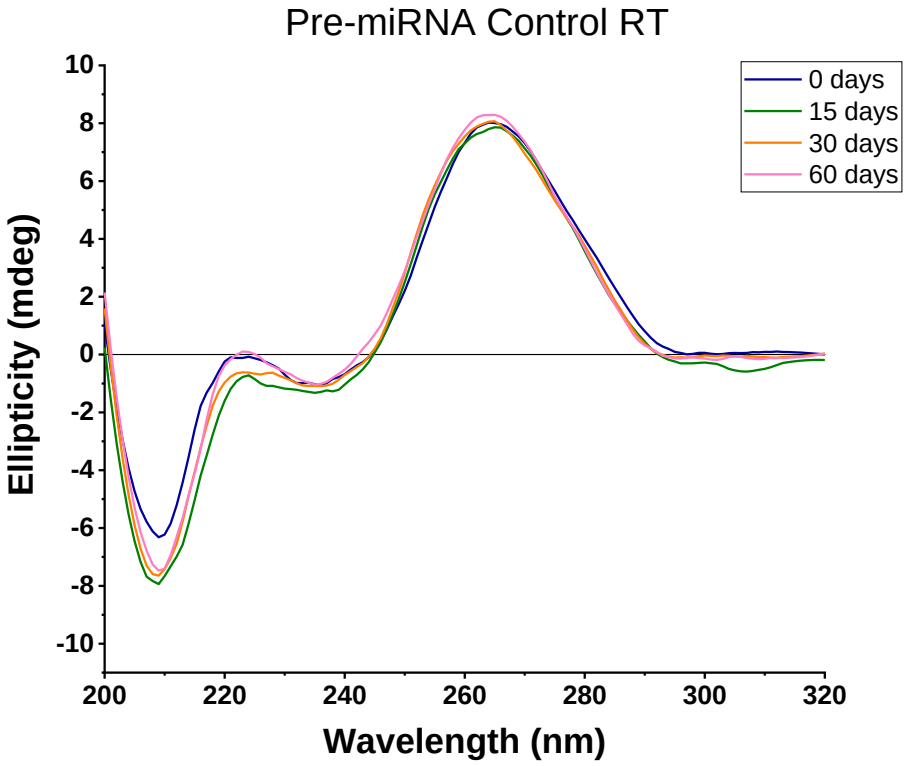


Figure 8 - Pre-miRNA control sample behavior over time, after storage at room temperature.

3.2 Influence of amino acids in RNA stability

Considering the instability of the RNA molecule, particularly when stored for more than 30 days at room temperature, it is important to establish innovative conditions to improve the stabilization of the molecule for long periods of time, which may also facilitate the development of therapeutic applications for RNA.

It is known the potential stabilizing effect that amino acids can have on biomolecules. In the present study, the amino acids tested were arginine and glycine both at concentrations of 1 and 4 mM. The selection of these amino acids was mainly related to their previous use in stability studies with proteins [65], and the concentrations were also established on the basis of literature data. A study focusing on proteins stabilization showed that amino acids concentrations of 1 mM were able to improve the thermal stability of myoglobin, however concentrations above 100 mM lead to destabilization of the protein [65]. Also, some previous results obtained by the group, demonstrated the potential of long-term stabilization of RNA using arginine, at low temperature. All this information lead to the selection of the concentrations of 1 mM and 4 mM to the study of the effect of amino acids in RNA stability. All conditions were maintained for the different samples in order to guarantee that any effect was directly related with the presence of the agent under study.

In general, RNA in contact of both amino acids seems to maintain its structure, as suggested by the similar spectra that were obtained, at the first time-point, in comparison to the RNA control sample. As shown in figure 9, only slight changes in ellipticity occurred, which may result from interactions between RNA and the amino acid.

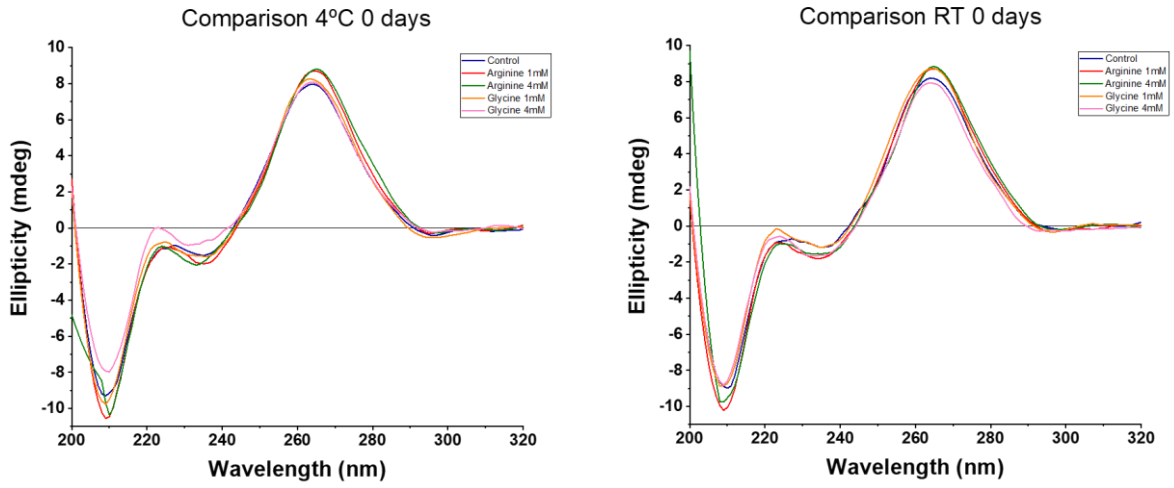


Figure 9 - RNA CD spectra in amino acids-containing solutions at 0 days

The structural behavior of RNA in solutions containing amino acids was then evaluated after 60 days of storage, at room temperature and 4 °C. At this time point it was not verified a significant impact on RNA stability, particularly for the samples stored at lower temperature (figure 10). However, at room temperature RNA in arginine 1 mM suffered some changes as demonstrated by the decreased ellipticity on CD spectra. This condition seems be less effective on RNA stabilization than glycine or more concentrated arginine, but it still shows some effect, particularly when comparing with the RNA control, stored for 60 days.

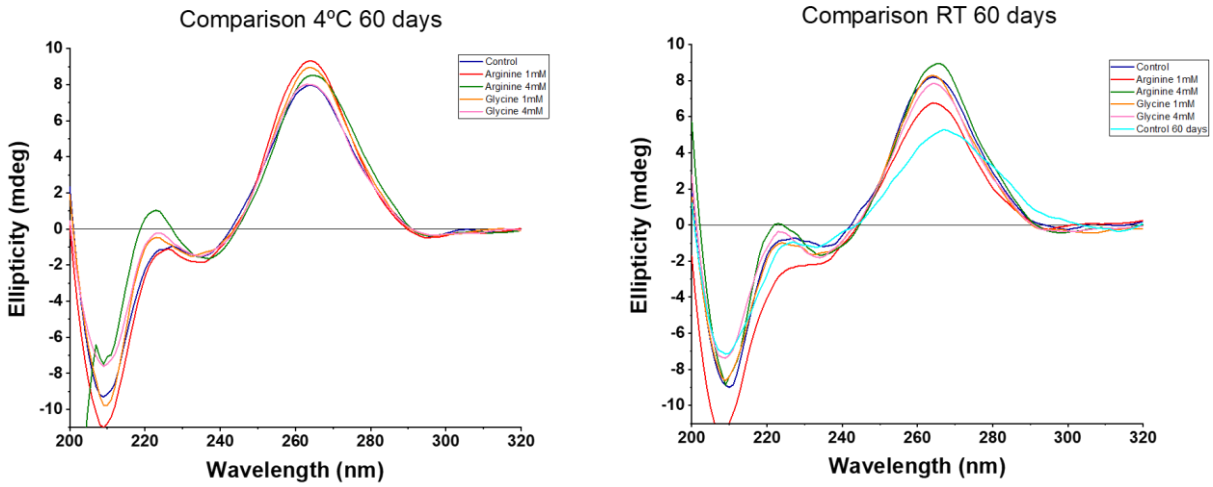


Figure 10 - RNA CD spectra in amino acids-containing solution at 60 days

The determination of melting temperature revealed that arginine could induce some thermal stabilization of RNA, while glycine had no real improvement, as suggested by the increase of T_m of RNA in the presence of arginine, with less pronounced effect when the glycine solution was used (table 5). It is also important to state that although

Table 5 - Melting temperature of RNA in amino acid containing solution, at 0 and 60 days.

	4ºC		Room temperature	
Excipients	**0 days**	**60 days**	**0 days**	**60 days**
Control	40.2 ± 1.4	40.2 ± 2.2	44.3 ± 1.5	44.9 ± 1.8
Arginine 1mM	40.0 ± 2.2	44.1 ± 1.4	40.0 ± 1.75	54.5 ± 1.0
Arginine 4mM	43.7 ± 1.1	43.1 ± 2.0	45.1 ± 0.9	47.1 ± 0.9
Glycine 1mM	35.8 ± 2.3	38.0 ± 2.2	40.8 ± 1.4	45.3 ± 0.9
Glycine 4mM	39.0 ± 2.7	40.0 ± 2.4	37.0 ± 2.8	46.0 ± 0.9

these amino acids did not induce a great improvement on RNA stability, they also did not present a harmful effect on the molecule.

The analysis of RNA structure over the various time points was performed by comparing the ellipticity values at 265 nm (figure 11), as it is one of the main characteristic bands of RNA in CD. The ellipticity variation at 215 nm, which represents the other characteristic CD band, was not analyzed due to higher interference on spectra. In figure 11 it is confirmed the small variation on RNA structure when it is stored in the amino acids solutions at 4 °C, for 7, 15, 30 or 60 days. The ellipticity is slightly higher in amino acids solution, but this is an effect of the formulation containing the respective amino acids. The analysis of RNA stored at room temperature is very similar, being important to highlight the difference achieved for the RNA combined with arginine or glycine in comparison with the RNA control stored for 60 days. These results indicate an improved stability in samples incubated with amino acids when compared with free RNA, as ellipticity values were mainly maintained throughout the 60 days. Actually, RNA stored in arginine 1 mM lost some stability between 30 and 60 days, but the structural change was not so significant as occurred in RNA control, as previously identified for the analysis of global CD spectra. These amino acids have already showed to have stabilizing effect in proteins [65], and these results in RNA substantiate their added value as stabilizers of biomolecules.

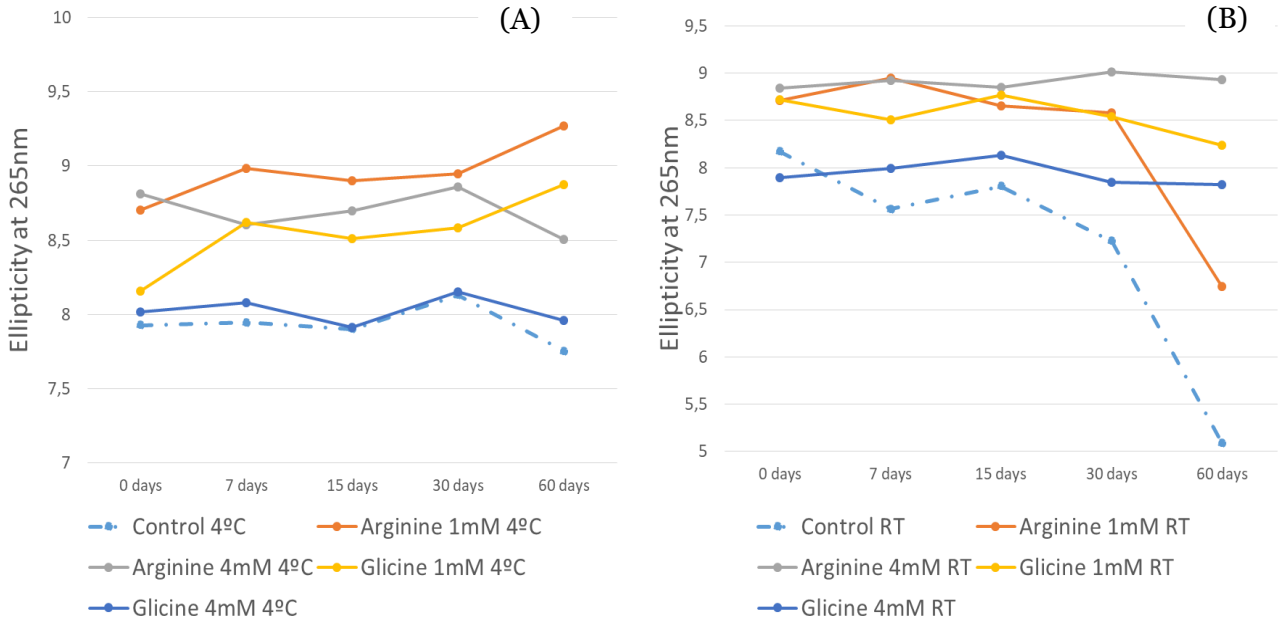


Figure 11 - Ellipticity variation at 265 nm over time, for RNA combined with amino acids and stored at 4 ºC (A) and room temperature (B)

3.3 Effect of N-acetyl amino acid N-alkyl cholinium-based ionic liquids in RNA stability

In recent studies some Good's buffers ILs, composed by a cholinium cation and a Good buffer derived anion have shown potential as RNA stabilizers [20]. Taking into consideration the results obtained with the amino acids tested it seemed reasonable to take advantage of the many characteristics that make ILs good solvents combined with the potential stabilizing effect of amino acids [66]. Moreover, while simple amino acids have already been reported as compatible anions for ionic liquids, acetylated amino acids have a greater thermal stability which in this case could even be more advantageous for usage as RNA stabilizing agents. One study found that ILs based on acetylated amino acids could induce an improvement in water solubility of two drugs [67], and another study also determined that choline based ILs are biocompatible [68]. All these characteristics seem to support the great potential of ILs as stabilizer candidates. Thus, in this study, the ionic liquids that were tested were composed of an N-alkyl cholinium cation (C_2Ch and C_5Ch) and an N-acetyl amino acid anion (acetylated-glycine, -phenylalanine, -methionine, -cysteine and -alanine). The concentrations used in this screening were the same used for the study of free amino acids (1 and 4 mM) for comparison purposes. RNA samples prepared in each formulation were kept at room temperature and 4 °C and analyzed at 0, 7, 15, 30 and 60 days.

3.3.1 ILs containing an acetyl glycine-based anion

Analyzing the CD results for the RNA sample incubated with ILs based on N-alkyl cholinium and N-acetylglycine, it was observed that only the RNA combined with [C_5Ch] [AcGly] at 4 mM maintained the stability at room temperature, for 60 days (figure 12). It is also worth noting that the rest of the samples lost their stability prior to 15 days (figure 13). On the other hand samples kept at 4ºC maintained the structural stability and little to none ellipticity variation was verified, except for [C_2Ch] [AcGly] at 1 mM, which showed a considerable decrease in ellipticity at 60 days, which might indicate that some sample was degraded. It would be more beneficial if samples could be kept at room temperature, due to easier and less expensive means of transportation and storage. Therefore, all stabilizers that are able to maintain RNA structure at room temperature bring added value to this objective. Taking this into consideration, as only the RNA combined with [C_5Ch] [AcGly] 4 mM maintained stability for 60 days at room

temperature, this would be the most cost effective stabilizer from the ILs containing an acetyl glycine-based anion.

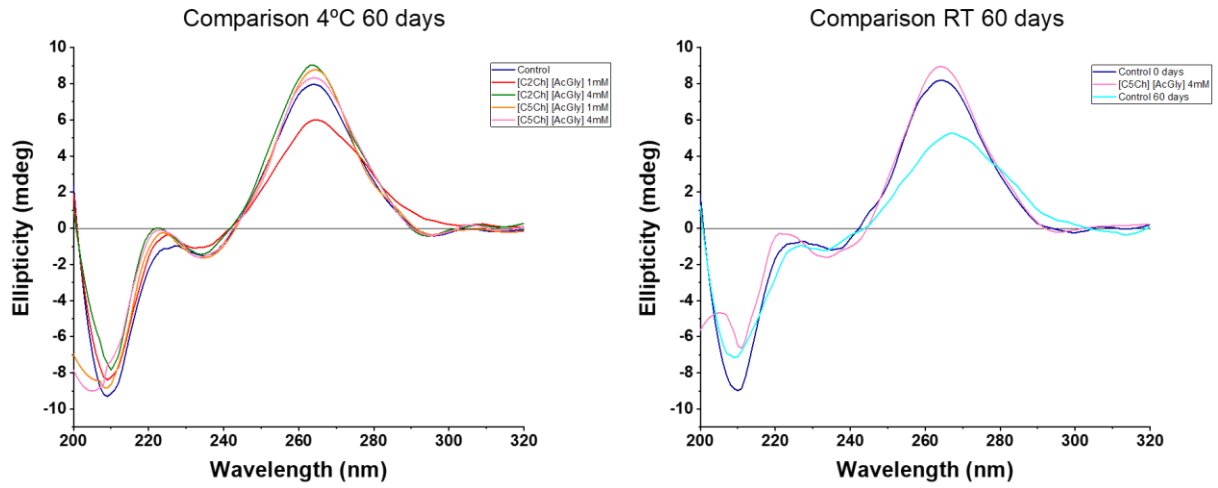


Figure 12 - RNA CD spectra in ILs containing an acetyl glycine-based anion-containing solution at 60 days

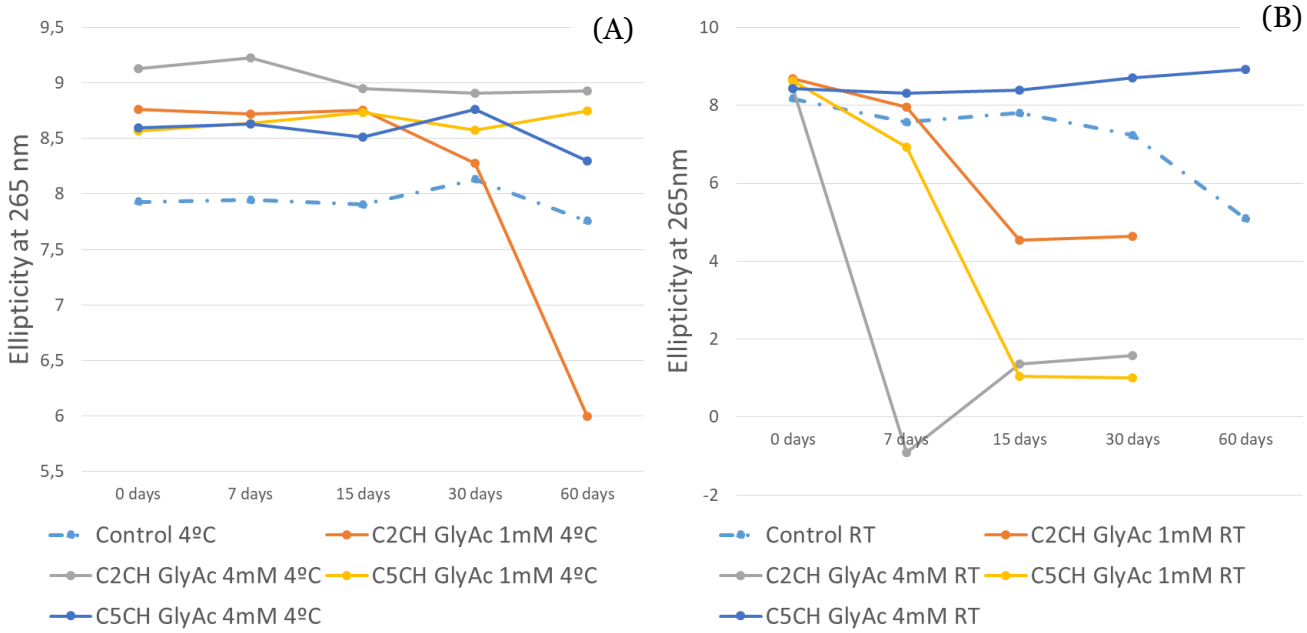


Figure 13 - Ellipticity variation at 265 nm over time, for RNA combined with ILs containing an acetyl glycine-based anion at 4 ºC (A) and room temperature (B)

When comparing these results with the ones obtained for the free amino acid glycine, it seems that there is not a significant improvement from using an IL, considering that most simple glycine samples maintained a characteristic RNA spectra, while some of the samples with the IL as stabilizer showed decreased ellipticity values, suggesting that the instability occurred at some extent (figures 14 and 15). Actually, the best conditions achieved with this IL induced a similar stabilization effect as free glycine

(figure 15) However, it must be taken into account the other properties that ILs possess, such as improved water solubility and good thermal stability which might bring forth other advantages when handling these samples, for example for longer storage periods.

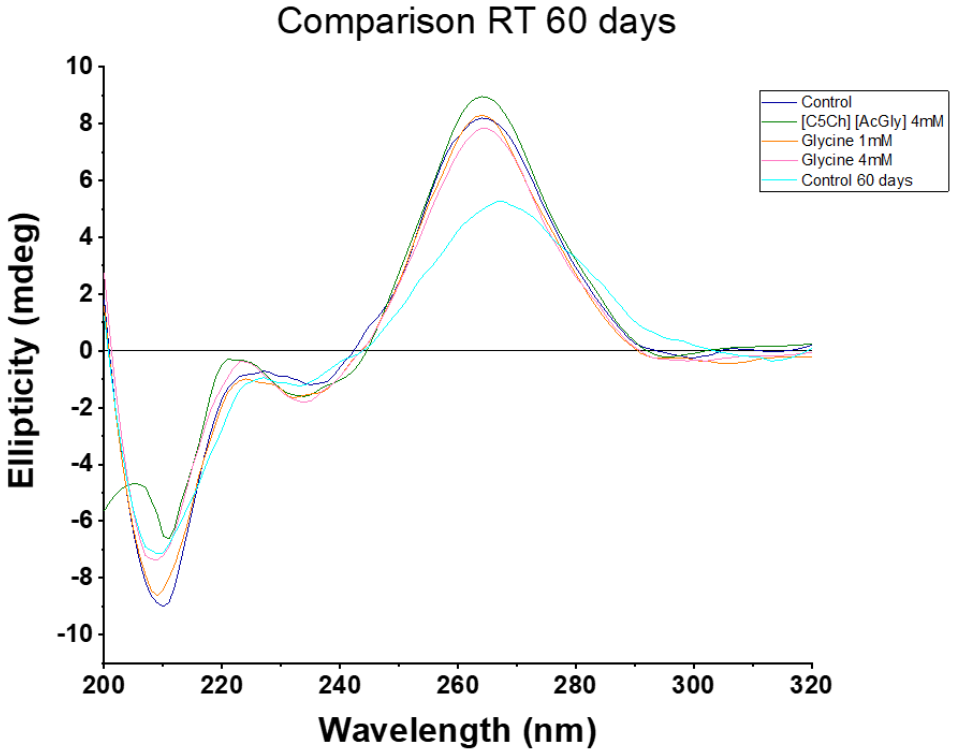


Figure 14 - Comparison of RNA stability in free glycine and in ILs containing an acetyl glycine-based anion, when stored at 4 ºC, for 60 days

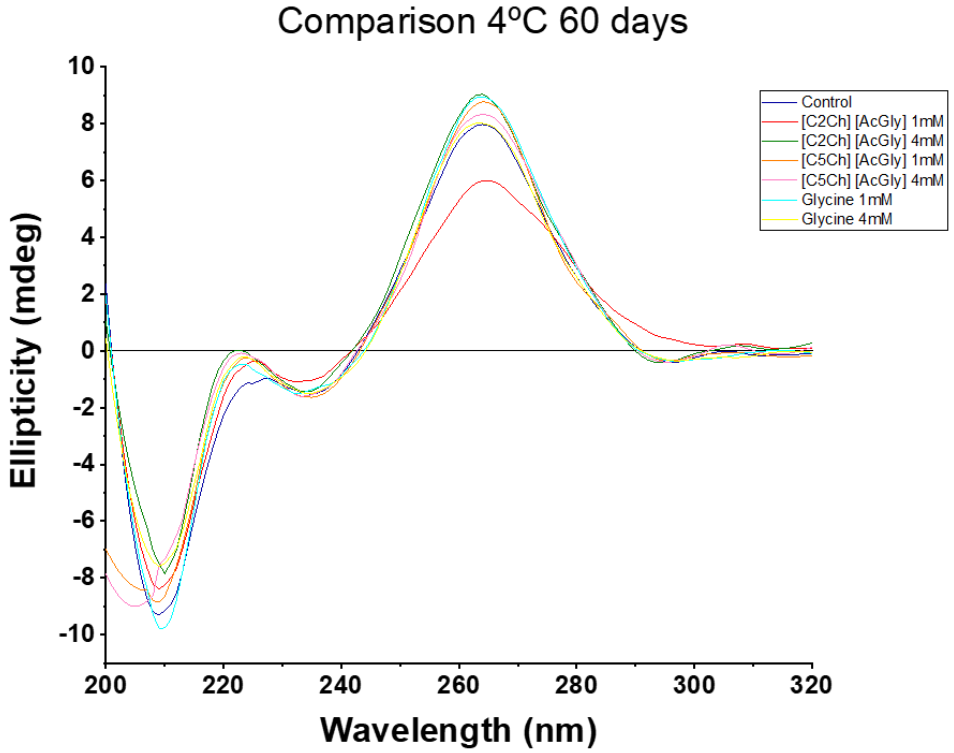


Figure 15 - Comparison of RNA stability in free glycine and in ILs containing an acetyl glycine-based anion, when stored at room temperature, for 60 days.

3.3.2 ILs containing an acetyl phenylalanine-based anion

CD results of RNA samples incubated in solution with ILs based on N-alkyl cholinium and N-acetylphenylalanine indicate that all samples maintained stability throughout the 60 days both at 4 °C and room temperature (figure 16), some of the samples have slight changes in ellipticity values, which is likely to occur due to the complex formed by the biomolecule and the stabilizer. Around 200 and 220 nm the spectra has a more irregular behaviour, which is related with the interference from the aromatic ring of phenylalanine which also absorbs light at this wavelength. For the RNA incubated in [C_2Ch] [AcPhe] 1 mM at room temperature, it was found a CD spectra with high ellipticity values along the entire range, what was also accompanied by a melting temperature of around 50 °C, for 60 days of storage, which when compared with the control represents an increase of almost 6 °C. This indicates that the molecule was more stable in the presence of the IL. In this case, it is suggested that the aromatic ring of phenylalanine may play an important role on RNA stabilization, as it is possible the establishment of ring stacking interactions with the nitrogenous bases of RNA, resembling an intercalating phenomena, which may result in an improved stability for RNA along storage. This could be a similar behaviour to what was previously described in literature for the interaction of histidine with nucleic acids [69].

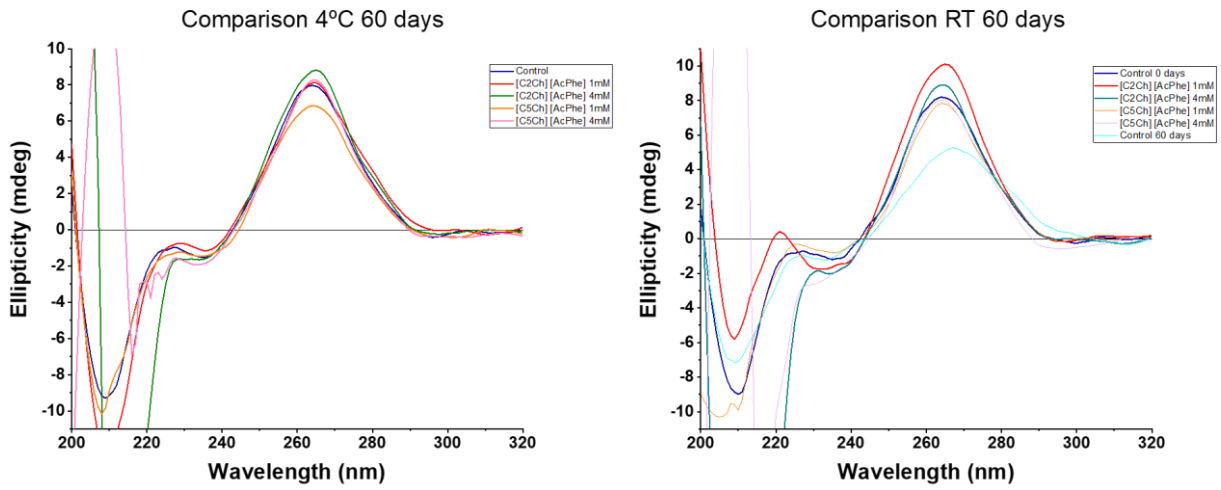


Figure 16 - RNA CD spectra in ILs containing an acetyl phenylalanine-based anion-containing solution at 60 days

3.3.3 ILs containing an acetyl methionine-based anion

In the case of RNA samples incubated with ILs based on N-alkyl cholinium and N-acetylmethionine and stored at 4 °C, it was observed the stabilization of the molecule all through the 60 days. However, RNA kept at room temperature did not have the same results, as only the samples stored in the IL composed by the cholinium cation modified with the 5 carbon alkyl chain ([C_5Ch] [AcMet] 1 and 4 mM) maintained some stability over the testing period. The sample incubated with 1 mM of [C_5Ch] [AcMet] at room temperature, comparetively with the control at 60 days, has similar ellipticity values but both display a decrease when compared with the control at 0 days, which indicates an alteration in RNA structure. On the other hand, RNA in the presence of [C_5Ch] [AcMet] 4 mM at room temperature maintains ellipticity values and the CD profile, all through the 60 days (figure 17), representing a more interesting result in the point of view of RNA stabilization. Moreover, similarly to the results obtained with N-acetylglycine, only the RNA sample incubated in the higher concentration of the IL composed by cholinium with 5 carbon alkyl chain maintained stability at room temperature. This might indicate that the longer alkyl chain might have a direct effect on stability, thus suggesting that hydrophobic effects may benefit for the stabilization of the RNA molecule, what would also corroborate the results achieved with the N-acetylphenylalanine-based IL.

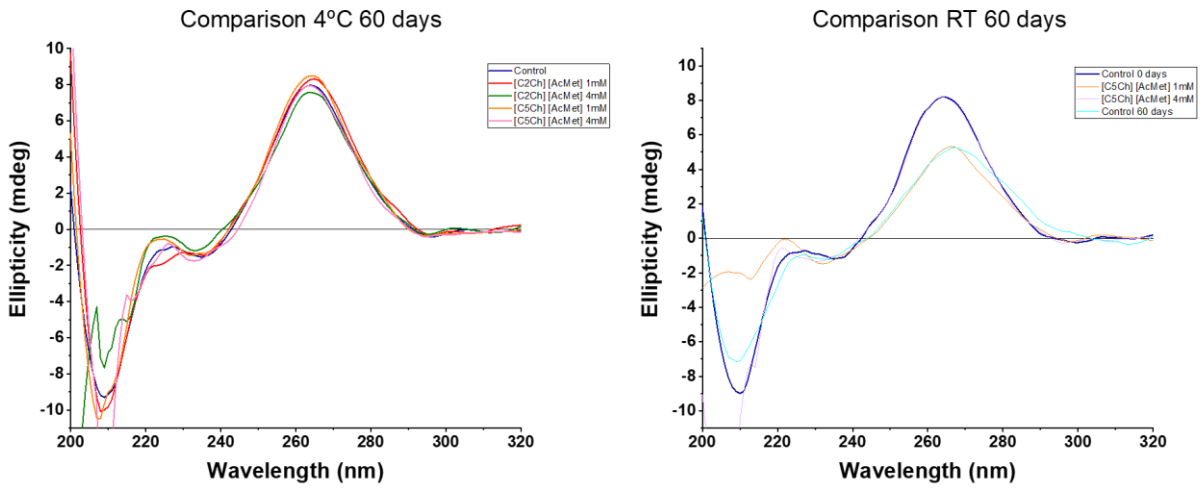


Figure 17 - RNA CD spectra in ILs containing an acetyl methionine-based anion-containing solution at 60 days

In regards to the melting temperature all samples at 4 °C showed some increase in Tm especially at 60 days which might indicate induced stabilization of the RNA molecule with this IL. At room temperature both RNA samples incubated in [C_5Ch] [AcMet] at 60 days showed some improvement in T_m also suggesting some thermal stabilization to some extent (table 6).

Table 6 Melting temperature of RNA in ILs containing an acetyl methionine-based anion solution, at 0 and 60 days.

	4ºC		Room temperature	
Excipients	**0 days**	**60 days**	**0 days**	**60 days**
Control	40.2 ± 1.4	40.2 ± 2.2	44.3 ± 1.5	44.9 ± 1.8
[C2Ch] [AcMet] 1mM	46.6 ± 0.8	46.4 ± 0.9	46.4 ± 1.0	denatured
[C2Ch] [AcMet] 4mM	39.8 ± 1.4	44.6 ± 1.1	44.8 ± 1.5	denatured
[C5Ch] [AcMet] 1mM	45.4 ± 1.3	42.8 ± 1.1	41.0 ± 1.2	49.0 ± 2.6
[C5Ch] [AcMet] 4mM	38.7 ± 1.8	41.3 ± 1.3	42.0 ± 1.5	43.6 ± 1.1

3.3.4 ILs containing an acetyl cysteine-based anion

N-acetyl cysteine is widely used as a pharmacological antioxidant and cytoprotectant [70, 71], and for this reason it was though that it could be interesting to evaluate the effect of the corresponding IL on RNA stabilization. However, analyzing the CD results it was not possible to directly conclude about the advantageous effect of the IL on maintaining the RNA structure, as all samples stored at room temperature had lost stability after the 60 days of testing. Furthermore, at 4 °C only one of the samples maintained a characteristic CD spectrum. When analyzing the RNA spectra at 0 days, which means only the RNA in contact with the N-acetylcysteine IL (figure 18), an immediate decrease in overall ellipticity is noticeable, which suggests that something may compromise the stability of RNA. Considering that this was not verified for any other RNA samples tested, the pH of the IL solution was tested. It was observed that the pH of

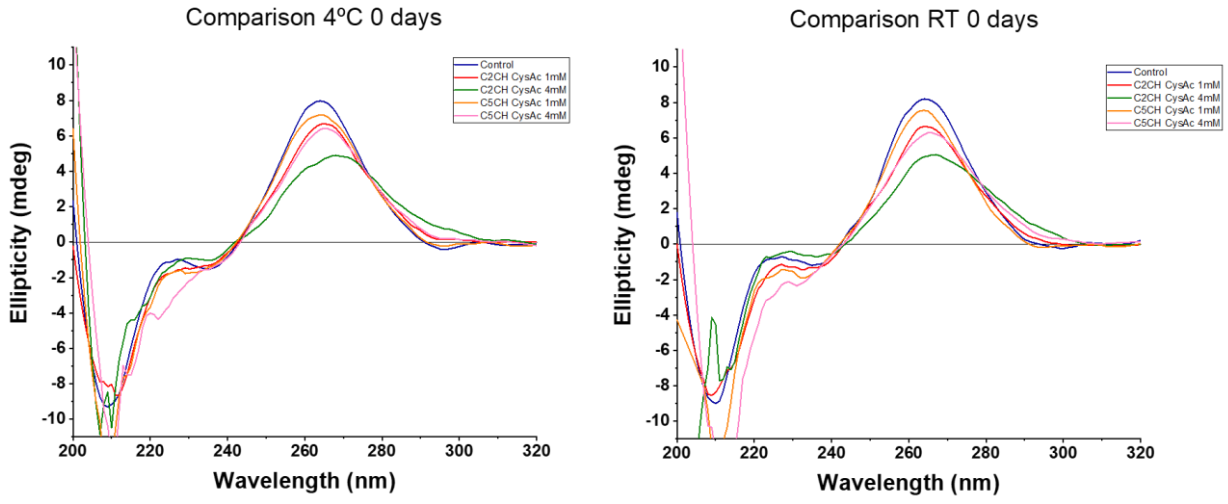


Figure 18 - RNA CD spectra in ILs containing an acetyl cysteine-based anion-containing solution at 0 days

the IL solutions was around 5 which might have led to structural instability of RNA, and therefore affecting the ellipticity values. As mentioned, cysteine has shown to be an effective antioxidant which might help protect the RNA molecule from free radicals [72], therefore in the future it would be interesting to neutralize the pH solution in order to verify if the IL can thus stabilize the biomolecule.

After the result obtained with the RNA samples incubated in IL containing an acetyl cysteine-based anion, a new study was performed where RNA samples were incubated with the free N-acetyl amino acid (without the cholinium cation) in order to determine the influence of each component of the IL in RNA stabilization. When the samples were tested at 0 days the ellipticity values were all around 1 and there was no characteristic RNA spectrum independently of the temperature of storage. Similarly to the IL with N-acetylcysteine, the pH values of the free N-acetyl amino acids solutions were low, being even lower (pH around 2-3) than the respective ILs. This demonstrated that the N-alkyl cholinium cation helped to neutralize the pH of the N-acetyl amino acids, demonstrating a direct advantage of using the IL over the free N-acetyl amino acids. Even though, further studies must be conducted to verify if other conditions may actually be used with this IL to promote RNA stabilization.

3.3.5 ILs containing an acetyl alanine-based anion

In regard to RNA samples incubated with ILs containing an acetyl alanine-based anion and stored at 4 °C, it was observed that all maintained stability throughout the 60 days of testing (figure 19). At room temperature however, similarly to the ILs with N-acetylglycine and N-acetylmethionine only the samples with the cholinium cation modified with 5 carbon alkyl chain maintained their stability along the 60 days of study. On the contrary, it is also important to mention that a room temperature both RNA samples incubated with the IL composed by the cholinium cation with 2 carbon alkyl chain had lost stability at 15 days. Once more, these results indicate the important role that the longer alkyl chain has on RNA sample stability, emphasizing the need to further explore the influence of the hydrophobic effects on RNA stability.

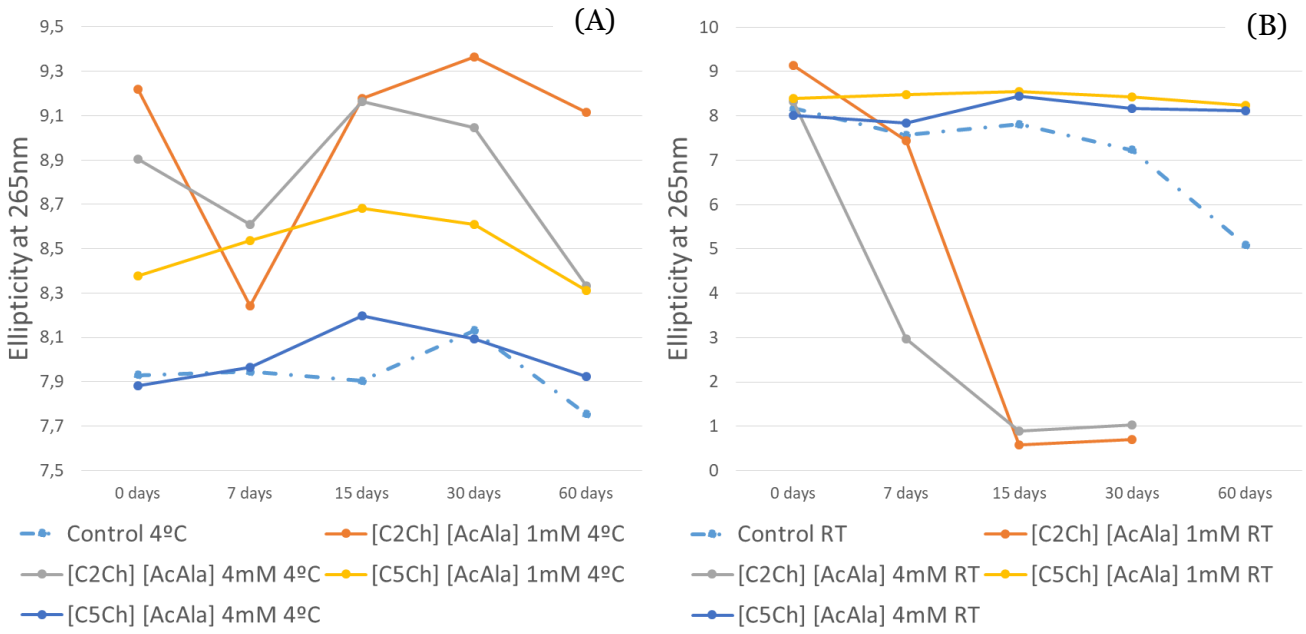


Figure 19 - Ellipticity variation at 265 nm over time, for RNA combined with ILs containing an acetyl alanine-based anion at 4 ºC (A) and room temperature (B)

3.4 Sugars

Sugars have had numerous reports regarding to their potential as cryoprotectants and have shown great results in that role of stabilizing biomolecules upon storage [35]. Some sugars such as trehalose and sucrose are also used as osmolytes in nature and can stabilize microorganisms under harsh environmental conditions such as high temperatures and low water environments [73]. Even though many stability studies in the solid samples have been conducted little is known about their potential as stabilizers in solution. Thus, in the present work, it was decided to evaluate the potential stabilizing effect of some of these sugars on RNA solutions. In these stability assays the sugars tested were glucose, sucrose and trehalose which have shown some potential in protecting proteins against thermal denaturation using concentrations of 10 and 20 % w/w [36]. The same concentrations were used for these tests on RNA stability, also studying their effect upon storage at 4 °C and room temperature for 60 days.

As can be observed in figure 20, most of the samples that were kept at 4 °C maintained stability for the 60 days, except for the samples incubated in sucrose 20 % and trehalose 20 %, which had lost stability before reaching the 60 day mark, particularly the RNA sample incubated in sucrose 20 % that lost stability before 30 days. Concerning the storage at room temperature sugars are not so effective stabilizers, as no sample was

able to maintain ellipticity levels and most samples had lost stability at the 15 days mark (figure 20).

Despite the results achieved for the storage at room temperature, as sugars show a great potential on RNA stabilization at refrigerated conditions, some additional studies were perfomred, trying to better understand the interaction mechanism occuring between sugars and RNA, that can lead to structural stabilization.

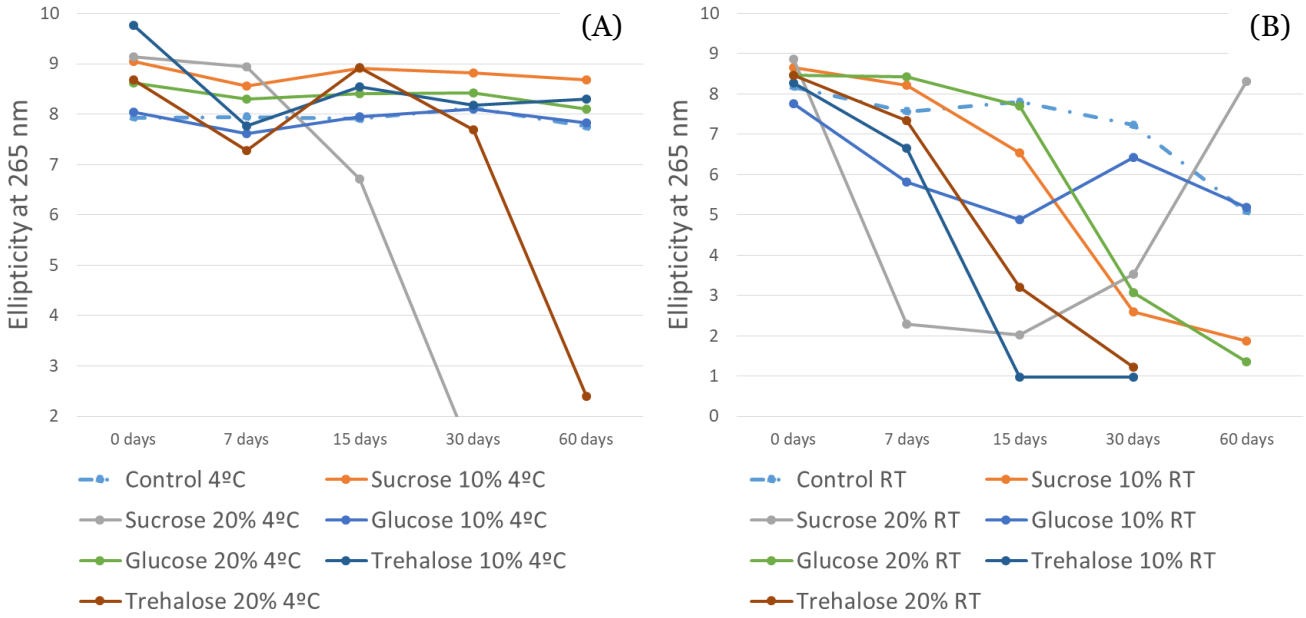


Figure 20 - Ellipticity variation at 265 nm over time, for RNA combined with sugar-based solutions at 4 ºC (A) and room temperature (B).

Thus, Molecular Docking was used to predict the preferred binding orientation of one molecule to the other. In this case docking simulations of sucrose, glucose and trehalose were conducted to verify the previous results. Since Hydrogen Bond Donors (HBD) and Hydrogen Bond Acceptors (HBA) have individual impact on RNA stability, identifying the major interactions could lead to a better understanding of the impact of agents on RNA stability. The results indicate that all sugar interact with the RNA molecule by hydrogen bonds and in most of those interactions they play the role of HBD. The binding energy of the sugars to the RNA was higher for trehalose-RNA (-9.0 kcal/mol) when compared with sucrose-RNA and glucose-RNA (-8.4 and -6.1 kcal/mol respectively). The preferential docking pose for each sugar is shown in figure 21. These results suggest that the enhanced stability of RNA should follow: Trehalose > Sucrose > Glucose.

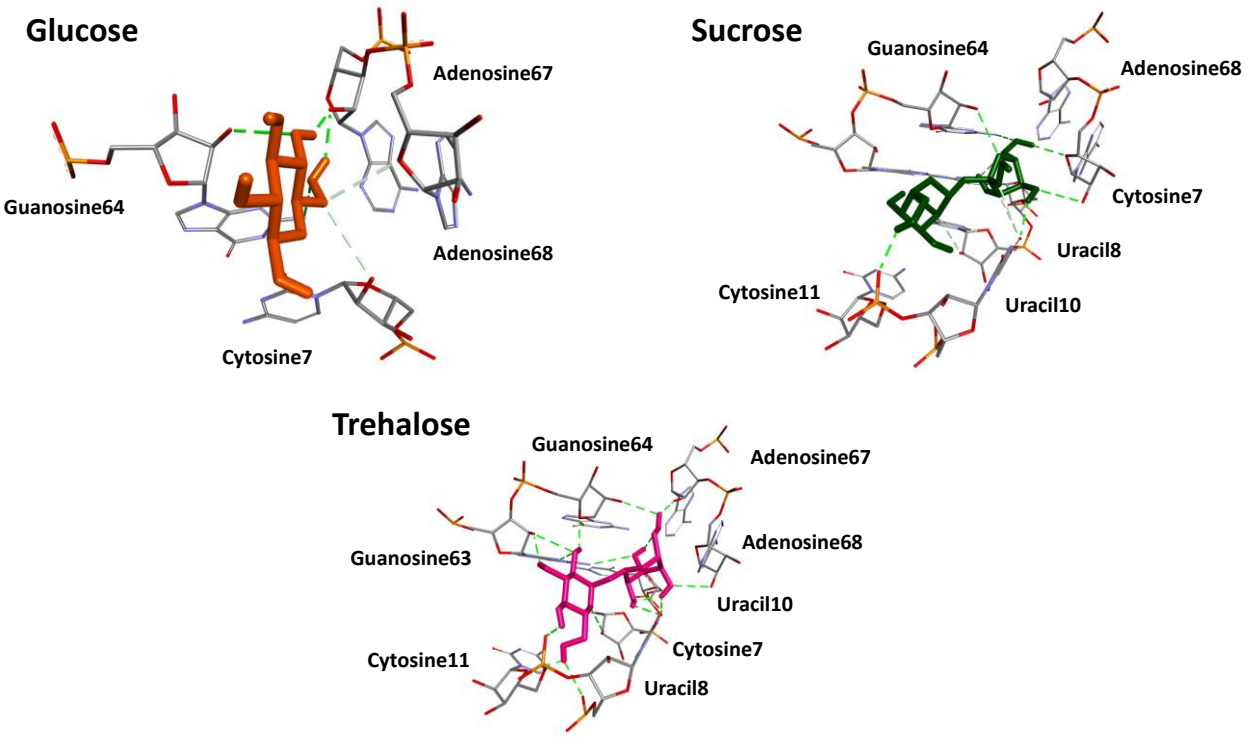


Figure 21 - Molecular interaction diagrams of Glucose-RNA, Sucrose-RNA and Trehalose-RNA.

Analyzing all the information it seems that there is no correlation between the results achieved by CD and molecular docking, however given that sugars mainly function as cryoprotectants, the stabilizing effect might happen at lower temperatures. Actually, molecular docking predicted several interactions that can occur between RNA and sugars, but maybe these are more evidenced at lower temperatures, what could also explain the improved results when going from room temperature to 4 °C. In the future, further studies at lower temperatures should be performed to verify if the stabilizing effect is improved with the temperature decrease. Taking into account the poor results obtained using sugars as stabilizers at room temperature, it might be necessary to conjugate sugars with other compounds to take advantage of their stabilizing effect.

3.5 Deep Eutectic Solvents

In recent studies Deep Eutectic Solvents (DES) have showed to have multiple applications in various fields [50]. In biologic applications DES can function as solubilizers or stabilizers, having been used in protein stability assays [51]. Moreover, some DES are biodegradable and more importantly biocompatible [74], making them great candidates for their study as RNA stabilizers. Furthermore a recent study as shown the potential of trehalose-based DES as a stabilizer of proteins [75]. Taking into account the ease of preparation of these stabilizers and the fact that it could be interesting to deepen the study of sugars as stabilizers, here conjugated with ChCl to form DES, it was planned the study of sugar-based DES on RNA stabilization.

The DES tested in these assays were composed of ChCl and a sugar, being the sugars used glucose and trehalose. The DES were obtained by continous mixing of the two solid components while being heated. The concentrations used were 10 and 20 % for a direct comparison with the study of free sugars as stabilizers, and RNA samples were stored at 4 °C and room temperature and analized at 0, 7, 15, 30 and 60 days.

3.5.1 Glucose-based DES

When preparing the glucose-based DES, various ChCl:glucose ratios were tested, however only ratios 1:2 and 2:1 formed a deep eutectic solvent. After the DES were formed they were weighted and dissolved in water to make the glucose-based DES solution, used to prepare the RNA sample. CD results of the samples stored at 4 °C show that only the sample incubated in glucose-based DES 1:2 ratio (ChCl:glucose) at 20 % concentration maintained the stability after the 60 days, and the same happened at room temperature, where the other samples lost stability before 15 days (figure 22).

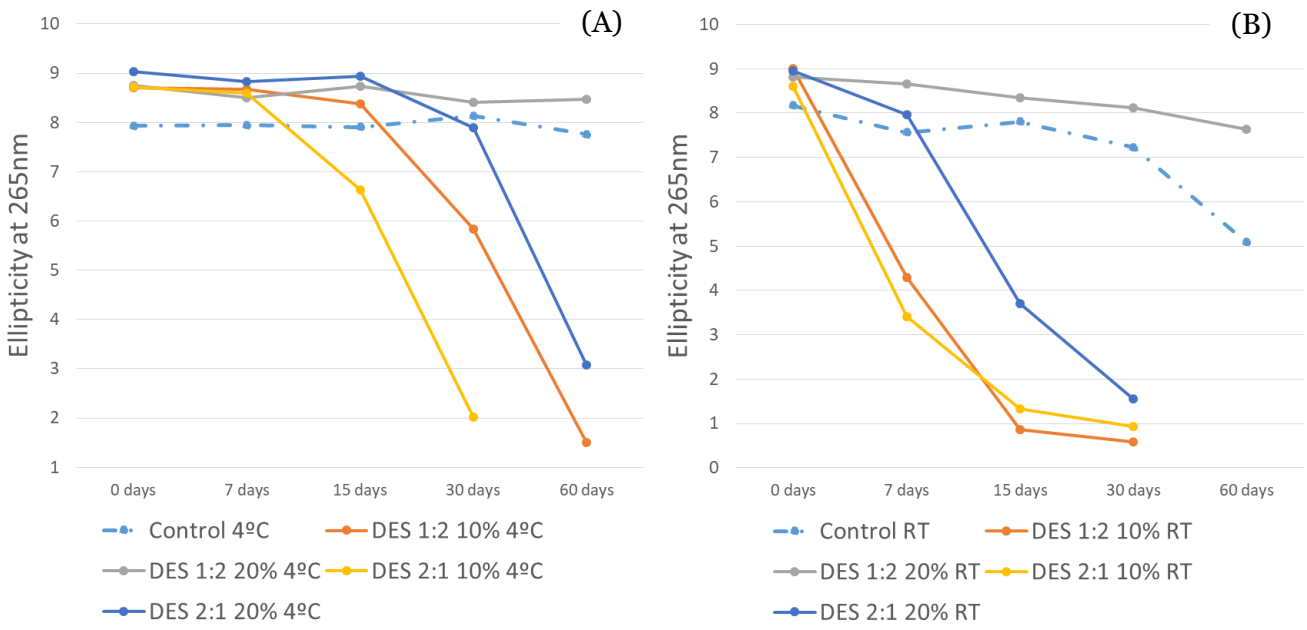


Figure 22 - Ellipticity variation at 265 nm over time, for RNA combined DES-glucose solution at 4 ºC (A) and room temperature (B).

The melting temperature of the sample that maintained stability had also a significant improvement of around +16 °C for the RNA sample stored at 4 °C, and around +9 °C when stored at room temperature (table 7), when compared with the control sample. This clearly indicates an improved thermal stability of the RNA molecule induced by the presence of DES (ChCl:glucose) at the ratio 1:2.

Table 7 - Melting temperature values for RNA stored for 0 and 60 days in the presence of DES-Glucose

	4ºC		Room temperature	
Excipients	**0 days**	**60 days**	**0 days**	**60 days**
Control	40.2 ± 1.4	40.2 ± 2.2	44.3 ± 1.5	44.9 ± 1.8
DES 1:2 10%	58.3 ± 0.6	denatured	56.9 ± 0.5	denatured
DES 1:2 20%	57.8 ± 0.4	56.5 ± 0.6	59.3 ± 0.4	53.6 ± 0.8
DES 2:1 10%	61.1 ± 0.4	denatured	62.0 ± 0.5	denatured
DES 2:1 20%	62.9 ± 0.4	denatured	61.8 ± 0.4	denatured

Results of glucose-based DES when compared with simple glucose show a slight improvement in long term stability. Actually, as DES is composed of glucose and cholinium chloride, both elements can contribute for the stabilization of RNA. A previous study performed in the group, based on the use of ILs, demonstrated the stabilizing effect of cholinium cation on RNA structure [20]. Therefore, in the present study it was compared the CD results for RNA samples incubated with only glucose, with glucose-based DES (ChCl:glucose) and the ChCl. In this way, it was expected to understand the influence of the sugar but also of the ChCl on RNA stability. When comparing the results at 4 °C it was verified that 2 samples incubated in DES lost their stability, while both free glucose and ChCl incubated samples maintained stability. However at room temperature the only samples that maintained stability through the entire testing period were glucose-based DES 1:2 20 % and ChCl 20 %. Taking into consideration the benefits of storing biologic products at room temperature this makes the glucose-based DES a more attractive stabilizer. Regardin the study of RNA in ChCl, it was observed that RNA long term stability when compared to free glucose, being a more pronounced effect at room temperature than at 4 ºC (Figures 23 and 24). These results might indicate that the improvement in long term stability observed in the sample incubated in glucose-based DES 20 % comes from the contribuition of ChCl.

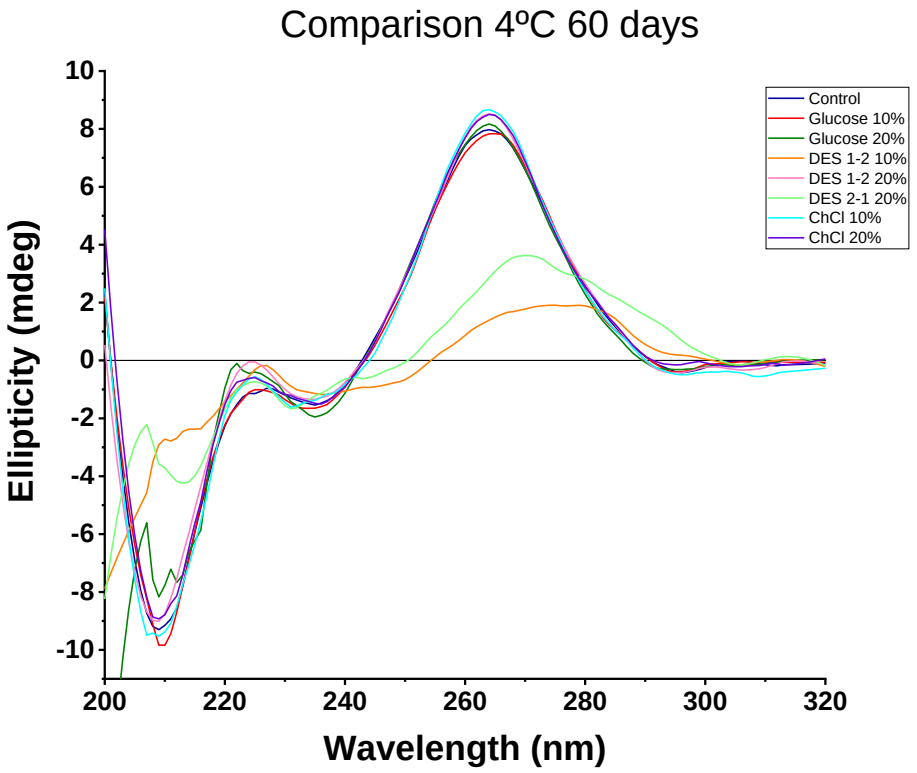


Figure 23 - Comparison of RNA stability in free glucose, in glucose-based DES and in free ChCl, when stored at 4 ºC, 60 days.

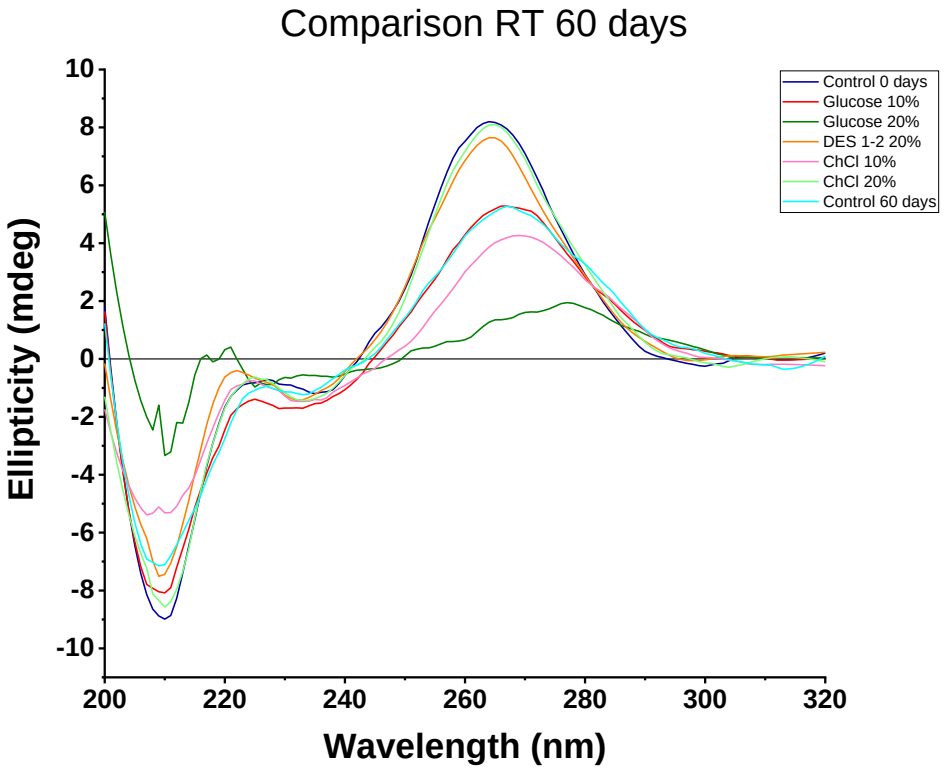


Figure 24 - Comparison of RNA stability in free glucose, in glucose-based DES and in free ChCl, when stored at room temperature, for 60 days.

As it was previously referred glucose-based DES had improved thermal stability, at both temperatures, in the RNA samples when compared with the control (table 8) and the same happens with ChCl which had resulted in a melting temperature of around 61 ºC at 0 days at both temperatures, however these values decreased to around 53 ºC after 60 days. Despite this decrease both glucose-based DES and ChCl show significantly better Tm than glucose in the conditions tested, also corroborating the stabilizing effect that can induce on RNA strucuture.

Molecular Docking was used to predict the interaction between the RNA molecule and the DES constituients. As previously mentioned glucose had a HBD binding energy of -6.1 kcal/mol whereas $[Ch]^+$ and Cl^- are HBA with binding energies of -3.4 and 0.8 kcal/mol, respectively. The $[Ch]^+$ cation binds to the the RNA chain through hidrogen bonds and eletrostactic interactions whereas the Cl^- anion does not bind to the RNA molecule but stays in close proximity of the cation. Taking into consideration that both HBD and HBA have individual impact on RNA stability, the improved stability from the DES-RNA complex comes from both the interactions between RNA-glucose and RNA-Ch.

3.5.2 Trehalose-based DES

Following the study of sugars, it was also evaluated the effect of using trehalose incorporated in a DES with ChCl, at inducing RNA stability for 60 days, for the comparison with the results achieved with the free trehalose. Similarly to glucose-based DES various ratios were tested to form a deep eutectic solvent, however with trealose only one of the ratios tested had the desired outcome, that was 2:1 choline to trehalose ratio.

The RNA samples incubated in a trehalose-based DES solution at 4 ºC maintained a characteristic ellipticity spectrum all throughtout the 60 days and showed a slight increase in ellipticity values when compared to the control (figure 25) which might happen due to the complex formed with the RNA molecule. At room temperature both samples maintained ellipticiy values during the testing period, indicating an improvement in stability in comparison with the RNA control.

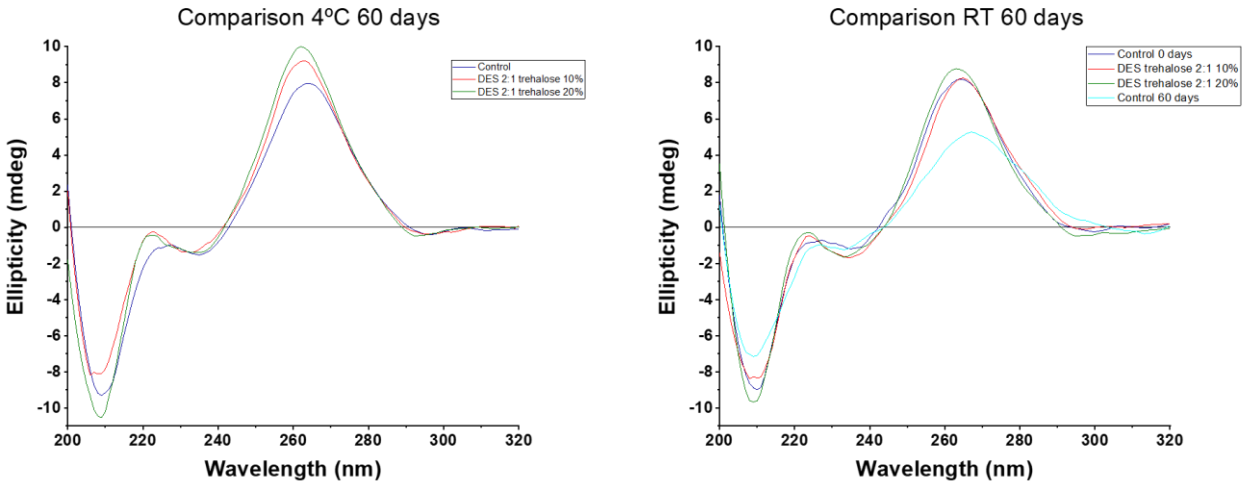


Figure 25 - CD spectra of RNA with DES Choline:Trehalose after 60 days of storage

Melting temperatures of RNA in trehalose-based DES were significantly higher when compared to the values of RNA control. At 10 % concentration a decrease in melting temperature happens over time. This, however, does not happen at 20 % concentration which leads us to believe that even though both concentrations of DES improve thermal stability, the higher concentration has a more lasting effect (Table 8).

Table 8 - Melting temperature values for RNA stored for 0 and 60 days in the presence of DES-Trehalose

	4ºC		Room temperature	
Excipients	**0 days**	**60 days**	**0 days**	**60 days**
Control	40.2 ± 1.4	40.2 ± 2.2	44.3 ± 1.5	44.9 ± 1.8
DES 2:1 trehalose 10%	60.6 ± 0.4	51.7 ± 0.5	58.2 ± 0.4	50.8 ± 0.6
DES 2:1 trehalose 20%	58.6 ± 0.4	57.7 ± 0.3	57.8 ± 0.4	57.9 ± 0.3

When comparing the results from the samples incubated in trehalose-based DES solution with the simple trehalose it becomes quite clear that there is an improvement in stability over time. Considering the RNA storage at both temperatures with trehalose-based DES it was verified that RNA maintained a characteristic ellipticity curve throughout the 60 days, whereas for free trehalose samples only trehalose 10 % at 4 °C maintained stability. The RNA incubated in [Ch]Cl also showed a better stability profile than isolated trehalose, especially at 4 ºC where both [Ch]Cl samples maintained stabillity. At room temperature however only one of the samples maintained the ellipticity levels throughout the 60 days (figures 26 and 27). This indicates that the stabilizing effect of the DES trehalose does not come only from the [Ch]Cl but rather

from the complex formed by the two compounds, with a real contribution from the cholinium ion and the trehalose.

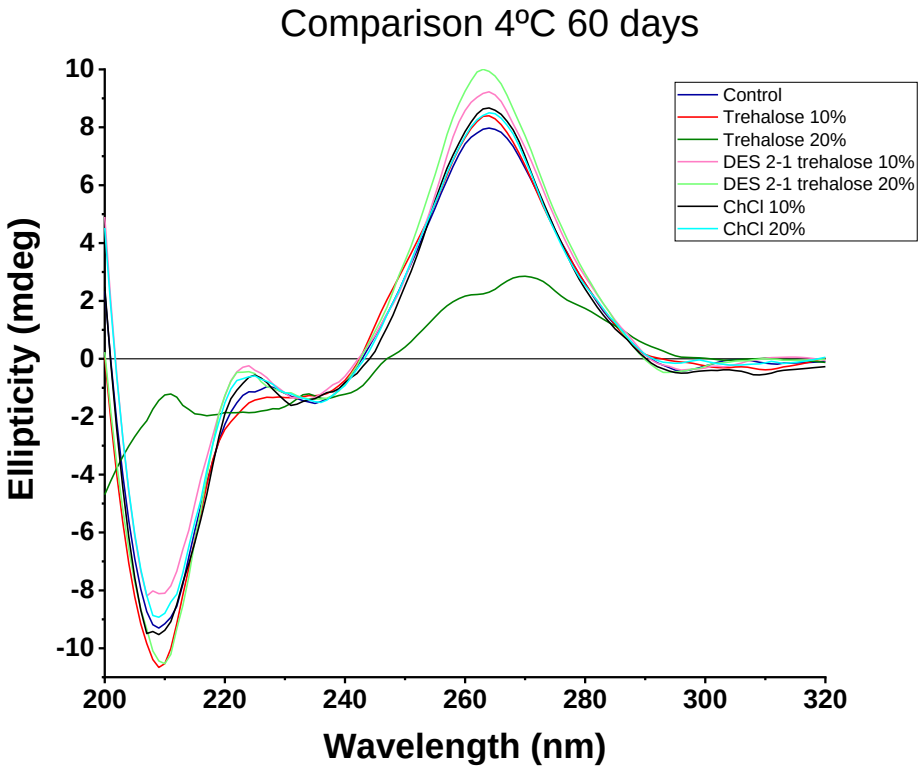


Figure 26 - Comparison of RNA stability in free trehalose, in with trehalose-based DES and in free ChCl, when stored at 4 ºC, for 60 days

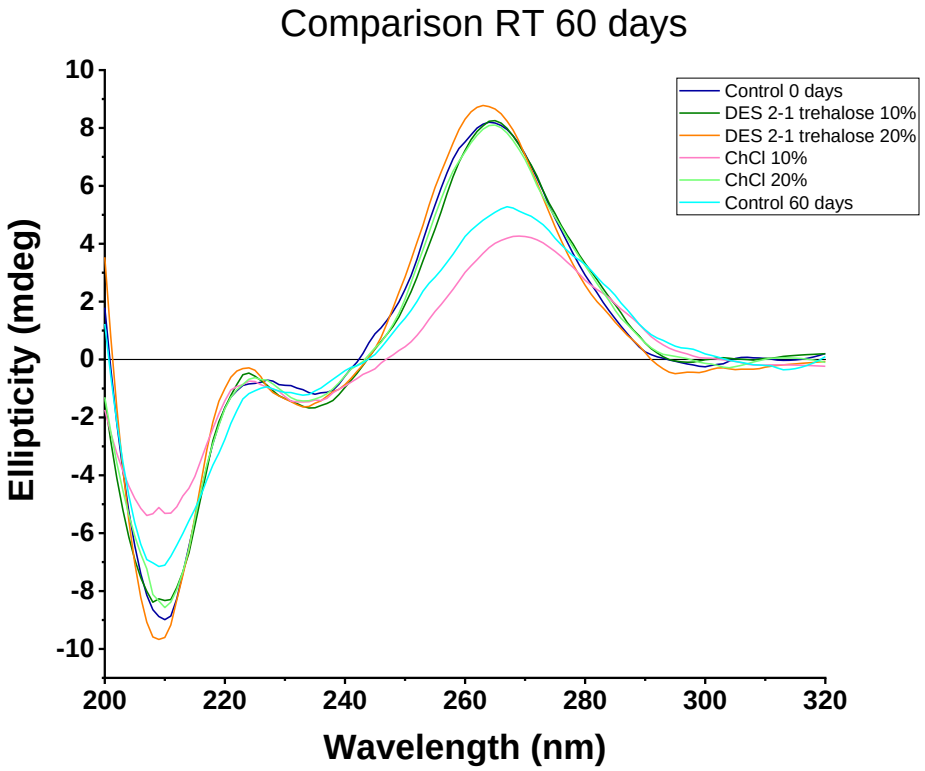


Figure 27 - Comparison of RNA stability in free trehalose, in with trehalose-based DES and in free ChCl, when stored at room temperature, for 60 days

Melting temperatures of RNA also showed an improvement when using DES trehalose compared to isolated trehalose. The melting temperature values vary from around 41 ºC at 0 days with trehalose to around 58 ºC with trehalose-based DES ,and 61 ºC with [Ch]Cl. Despite the higher values obtained with [Ch]Cl these seem to decrease over time at both concentrations, which does not happen at 20 % DES trehalose indicating that this solvent may induce a better thermal stability of RNA over time.

These results are also in acordance with the data obtained by molecular docking simulation which indicated that the complex formed by DES-RNA should have higher stability following this rank: [Ch]Cl:Trehalose > [Ch]Cl:Sucrose > [Ch]Cl:Glucose. The improved stability found for RNA solubilized in the trehalose-based DES in comparison to the glucose-based DES might be due to the number of interactions that occur between the two molecules. Trehalose is a bigger molecule and has more points of interaction making it a better candidate to stabilize the molecule. The high binding energies of HBA and HBD together, without superimposed bind spot on the RNA surface and an improvement of hydrogen bond ability on RNA nitrogen bases, induces stabilization of RNA structural motifs.

Overall free sugars did not have the best results as stabilizers at the temperatures tested, however when conjugated with ChCl to form a DES there was a clear improvement of RNA stability over time. This improved stability was particularly relevant in the trehalose-based DES where RNA samples not only maintained stability over the testing period but also had significant improvements in melting temperature as it was indicative by the data from the molecular docking simulations. The results from this work using innovative solvents as stabilizers opens a new door to various new formulation possibilities.

Chapter 4

4. Conclusion and Future perspectives

In recent years, the study of the RNA molecule has had an immense growth and has shown the potential of RNA as a therapeutic agent. Numerous RNAs, which have distinct structures, have been discovered and new functions have been identified. These can be used to regulate different cellular mechanisms and pathways, leading to gene expression or silencing. For the RNA molecule to exert its function, its stability must be maintained. RNA is considered to be unstable and susceptible to outside factors that can lead to molecule denaturation. Therefore, it has never been so important to understand the mechanisms that will help to maintain its stability, and which compounds will ultimately improve RNA stability in order to ensure its biological activity.

The present work had as a main objective to find stabilizers that would improve RNA stability for long term storage at easy to sustain temperatures. Circular Dichroism was used to attest RNA structural stability while in contact with various types of stabilizers, namely, amino acids, ionic liquids, sugars and deep eutectic solvents at temperatures of 4 °C and room temperature, for a 60 days period. Analyzing CD spectra of RNA incubated in amino acids containing solutions at concentrations of 1 and 4 mM it was found that the samples maintained the stability for 60 days at 4 °C and room temperature. Amino acid-based ILs, similarly to free amino acids, proved to have stabilizing capabilities, given that many of the samples tested also maintained stability for 60 days at both temperatures. On the other hand, samples incubated in sugar containing solutions did not maintain stability at room temperature, but some positive results were obtained at 4 °C, particularly for the concentration of 10 %. Given their common use as cryoprotectants, these results seem to suggest that the stabilizing effect might be greater at lower temperatures. Sugar based-DES also had promising results as stabilizers, as some of the RNA samples incubated in sugar-based DES were able to maintain stability through the 60 days of testing at room temperature, showing a clear improvement in comparison to free sugars.

Ultimately, the CD results indicate that the stabilizer candidates tested do have an effect on the stability of RNA and most of them positively influence molecule stability for long term storage at the temperatures tested. Overall, it is possible to say that the main goal of this work was achieved as many of the stabilizer candidates tested were able to stabilize the RNA molecule. However, there is room for improvement as not all of the

interactions between the molecule and the stabilizer are known and stabilizing mechanisms are not fully understood. Moreover, in this work we were also able to verify that RNAs with different structures may have diverse stability profiles over time, as it was evidenced by the dissimilar profile of sRNA and pre-miRNA at room temperature. This indicates that when formulating RNAs-based biopharmaceuticals, the structure of the biomolecule must be taken into account.

Thus, the next challenge can be the study of the stabilizing effect of these agents when in contact with other RNA molecules, differing on their native structure. Moreover, in the future it would be interesting to further confirm these results using other techniques to ensure molecule stabilization. Prolonging these studies would also be of value, taking into account the fact that the control RNA sample did not lose stability after 60 days at 4 °C. So, it would be important to determine, at which time point RNA is no longer stable, for a better understanding on the molecules long term stability. Furthermore cytotoxicity assays of the stabilizer candidates will be critical to validate their applicability in future biopharmaceutical formulations.

Chapter 5

5. Bibliography

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