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Nanodiamonds selectivity towards DNA and RNA: exploiting a tool for biopharmaceuticals capture

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Resumo

O potencial terapêutico do RNA tem vindo a ser estudado desde a sua descoberta no início do século XX. Não obstante, para que o RNA possa ser usado em tais aplicações tem de apresentar elevada pureza, integridade, assim como atividade biológica.

A produção recombinante de ácidos nucleicos é um processo complexo que envolve múltiplas etapas, e pode dividir-se em processamento upstream e downstream. Embora tenham sido feitas melhorias no processamento upstream, o mesmo não se observa diretamente no processamento downstream.

O processamento downstream é a parte de um bioprocesso no qual o produto obtido do processamento upstream é recuperado, concentrado e purificado. Este inclui três etapas principais: recuperação inicial ou purificação de baixa resolução, purificação de alta resolução, e polimento. Quanto maior o nível de pureza do produto após a fase de purificação de baixa resolução, maior a probabilidade de minimizar o número de passos de purificação de alta resolução, permitindo assim que haja uma redução de custos, uma vez que o processamento downstream representa cerca de 80% dos custos totais de produção de biofármacos.

Para avançar no estado-de-arte atual relativo à purificação de RNA, procedeu-se a uma otimização de um método de adsorção para a pré-purificação de RNA quando este está presente em amostras complexas de lisado de *E. coli*, utilizando microdiamantes, nanodiamantes e nanodiamantes funcionalizados como adsorventes.

Foi desenvolvido um método simples e eficiente, utilizando os materiais de carbono para a captura seletiva e recuperação de RNA de lisados de *E. coli* contendo impurezas como pDNA e proteínas. Em particular, os nanodiamantes oxidados foram os materiais que mostraram ser os mais promissores, permitindo alcançar uma capacidade de adsorção de 86.9 mg RNA/g de CM, não havendo presença de pDNA na amostra de RNA recuperada no final do procedimento, e atingindo uma eliminação de 91.28 % de proteínas solubilizadas da amostra recuperada relativamente à inicial.

Globalmente, os resultados deste trabalho demostraram que os nanodiamantes oxidados podem ser utilizados como adsorventes em extração de fase sólida dispersiva, sendo capazes de capturar seletivamente RNA e permitindo a sua completa recuperação sem contaminação.

Palavras-chave

Biofármacos; RNA; Extração de fase sólida; Purificação; Nanodiamantes; Microdiamantes; Recuperação; Seletividade.

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Abstract

The therapeutic potential of RNA has been unveiled since its discovery in the beginning of the 20th century. Notwithstanding, for RNA to be used for such applications, it has to present high purity, integrity, and biological activity.

The recombinant production of nucleic acids is a complex process involving multiple steps. It can be divided into upstream and downstream processing. While improvements have been made in upstream processing, the same level of progress is not directly observed for downstream processing.

Downstream processing, a crucial part of a bioprocess, involves the recovery, concentration, and purification of the product from the upstream process. It encompasses three main steps: initial recovery, high-resolution purification, and polishing. The higher the product purity level achieved in the low-resolution purification phase, the greater the potential for reducing high-resolution purification steps, thereby enabling cost reduction. This is particularly important, as downstream processing accounts for nearly 80% of the total biopharmaceutical production costs.

To advance on the current state-of-art for RNA purification, an optimization of an adsorption method for the pre-purification of RNA when it is present in complex *E. coli* lysate samples, using MDs, NDs, and functionalized NDs as adsorbents was carried out. A simple and efficient method, using the CM that showed to be the most promising, for the selective capture and recovery of RNA from *E. coli* lysates containing impurities such as pDNA and proteins, was developed. Particularly, ND-ox were the materials that showed to be the most promising, allowing the achievement of an adsorption capacity of 86.9 mg of RNA/g of CM, not being present pDNA in the RNA sample recovered at the end of the procedure, and achieving an elimination of solubilized proteins of 91.28 % relatively to the initial sample.

Globally, the results of this work demonstrated that ND-ox can be used as adsorbents in DSPE, being capable of selectively capturing RNA and enabling its complete recovery without contamination.

Keywords

Biopharmaceuticals; RNA; Solid-phase extraction; Purification; Nanodiamonds; Microdiamonds; Recovery; Selectivity

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List of Acronyms

asRNA	Antisense RNA
CD	Carbon Dots
CM	Carbon Material
CMV	Cytomegalovirus
CNH	Carbon Nanohorns
CNM	Carbon Nanomaterial
CNO	Carbon Nanoonion
CNT	Carbon Nanotubes
CO	Carbon Onions
CVD	Chemical Vapor Deposition
DND	Detonation Nanodiamond
DSPE	Dispersive Solid-Phase Extraction
E. coli	*Escherichia coli*
ELISA	Enzyme Linked Immunosorbent Assay
FDA	Food and Drug Administration
FI	Fluorescence Imaging
GO	Graphene Oxide
GQD	Graphene Quantum Dot
HCPs	Host Cell Proteins
HPLC	High- Performance Liquid Chromatography
IEC	Ion-Exchange Chromatography
IU	International Unit
Li-ion	Lithium-ion
LLE	Liquid-Liquid Extraction
lncRNA	Long non-coding RNA
LPS	Lipopolysaccharide(s)
MD	Micro-diamond
MEF	Mouse Embryonic Fibroblast
miRNA	microRNA
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MWCNT	Multi-walled Carbon Nanotubes
ncRNA	Non-coding RNA
ND	Nanodiamond
NIR	Near-Infrared
OD	Optical density
PCR	Polymerase Chain Reaction
pDNA	Plasmid DNA
Ph. Eur	European Pharmacopeia
PTT	Photothermal Therapy
rGO	Reduced Graphene Oxide
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
SCs	Solar Cells

SDS	Sodium Dodecyl Sulphate
siRNA	Small interfering RNA
snoRNA	Small Nucleolar RNA
snRNA	Small Nuclear RNA
SPE	Solid-Phase Extraction
SWCNT	Single-walled Carbon Nanotubes
tRNA	Transfer RNA
UV	Ultraviolet

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Chapter 1

Introduction

1.1. Biopharmaceuticals

Biopharmaceuticals, alternatively referred to as biologics or biotechnology medicines, are biological compounds with therapeutic activity (pharmaceuticals) produced by or extracted from living organisms through biotechnological processes [1].

In contrast to traditional drugs synthesized through chemical processes, biopharmaceutical products derive from biological processes. They are produced through recombinant DNA technologies and extracted from living systems [2]. Different organisms are commonly used to produce biopharmaceuticals, such as bacteria, yeast, viruses, animal or human cells, animals, and plants that have been genetically modified [1, 2].

Therapeutic insulin is an example of a biopharmaceutical currently produced using recombinant DNA technologies. In the past, it was extracted from porcine pancreatic islets and, at present, is produced by *Escherichia coli* (*E. coli*) or by the yeast *Saccharomyces cerevisiae*. Notably, the recombinant human insulin produced by *E. coli* was the first biopharmaceutical approved by the U.S. Food and Drug Administration (FDA) in 1982. Biopharmaceuticals produced by recombinant DNA technologies encompass:

- Fusion proteins [3, 4];

- Monoclonal antibodies [5, 6];

- Interferons [7, 8];

- Therapeutic Enzymes [9, 10];

- Hormones [8, 11, 12];

- Growth factors [13];

- Vaccines [14, 15];

Biopharmaceuticals development and clinical application are rapidly growing, thus confirming their potential as new options for severe disease treatment [16].

According to the latest survey of biopharmaceutical approvals conducted by Gary Walsh and Eithne Walsh, between January 2018 and June 2022, 197 biopharmaceutical products were approved in the US and/or EU. In 2022, the number of biopharmaceutical products with active licenses is estimated to be 443 [17].

1.2. RNA as a biopharmaceutical

Nucleic acids, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) are natural biomolecules present in all living organisms that play fundamental roles in various biological processes. They have been studied for several years to be used as therapeutic agents for the treatment of human diseases related to genetic disorders, including cancer and genetic diseases, as well as in the production of highly effective vaccines to prevent infectious diseases [18].

RNA has a broad spectrum of functions, which include the participation in protein synthesis (messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA)), regulation of gene expression (small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), and long non-coding RNA (lncRNA)), and post-transcriptional gene silencing (microRNA (miRNA) or anti-sense RNA (asRNA)) [19]. Some RNA molecules can control the expression and activity of specific target molecules, thus providing the means to treat diseases that do not respond to conventional drug therapies. The use of RNA-based molecules to modulate biological pathways to treat or prevent diseases is known as RNA therapy [20].

Unlike traditional therapeutics, RNA-based therapies offer a paradigm shift in healthcare. They are designed to fix the underlying pathology instead of merely treating the symptoms due to the possibility of reestablishing a biological function. Depending on the methods used, RNAs as therapeutic agents can be more cost-effective and easier to develop than traditional small molecule- or protein-based therapeutics. This not only makes them a promising option for the future of medicine but also holds the potential to revolutionize healthcare [21, 22].

The first RNA therapy, Fomivirsen, was approved in 1998 by the FDA to treat cytomegalovirus (CMV) retinitis [23].

Fomivirsen is an antisense oligonucleotide used for the intravitreal treatment of CMV retinitis in HIV-infected patients who cannot tolerate or have not responded to other drugs [24]. Since then, several RNA molecules have been studied and are currently being evaluated in pre-clinical or clinical trials with different therapeutic applications.

RNA therapy can be categorized based on the chemistry of RNA molecules as [20]:

1- **Antisense RNA (single-stranded RNA) (asRNA)** is used to silence genes and inhibit their expression [25]. asRNA has complementary sequences to the target molecules, such as mRNA. By binding to it, it modulates the splicing of pre-mRNA or induces the degradation of mRNA [26, 27].

2- **Small interfering RNA (double-stranded RNA) (siRNA)**, also known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules containing 20–25 nucleotides. They contribute to the post-transcriptional regulation of gene expression [28]. siRNA is used as a technique for the knockdown of lncRNAs when it is intended to determine their function [29]. The first siRNA agent, Patisiran, received the FDA approval in 2018. To date, the FDA has approved four siRNA agents: Patisiran, Givosiran, Lumasiran, and Inclisiran [27, 30].

3- **RNA aptamers** are short RNAs that bind nucleic acids or proteins and can thus modulate intracellular processes [31]. They can bind to a specific protein and block its function [32]. Similar to antibodies, they can inhibit, modulate, and effectively disrupt target proteins functions, turning them into promising therapeutic agents for treating various diseases and targeted drug delivery [33].

4- **mRNA:** after being introduced into the cell, cellular machinery, translates mRNA into a protein [27]. That protein can act as an antigen and induce an immune response or replace a mutant protein whose function is compromised. mRNA is also used in immunotherapy (mRNA-based immunotherapy) [34]. Furthermore, the development of scientific areas such as biotechnology, molecular biology, immunology, and genetic engineering enabled the creation of prophylactic nucleic acid-based vaccines. Nucleic acid-based vaccines are non-live vaccines based on nucleic acids such as DNA and RNA. These vaccines mimic infection by a pathogen and safely induce immune responses in the organism. RNA vaccines are potentially effective in cases of emerging and re-emerging infectious diseases, as demonstrated by the prevention and attenuation of COVID-19 with the mRNA vaccines BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna) [21, 35]. As the name implies, RNA vaccines are composed of the nucleic acid RNA, which encodes genes of an infectious agent. When administered to host cells, the RNA is translated into antigenic proteins that trigger the immune system to produce a protective response against the infectious agent [36].

Each type of RNA molecule targets different genetic material within the cells [27]. Consequently, a diverse array of different RNA therapies can be applied for different purposes. Figure 1 is a schematic representation of the mechanisms mentioned above.

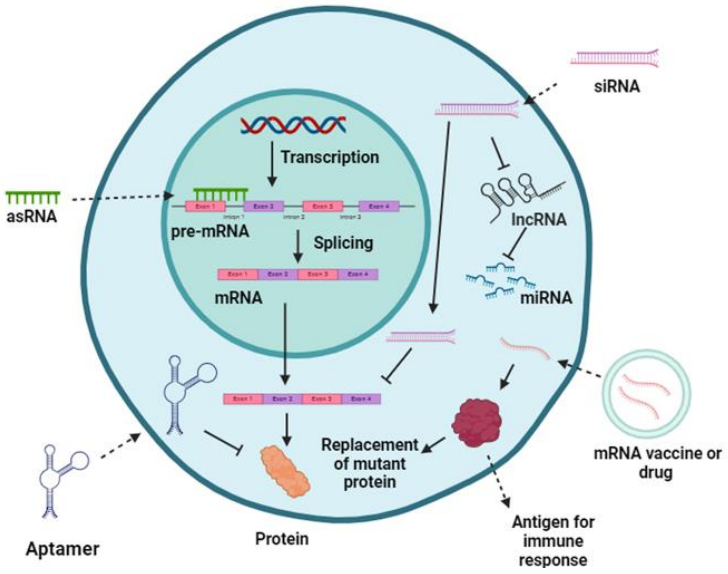

Figure 1. Main cellular events associated to RNA therapy. Adapted from [20].

tRNA is another type of RNA that has gained significant attention in recent years due to its therapeutic potential for the treatment of genetic diseases associated with mutations that alter mRNA translation, as they have crucial roles in protein synthesis. The tRNA molecule plays a crucial role in protein synthesis by linking a specific codon in mRNA with its corresponding amino acid [37]. There is a type of mutation that leads to premature termination of mRNA translation. Research has been done to engineer tRNAs to read through these mutations and restore protein synthesis and function. This innovative approach shows promise in the field of genetic disease treatment [38]. Different legal criteria cover RNA drugs, yet harmonization needs to be improved. According to the legislation followed in the European Union (EU), RNA-based products used as vaccines can be categorized depending on their targets (infectious or non-infectious diseases), and for non-vaccine drugs, the classification is determined by the type of RNA substance and its production into chemical (currently chemically synthesized [39]) or biological medicinal products (products whose active substance is a biological substance). A biological substance is defined as "a substance that is produced by or extracted from a biological source and that needs for its characterization and the determination of its quality a combination of physical-chemical-biological testing, together with the production process and its control". Two subcategories of biological medicinal products related to RNA drugs are Immunological medicinal products including vaccines, and Advanced Therapy Medicinal Products. An immunological medicinal product is defined as "any medicinal product consisting of vaccines, toxins, serums or allergen products." As for vaccines, they are defined as "agents used to produce active immunity". Advanced therapy medicinal product (ATMP) is defined

as any of the following medicinal products for human use: a gene therapy medicinal product (GTMP), a somatic cell therapy medicinal product (sCTMP), or a tissue-engineered product (TEP) [39].

Figure 2 represents which types of RNA are included in each category.

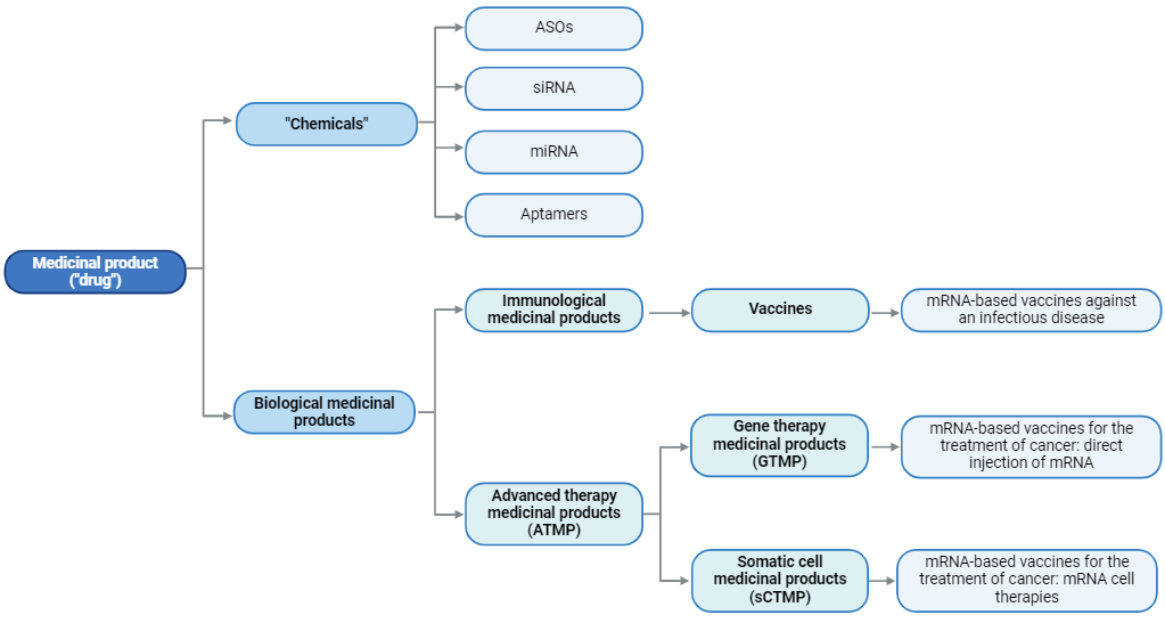

Figure 2. Categorization of RNA-based products according to the type of RNA substance, its production, and target.

1.3. Biopharmaceutical production

Biopharmaceutical production, known as bioprocess, is a multidimensional and complex process that comprises various steps. These, involve cell culture systems based on bacteria, yeast, other living organisms or animal cell culture, the recovery and purification of the product of interest, and a wide range of techniques [1, 16]. Globally, the production of biopharmaceuticals can be divided into upstream and downstream processing as represented in Figure 3.

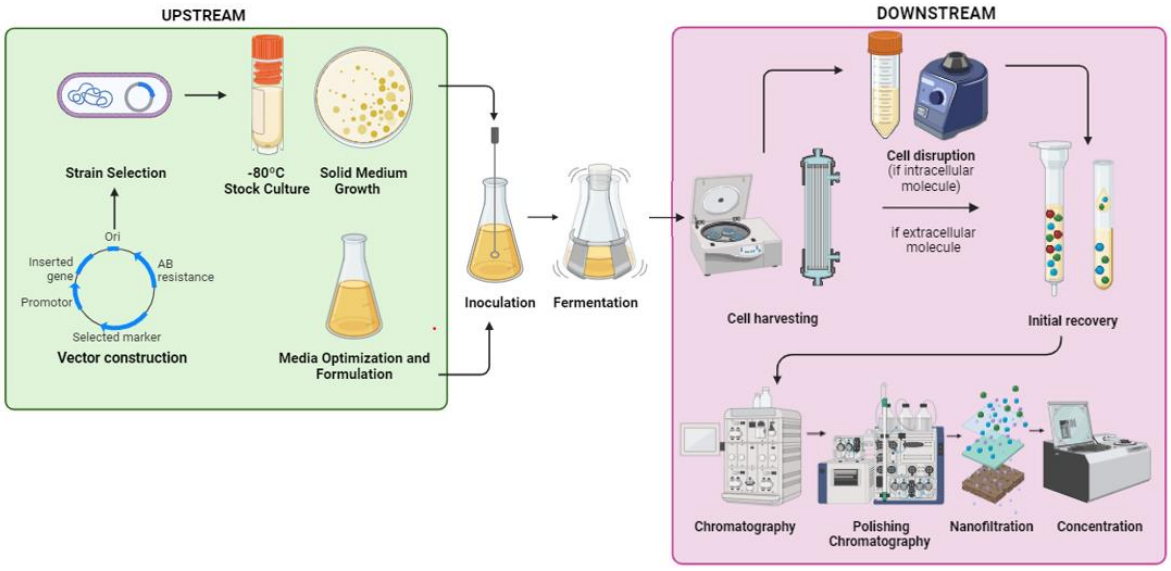

Figure 3. Upstream and downstream procedures used in biopharmaceuticals production.

1.3.1. Upstream processing

The upstream process is mainly focused on the microbial growth required to produce recombinant biopharmaceuticals or other biomolecules [40]. When referring to biopharmaceuticals, the upstream process includes: i) selecting the bacterial strain or cell line to be used as a host for the heterologous production of the biopharmaceutical of interest; ii) selecting the adequate culture media and growth parameters (temperature, pH, oxygen percentage, mode of operation); iii) growing those cells; and iv) optimizing the process to obtain optimal conditions for cell growth and biopharmaceutical production [41].

1.3.2. Downstream processing

Downstream processing is the part of a bioprocess in which the product from the upstream process is recovered [42], concentrated, and purified [43]. It comprises the following steps:

- initial recovery (harvesting and low-resolution purification, also referred to as extraction, isolation, or capture);
- high-resolution purification ((or intermediate purification) that consists in the removal of most contaminants);
- polishing (removal of specific contaminants and unwanted forms of the target biomolecule that could have been formed during extraction and purification).

The goal of downstream processing is to isolate the target product from a mixture while also removing any contaminants that could interfere with its intended use [44]. This creates a final purified product that meets stringent safety and quality standards [1, 40].

While the upstream process mainly depends on biological limits, such as cell line and media optimization, which can be raised without an increase in costs [45], downstream processing is considered one of the most challenging stages of biopharmaceutical manufacturing, accounting for nearly 80% of the total production costs [46], mainly due to the high purity required for biopharmaceuticals (for pharmaceutical applications, product purity mostly is >99 %) [16]. For that reason, an ineffective downstream process can significantly increase the cost of the whole manufacturing process [47].

Although process improvements have been made in the upstream processing, the same is not directly observed for downstream processing [48]. Thus, there is an urgent need to develop efficient and economical downstream processing strategies with fewer steps [49], maximize product recovery and purity, and minimize production costs [50]. The selection and optimum combination of purification techniques for low-resolution purification, high-resolution purification, and polishing steps is crucial to balance recovery, purity, time, and costs [51].

Depending on the nature of the product and its synthesis method, different techniques or combinations of them can be applied in each step of downstream processing [52]. The following paragraphs summarize such techniques.

- **Initial recovery or low-resolution purification**
  - Harvest
    - ✓ Cell lysis is only performed if the recovery of intracellular components is intended. It is crucial to release intracellular components such as nucleic acids, proteins or metabolites [53].
    - ✓ Centrifugation is a method of separating molecules with different densities by spinning them in solution around an axis (in a centrifuge rotor) at high speed [54]. It is also a very efficient cell harvesting technique [55].
    - ✓ Filtration, in downstream processing, refers to any process in which a liquid feedstock is forced to pass through a selectively permeable medium so that only specific feed components pass through (into the permeate or filtrate). In contrast, other components are retained (the retentate) [56], enabling the separation of solid particles from a fluid [57].
  - Isolation

- ✓ Solid-phase extraction (SPE): is employed to isolate and concentrate analytes from a liquid sample by their transfer to and adsorption onto a solid stationary phase [58].
- ✓ Liquid-liquid extraction (LLE): is used to separate chemicals from one solvent to another based on the different solubility of a solute in two solvents that are immiscible or partially miscible with each other [59].
- ✓ Precipitation is a process used to separate compounds from a mixture [60] by their differential solubility in a solvent [61]. The precipitation of the desired product can be accomplished by pH [62] or temperature variations [63], or by adding a precipitant agent, such as salts, polymers or organic solvents [64].

- Buffer exchange and up-concentration
  - ✓ Ultrafiltration: is a membrane filtration technology in which membranes with a pore size between 0.001–0.05 μm [65] separate microscopic particles and dissolved molecules from fluids.
  - ✓ Diafiltration: is an operation mode of a pressure-driven membrane filtration process in which a solvent is added to the solution to be processed to facilitate the separation of macrosolutes from microsolutes [66]. It is used to adjust buffer concentrations before or after a chromatography step [67].

- **Intermediate recovery or high-resolution purification**
  - Chromatography
    - ✓ Ion exchange chromatography (IEX): is a type of chromatography where ions or polar molecules are separated by their interactions with oppositely charged ion exchange groups immobilized on a support [68]. The separation is based on differences in charge properties [69].
    - ✓ Hydrophobic interaction chromatography (HIC): is a liquid chromatography modality used to separate and purify biomolecules by their hydrophobic interaction with the hydrophobic ligands coupled to porous media [70].

- ✓ Reversed-phase chromatography (RPC): the interaction (partitioning) between the stationary phase and solutes is controlled by changing the polarity of the mobile phase [67]. The compatibility with the biomolecules integrity must be assessed due to the use of organic solvents.
- ✓ Affinity chromatography (AC): is a liquid chromatographic method in which a biological agent or biomimetic ligand is used to selectively retain biorecognized compounds [71].

- **Polishing**
  - Polishing chromatography
    - ✓ Affinity chromatography (AC)
    - ✓ Size-exclusion chromatography (SEC) separates individual components from a mixture based on their sizes [72], with particular relevance on the elimination of fragments or aggregates.
    - ✓ Ion exchange chromatography (IEX)
    - ✓ Hydrophobic interaction chromatography (HIC) [73].

1.3.2.1. Precipitation

Precipitation is a simple, scalable and relatively economical procedure for the recovery and purification of products [16, 64].

This method is used to separate biological products from a complex medium. The precipitation of a biological product can be achieved by adding a precipitation agent (salts, organic solvents, polyelectrolytes, non-ionic polymers, affinity ligands and metallic ions) or by changing the solubility conditions of the solution, such as pH, temperature and ionic strength [74]. Over recent years, the method has gained interest in the downstream processing of high-value biopharmaceutical products, such as nucleic acids [64].

The precipitate can be the target product or an impurity, which can be recovered or removed by a solid-liquid separation step [74].

Precipitation is generally induced by high salt concentrations [75] or by the addition of either isopropanol [76] or ethanol [77], being commonly used to concentrate [78], desalt [79], and recover nucleic acids [80].

1.3.2.2. Liquid-liquid extraction (LLE)

LLE, also known as partitioning, is a separation process based on the principle of differential solubility of a solute between two solvents. Frequently, one of the solvents is an organic liquid (non-polar) and the other is water or an aqueous solvent (polar) [81]. It consists of the transfer of the solute from one solvent to another, being the two solvents immiscible or partially miscible with each other [82].

This process is widely employed in the biotechnological, pharmaceutical, and chemical industries in sample preparation (for cleanup of biological samples, such as blood, serum, and urine, before their analysis by LC or GC [83], in the purification of biomolecules [84] and isolation of antibiotics from fermentation broths [85].

The purification of biomolecules using LLE has been successfully carried out on a large scale for over a decade. An advantage of using this system is the low cost of chemicals [84]. The phenol-chloroform extraction of RNA performed in this work is an example of an LLE method [86].

LLE provides high extraction efficiency [87] and recovery [88, 89]. Nevertheless, some practical drawbacks associated with LLE exist, such as large consumption of solvents that potentially threaten human health and the environment [90], long extraction times [91] and emulsion formation [90].

1.3.2.3. Solid phase extraction (SPE)

Solid phase extraction (SPE) is a procedure developed to rapidly separate a target analyte from a complex sample before chromatographic analysis, such as HPLC and GC [92]. SPE is used for the purification of different types of samples: biological [93], pharmaceutical [94], clinical [95], food [96], and environmental ones [95]. This technique is widely used to extract emerging contaminants from solid and liquid samples [97] or to isolate and concentrate the analyte of interest [98].

An SPE method typically consists of three or four successive steps, as represented in Figure 4. The first step is equilibrating the solid-phase materials (silica-based, carbon-based, or clay-based)[99] by passing water or other solvents through the column [100], to remove possible contaminants from the sorbent and ensure good wettability of the packed material. The second step consists of loading the sample, which passes through a column, a tub, or a disk containing an adsorbent that retains the analytes. The optional washing step aims to

selectively remove unwanted interferences co-extracted with the analyte that the solid sorbent has retained without eluting the analyte of interest. After all of the sample (or washing solution - if the washing step is performed), has been passed through the sorbent, retained analytes are recovered upon elution with an appropriate solvent [101].

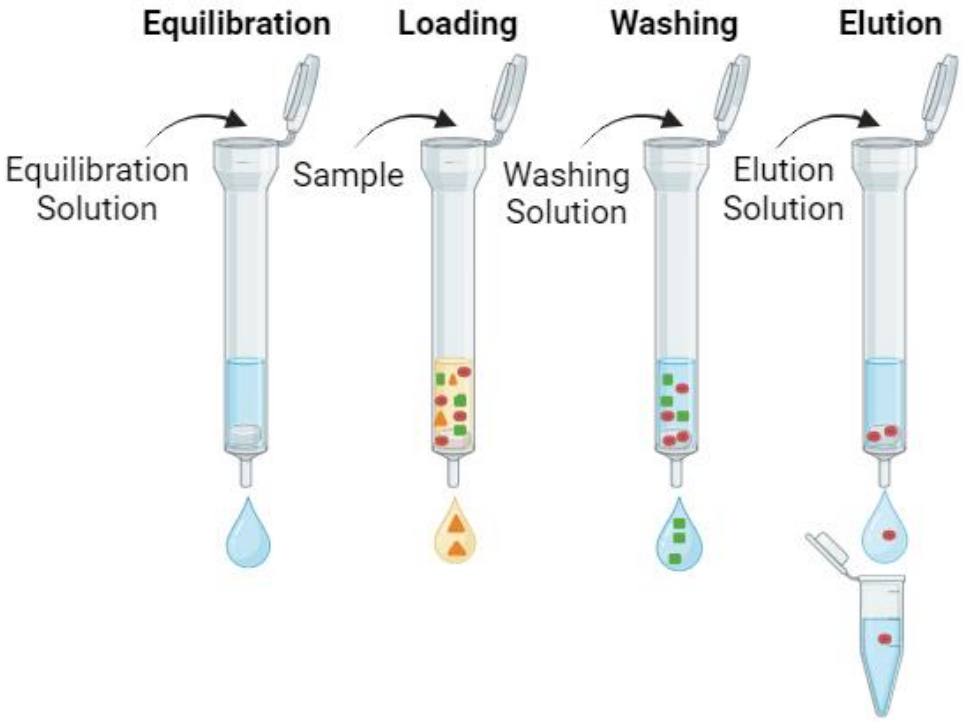

Figure 4. Steps of the conventional SPE procedure: equilibration, loading, washing, and elution.

Between different extraction /desorption cycles, a regeneration step should be performed with an appropriate solution (regeneration buffer) to avoid incomplete elution of the target analytes that could limit subsequent applications of the material [102].

The recovery of the molecule of interest is the objective of the elution step of extraction procedures such as solid-phase extraction (SPE). The more selective the step of SPE, the more sensitivity will be acquired, so it is essential to develop and optimize SPE procedures [103]. Employing SPE as a sample preparation method before chromatographic analysis can reduce harmful compounds introduced into the chromatography system [104], thereby extending the lifespan of the analytical column and instrument [105].

Despite the widespread use of the technique, SPE approaches can result in poor recovery, consistency issues, and insufficiently clean sample extracts. These problems may cause further challenges to the downstream processing. For that reason, it is fundamental to optimize SPE techniques [58].

SPE has various modes, namely, conventional SPE, dispersive SPE (DSPE), solid-phase microextraction (SPME), magnetic SPE (MSPE), pipette-tip SPE (PT-SPE), matrix solid-phase dispersion (MSPD), and stir-bar sorptive extraction (SBSE).

With regards to conventional SPE, DSPE is considered a beneficial sample preparation method due to its simplicity and high recovery. The main difference between these two modes is that in conventional SPE, a column is packed with a solid adsorbent, the sample to be tested is loaded to flow through the column, and the target compounds can be adsorbed and separated. DSPE is an alternative to conventional SPE and does not need an SPE column, extraction disc, or cartridge since the adsorption is based on the dispersion of a solid sorbent in a liquid sample [106]. The washing step is unnecessary, making the extraction process more environmentally friendly with less solvent consumption [107].

Additionally, the extraction time is shorter, and the adsorbent can be more fully dispersed into the sample solution, which improves the adsorption effect. The supernatant can be removed or analyzed after the purified sample is shaken and centrifuged. This method proves to be quick, easy, cheap, effective, rugged, and safe and is also known as the QuEChERS method (Quick, Easy, Cheap, Effective, Rugged, and Safe) [106].

In 1989, McCormick *et al.* introduced a new DNA extraction method involving SPE. Nowadays, SPE is one of the most efficient nucleic acid extraction techniques available in the market [108]. Nevertheless, developing novel solid-phase materials with nucleic-acid-binding ability is required [109]. In 2023, Ferreira *et al.* established a DSPE method to capture RNA and other impurities from complex *E. coli* lysates using pristine MWCNTs, to purify pDNA extracts [110]. In the present work, DSPE was chosen to capture and recover RNA from complex *E. coli* lysates using oxidized NDs. Figure 5 represents the DSPE steps: dispersion and adsorption, separation, desorption and separation.

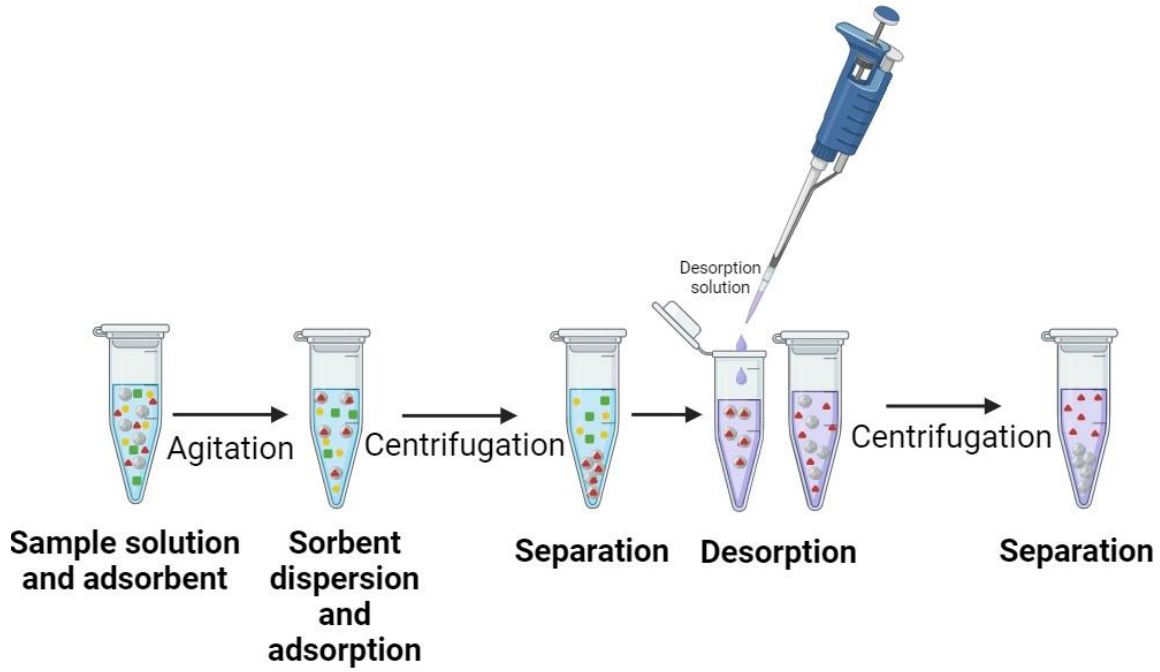

Figure 5. DSPE steps: dispersion and adsorption, separation, desorption, and separation.

1.3.2.4. Differences between SPE and LLE

The basic principles of SPE and LLE are similar. Both methods involve the distribution of dissolved species between two phases [99]. Nevertheless, SPE offers better selectivity and versatility than other sample preparation techniques. Compared with LLE, it is quicker, requires less solvent, avoids the use of organic solvents, sample volumes can be smaller, prevents the possible formation of emulsions, and is much more amenable to automation [94].

Regarding the analysis of biological samples, SPE has a crucial advantage over LLE since it enables a higher recovery (80-100%) with high reproducibility. The higher recovery makes the assay more sensitive, enabling a smaller sample to be successfully measured. In addition, biological samples may undergo the SPE procedure directly without any pretreatment, thus simplifying the extraction procedure [94].

The main differences between these two processes are summarized in Table 1.

Table 1. Differences between solid-phase extraction and liquid-liquid extraction (adapted from [111]).

	SPE	**LLE**
Separation	Involve partition between a liquid phase and a solid phase (adsorbent)	Two immiscible liquid phases

Cost	Expensive cartridge	Less expensive
Handling technique	Less manual effort	Labor intensive
Rate of separation	Faster	Slower
Selectivity	High	Lower
Sustainability	Low solvent consumption minimizing the environmental impact	Extensive organic solvent usage poses a potential threat to human health and the environment

1.3.2.5. Adsorption

Adsorption is a phenomenon of solute attachment to a surface [70]. The molecules that interact and attach to the solid surface are called adsorbates, while the solid surface is called adsorbent. In adsorption, species are selectively transferred toward the adsorbent surface based on the nature of the bonds created between the adsorbed species and the functional groups present on the sorbent structure [112]. Depending on the nature of the bonds, the adsorption is classified as physical or chemical. Physical adsorption is based on weakly intermolecular attractive forces like van der Waals forces. Chemical adsorption relies on the establishment of chemical attachments between the adsorbing molecule and the functional moieties of the surface of the adsorbent [113, 114].

Generally, adsorption is reversible, being the reverse process called desorption [115]. Adsorption operations are widely applied to remove or recover specific substances [70]. This phenomenon is the base of SPE techniques since the separation of analytes occurs through their adsorption on the surface of sorbents. When the molecules to be adsorbed are neutral or nonpolar, van der Waals forces may be responsible for their adsorption. Adsorption has been used to remove contaminants since it is relatively cheap and does not produce secondary contamination [112].

Figure 6 represents different adsorption mechanisms used for the removal of contaminants.

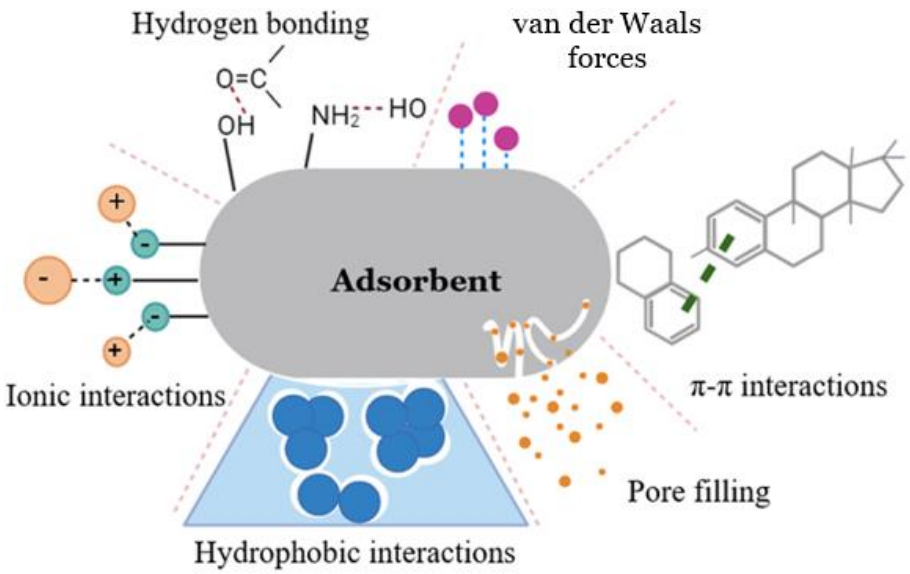

Figure 6. Different adsorption mechanisms. Adapted from [112].

1.3.2.6. Desorption

After the adsorption process, the primary step is desorption, which can be achieved by solvent or thermal changes to efficiently desorb the analyte from the sorbent [116]. The selection of a desorption method depends on different factors, such as the physical and chemical characteristics of the material used as adsorbent, the chemical nature of the adsorbate, and mainly the adsorption mechanism that governs the adsorbate–adsorbent interaction [112].

If electrostatic interactions mainly govern the adsorption, two strategies can be employed:

- a pH modification to neutralize compound and/or sorbent functional groups;
- an increase in salt concentration (ionic strength) to compete for ion-exchange binding sites [117].

On the other hand, if the adsorption mechanism is based on hydrophobic interactions, desorption may be accomplished through:

- a decrease in salt concentration [118] or eluent polarity [119];
- the addition of surfactant agents (detergents) [120] or chaotropic salts;
- a pH variation;
- a temperature modification.

In any case, it is necessary to consider the stability of the target molecule. For instance, when the desorption of nucleic acids is intended, high temperatures do not only unfold RNAs but can also affect their primary bonds by hydrolyzing the RNA, irreversibly damaging its integrity [121]. For that reason, other desorption methods must be employed. In this work, the effects of varying salt concentration as well as pH were tested when promoting electrostatic interactions. When hydrophobic interactions were promoted, two out of the four strategies referred to were used, namely by controlling the ionic strength of the buffer, and by adding a surfactant agent.

1.4. Biopharmaceuticals quality control

The manufacturing of pharmaceutical products is one of the most strictly regulated and rigorously controlled [122]. Quality control (QC) refers to all procedures undertaken to ensure the purity and identity of a pharmaceutical, with a particular focus on the sample composition, concerning any of its components, and dosage [123]. Manufacturers must demonstrate the elimination of host cell impurities and contaminants during biopharmaceutical development to ensure drug purity, manufacturing process consistency, and patient safety [124].

Impurity is any component of the drug product that is not the chemical entity defined as the drug substance or an excipient in the drug product [125]. Contaminants can be broadly defined as any adventitiously introduced materials (chemical, biochemical, or microbial species) that are not part of the manufacturing process. Potential impurities and contaminants can come from host cells, the raw materials, and the manufacturing process. Impurities and contaminants, depending on their nature, could be toxic and might induce an unwanted biological response or alter the integrity, efficacy, potency, stability, and hence the quality of the product [126].

Nucleic acid contaminants have theoretical adverse effects, such as potential integration into the host genome [127]. Other living cell wall components, such as polysaccharides, can also sometimes be of regulatory authority concern [128].

Both chemically synthesized drugs and biopharmaceuticals must be assessed for the safety risk of process-related impurities. Nevertheless, compared to chemically synthesized drugs, biopharmaceuticals present more impurities derived from the living organism used as an expression system. For that reason, there are special quality control concerns in biopharmaceutical manufacturing and the quality of biopharmaceutical products [128].

Besides toxicity concerns of the use of organic solvents, especially in chromatographic processes to purify the biopharmaceutical, and the possibility of leakage of metallic elements from product-containing surfaces, there are safety concerns to be considered about mutagenicity associated with the integration of residual cellular DNA into the host genome or the transmission of activated oncogenes or latent infectious viral genomes [129], immunogenicity induced by residual host cell proteins or cell membrane components, like endotoxins in the case of gram-negative bacteria, and undesired biological activity if residual antibiotics from the cell culture process are present [128].

It is of utmost importance that pharmaceutical analysis and quality control testing comply with international pharmacopeia specifications from the beginning of drug development until their release on the market [130]. Table 2 summarizes the quality control parameters that a biopharmaceutical must meet before being released on the market.

Table 2. Quality control parameters of biopharmaceuticals. Adapted from [131].

Quality control parameter	**Laboratory assessment method**	**Acceptance criterion**	**Guideline**
Purity	-	>98.5% or >98%	USP<891> Ph. Eur. 2.2.34
Sterility	Culture	No growth	Ph. Eur. 2.6.1 USP <71>
Endotoxin (LPS)	LAL Test (Rabbit) pyrogen test Monocyte activation test	<5.0 IU/Kg/h (body weight) <0.5°C increase in 3h Reaction less than the reaction induced by the allowed endotoxin contamination	Ph.Eur. 2.6.14, USP <85>
Host cell impurities	Identification and quantification		

Residual DNA	PCR/hybridization	<10 ng/dose/upper limits, as appropriate	Ph.Eur. 01/2008:0784, USP <1045>
Proteins (HCPs)	Immunoassay (ELISA), Western Blot	Strict upper limits, as appropriate	Ph.Eur. 01/2008:0784, USP <1045>
Virus	-	Maximal clearance	Q5A, Ph.Eur. 01/2008:0784, USP <1045>

1.5. Carbon materials

Carbon is a chemical element considered the backbone of all life forms and is one of the most notable, abundant, and versatile periodic table elements. The carbon atom possesses an unequal ability to form covalent bonds with up to four different atoms, making it a versatile element and a central part of biological molecules [132]. When one or more carbon atoms are covalently linked to atoms of other elements, most commonly hydrogen, oxygen or nitrogen, they constitute an organic compound. Nevertheless, certain carbon compounds (such as carbonates and cyanides) are classified as inorganic [133].

Carbon, in addition to its role in organic chemistry, also plays a crucial role in the creation of various inorganic materials, such as carbon materials and composites [134]. This unique property of carbon allows it to act as a bridge, facilitating interactions between inorganic and organic compounds. This bridging function is particularly relevant in the context of biomedical applications as highlighted by Hurtado and coworkers (2020) since carbon is the main constituent of living organisms [135].

Regarding its electronic structure, the carbon atom has the capability to organize its four valence electrons in different hybridization states: sp-, sp^2-, and sp^3, thus giving rise respectively to linear, planar and three-dimensional configurations in carbon-based molecules (such as alkynes, alkenes, and alkanes respectively), as well as in condensed matter (isotropic and anisotropic structures of diamond and graphite) [136, 137].

The different hybridizations enable carbon atoms to assume various allotropic forms, stable from zero to three dimensions, which include fullerenes (0D), carbon nanotubes (1D), graphene (2D), graphite (3D), diamonds (3D), among others [137]. Combining different hybridization states or incorporating foreign atoms into carbon hybrids enables researchers to tailor and modify the desired properties. Researchers who work with carbon materials

have implemented this strategy for a considerable time [132]. Over recent years, research on carbon-based materials, especially those of nanometre dimensions, has experienced exponential growth [138].

1.5.1. Synthesis methods

The synthesis of Carbon Materials (CMs) can be accomplished using several top-down and bottom-up synthesis approaches [42]. The top-down approach refers to the division of the starting material to generate nanosized structures and the bottom-up approach implies starting with precursor atoms or molecules and assembling them to form nanostructures [139]. Synthesis methods commonly used to synthesize CMs are represented in Figure 7.

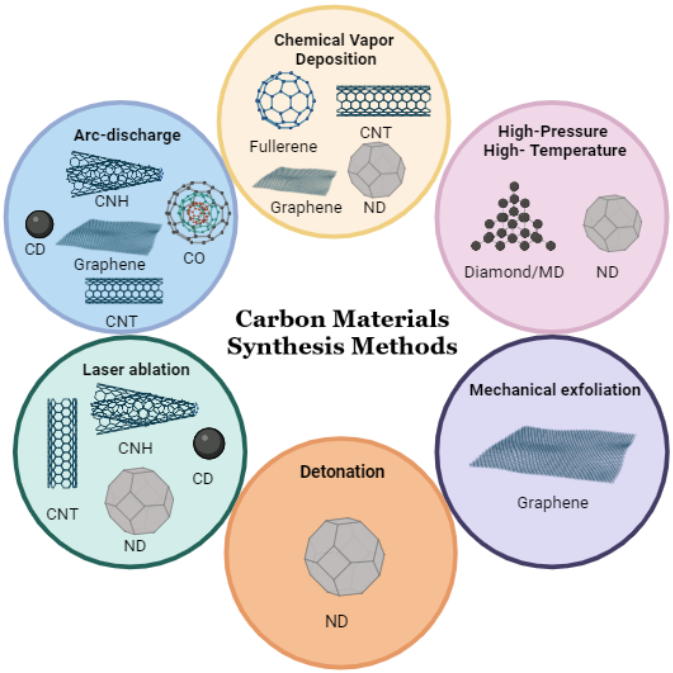

Figure 7. Different methods for the synthesis of carbon materials.

Arc discharge, laser ablation [140], and mechanical exfoliation [141] are top-down approaches. Among top-down approaches, the arc discharge method was one of the first techniques used to produce high-quality carbon-based nanostructured materials, such as carbon nanotubes (CNT), carbon dots (CD), graphene, carbon onions (CO) and carbon nano horns (CNH). It was introduced in 1991 in the production of CNT [142, 143]. The conditions under which arc discharge is performed are fundamental to achieving new forms of nanomaterials. This technique involves establishing a direct current between a pair of

graphite electrodes under an inert gas (helium or argon) atmosphere at about 500 torr [144].

Laser ablation is the most employed laser method for the synthesis of nanomaterials. It is a top-down approach that can be performed in a vacuum, liquid, or controlled gaseous atmosphere. It allows precise control of the dimensions and shapes of the produced nanomaterials and tuning their properties by changing the laser characteristics [145]. CNT [146], CNH [147], CD [148], and ND [149] can be synthesized through this method.

Mechanical exfoliation is a representative top-down strategy widely used for preparing high-quality 2D materials such as graphene [141]. The exfoliation of graphite to give graphene is one of the most promising ways to achieve large-scale production at a low cost [150]. In this approach, a thin metal film evaporated on graphite induces tensile stress resulting in the exfoliation of graphene [151]. The ideal case is that graphene can be peeled from the bulk graphite layer by layer [150].

Chemical vapor deposition (CVD) is a bottom-up approach extensively used to generate carbon-based nanomaterials. In this technique, a chemical reaction of vapour-phase precursors and the formation of a thin film of material occurs. Variations of this method enable the synthesis of different carbon-based materials, such as fullerenes, CNTs, NDs and graphene [142, 152, 153].

High Pressure High Temperature (HPHT) is a bottom-up approach. It uses graphite as a starting material. It is then subjected to pressure between 12 and 15 GPa and temperature up to 3500 ºC by direct heating through the sample by electric current for short periods, thus enabling the synthesis of the diamond from direct conversion of graphite [152, 154]. It resembles the natural process of diamond formation, where a carbon precursor, usually graphite, is brought to a state of high pressure and temperature. Inside a chamber, the temperature is brought to ~2000 °C, and the pressure is up to several GPa. This technique allows for the formation of MDs, which can be milled to obtain NDs [155].

NDs can also be produced by detonating carbon-containing explosives, so-called detonation nanodiamonds (DNDs) [156]. When an explosive blasts in a water environment, the detonation products contain MDs and NDs in a gaseous medium. The significant differences between HPHT NDs and DNDs, which influence their applications, are the size and content of nitrogen impurities in the core [157].

1.5.2. Properties

CMs have a wide range of structures, properties, and textures which enable their classification according to their C-C bonding, based on sp, sp^2, or sp^3 hybrid orbitals [158]. A CM that presents a significant proportion of sp^3 orbitals exhibits improved mechanical properties. For instance, a diamond, made of pure sp^3 bonds, is known for its excellent hardness. Moreover, the degenerated state of sp^2 presents good compatibility with other atoms, which could contribute to developing one- dimensional or two-dimentional CMs with excellent optical, catalytic, and electronic properties.

The chemical and physical properties of CMs can be adjusted through their surface functionalization. Some enhance their drug loading/release capacity, thus enabling targeted drug delivery and applicability in biological systems [132]. CMs present a high surface energy and, as a result, tend to agglomerate and have a bad dispersion in water [159]. Although some studies refer to CMs as biocompatible [160, 161], cytotoxicity was reported in other studies aiming to characterize the toxicity of CMs *in vitro* and *in vivo*. Some cytotoxic effects observed by Yuan *et al.* (2019) include reactive oxygen species (ROS) generation, lysosomal damage, mitochondrial dysfunction, DNA damage and even cell death [162]. However, in some cases, surface modification of CMs can reduce cytotoxicity.

1.5.3. Applications

Over recent years, CMs have been the object of extensive research from scientific and industrial points of view [137] and applied in different areas ranging from electronics [163] and engineering [164], to water treatment [165] and air purification [166].

Due to their high conductivity, thermodynamic and chemical stability, and outstanding robustness and flexibility, CMs are considered highly promising materials for the construction of energy storage devices [167] such as batteries [168] and solar cells [169].

In addition, the inimitable features of CMs have been widely exploited in diverse biomedical applications, including drug delivery [170, 171], cancer therapy [172], bioimaging [137], biosensing [139, 173], tissue engineering [174, 175], and on the removal of contaminants and purification processes [1].

Nanomaterial interactions with biological systems are governed by physicochemical properties such as size, shape, chemical composition, and surface chemistry [176]. Regarding size, nanomaterials must be smaller than the average eukaryotic cell and be able to pass through biological barriers such as cell membranes, tissues, and organs. In the case of carbon-based nanomaterials, their size must be between 0.7 and 300 nm for their possible application in drug and gene delivery, imaging and diagnostics, biosensing, and

tissue engineering [177]. Despite their easy cellular uptake and high drug loading capacity, questions remain about CNMs toxicity, pharmacokinetics, biodistribution, and fate in biological systems [178]. Actually, the colloidal stability of nanomaterials should also be maintained under physiological conditions, ideally across a wide pH range [138].

In the area of biotechnology, CMs have been employed in extraction protocols (solid-phase extraction (SPE)) and chromatography due to their large surface area, chemical and thermal stability and high adsorption capacity [179].

The following topics briefly describe some carbon nanomaterials and their biomedical applications:

- Carbon nanotubes (CNTs) are extensively used as scaffolds for cell culture growth, thanks to their porous structure that offers ample space for adherence, proliferation and differentiation of cells [180].
- CNHs may aid in entrapping drugs, biomolecules and other chemical substances and are effective in phototherapy because they are highly light-absorbent [181].
- GO and rGO strongly absorb light in the near-infrared (NIR) region, which makes them useful in photothermal therapy (PTT) for cancer treatment [172].
- Graphene quantum dots (GQDs) have been successfully used as fluorescent probes for *in vivo* and *in vitro* tumor and cellular imaging, due to their non-toxicity, fluorescence and improved renal clearance [182].
- Fullerene derivatives can be used as contrast agents for magnetic resonance imaging, in photodynamic therapy [154] and as sensors to detect analytes because of their improved conductivity [183].
- Carbon nanoonions (CNOs) have been studied as potential drug delivery systems [184] due to their high cellular uptake and good biocompatibility upon functionalization [185].
- The irradiation of carbon dots (CDs) triggers the production of ROS, rendering them able to kill nearby pathogens [186].
- Lastly, NDs, with their tetrahedrally bonded carbon atoms in a 3D cubic lattice, offer a platform for a multitude of applications such as drug delivery [182], tissue engineering [187], bioimaging [188] and sensing [189].

1.5.4. Microdiamond

MDs are defined as diamonds having the largest dimension smaller than 0.85 mm [190]. They are synthesized at high pressure and high temperature (HPHT MDs), have high thermal conductivity (800-2300 $Wm^{-1}K^{-1}$) due to the combination of strong covalent C–C

bonds [191], exceptional hardness (10 on the Mohs scale), and a high density (3.5 gcm^{-3}) [192, 193]. For those reasons, MDs are used to prepare robust, thermally stable and conductive composite adsorbents. Their surface contains carboxyl (-COOH) and hydroxyl groups (-OH), which renders them moderately polar [192].

The interaction mechanism between cells and diamond particles (uptake, transport, and final localization within cells) must still be understood [194]. In a study, Ebrahimi and coworkers (2024) studied the initial stages of MD cellular uptake, to understand the biological impact of MDs and assess their potential as cellular delivery platforms. The results suggested that cells actively transport MDs to their center along actin filaments. Moreover, it was noticed that after 24h of incubation of mouse embryonic fibroblasts (MEFs) with MDs, most of the absorbed particles accumulated around but not inside the nucleus. The results also confirmed the biocompatibility of MDs in a concentration up to 100 μg/mL as they neither caused apoptosis nor altered the morphology of MEFs [194].

1.5.5. Nanodiamond

NDs are diamond particles of nanometric dimensions (<100 nm [195] and thickness ranging from 2 to 10 nm) that bring most of the unique properties of diamonds down to the nanoscale [155, 196]. They stand out among nanomaterials not only for being relatively easy to produce [197] but mainly due to their outstanding properties, such as:

- exceptional biocompatibility [197, 198];
- versatile surface chemistry which facilitates its functionalization [198];
- stability and chemical inertness [195].

They present a covalent bonding carbon structure [195] composed of an sp^3 carbon core [199] and an sp^2 hybridized surface [200]. The sp^3 carbon core renders them chemically inert and highly stable under strong acidic or basic conditions [195, 199] and is responsible for their high refractive index, which leads to strong light scattering and makes them useful as non-toxic UV shielding additives in polymer coatings and sunscreen [201]. The sp^2 hybridized surface confers high electrical conductivity to NDs [202] and enables the establishment of interactions with aromatic rings through π-π stacking [200]. sp^2 carbons can be easily combined with diverse functional groups via hydrogenation, oxidation, reduction, amination and halogenation [195]. The presence of different functional groups enables the establishment of covalent and hydrogen binding with therapeutic agents, thus making it possible to use them in drug delivery applications [197]. Furthermore, compared with other CMs, NDs exhibit higher

biocompatibility and have shown potential to deliver bioactive agents due to their high surface area-to-volume ratio [203].

In 2019, Křivohlavá and coworkers (2019) demonstrated that HPHT NDs are effective carriers for short asRNAs both in 3D organoids and *in vivo,* and present good characteristics like cytocompatibility, cytoplasmic membrane penetration, easy surface functionalization and traceability [204].

Given their paramagnetic and ferromagnetic properties and fluorescence, NDs can also be used as diagnostic tools [197] as magnetic resonance imaging (MRI) and fluorescence imaging (FI) probes [195, 205].

In addition, NDs have been proven beneficial in manufacturing biodegradable medical instruments and scaffolds for tissue engineering [197], namely in wound healing and neural regeneration due to their biocompatibility, excellent physical and mechanical properties, chemical stability, highly functional surfaces, and highly resistant [206].

1.5.5.1. Structure

The structure of NDs is rather complex [207]. It is a unique nanometre-sized carbon structure [208] considered truncated octahedral with electrostatic fields on the surfaces, as depicted in Figure 8 [197, 205].

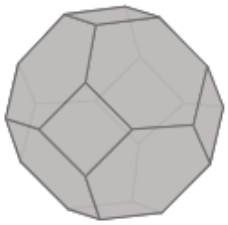

Figure 8. Pristine nanodiamond truncated octahedral structure.

Based on the hybridization of carbon atoms, NDs are supposed to have a core-shell structural design consisting of three layers: the diamond inner core made up of sp^3 carbon atoms, a fullerene-like shell of sp^2 carbon atoms and an outer shell consisting of carbon atoms, which forms functional groups by terminating with hydrogen and oxygen atoms [197]. The accurate nature of the outer shell remains to be determined, but two general models have emerged. One is an amorphous shell with a mixture of sp^3 and sp^2 carbon [209] content as represented in Figure 9A, and the other is a sp^2 graphene-type sheet of a fullerene

structure, giving rise to a structure described as 'bucky-diamond' as depicted in Figure 9B [205].

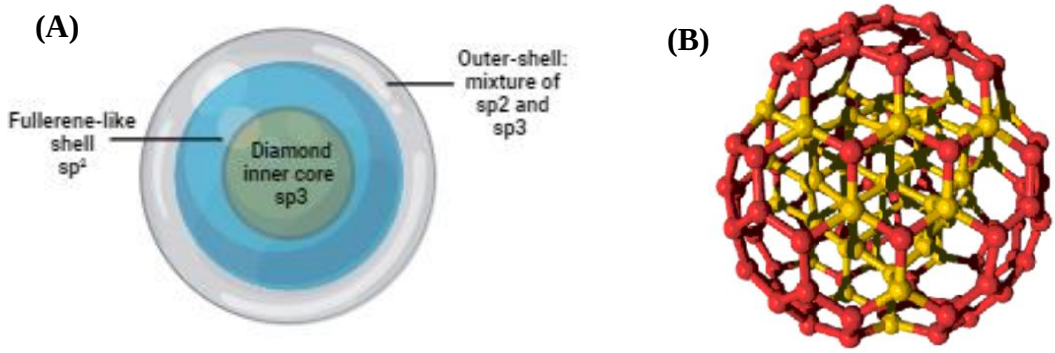

Figure 9. (A) Nanodiamond core-shell structural design (amorphous shell); **(B)** Bucky-diamond. Adapted from [210].

The truncated octahedral architecture and, thus, the large surface area of NDs renders them great potential for drug loading [205].

Depending on the production method and subsequent purification steps [211, 212] the surface of pristine NDs can present a multitude of different functional groups with different relative abundances and, for that reason, tend to be heterogeneous [209].

The surface of NDs mainly consists of carbon and other functional groups, including phenols, pyrones and sulfonic acid. Moreover, they can also contain carboxylic acid (-COOH), carbonyl (-C=O), ether (-C-O-C-), lactone (-O-C=O), anhydride, hydroxyl (-OH), ketone and epoxide groups [205, 209]. The presence of functional groups enables the conjugation of NDs with molecules such as drugs and genes for their intracellular and extracellular delivery.

Certain NDs naturally present nitrogen-vacancies (N-V), responsible for conferring their fluorescence properties. For that reason, they are known as fluorescent nanodiamonds (FND) and serve as biolabeling agents and cellular markers [205].

1.5.5.2. Synthesis methods

As previously mentioned, NDs can be synthesized through different techniques [212]. The properties of NDs change according to the method employed for their production [213]. NDs synthesis was discovered accidentally three times by scientists aiming to explore diamond synthesis. The first method to be discovered was detonation, in 1963 by Volkov and coworkers, followed by Staver and Lyamkin, in 1982, and by Savvakin in the same year [214]. Besides detonation, mass production of NDs can also be achieved by milling

microdiamond powders produced by high-pressure high-temperature (HPHT), and chemical vapor deposition (CVD) [215].

It was only in 1998 that NDs were first obtained by laser ablation of graphite in water using pulsed laser YAG [216].

Table 3 summarizes the advantages, disadvantages, the dimensions of the obtained particles and examples of applications of NDs obtained by each of the four mentioned methods.

Table 3. Advantages, disadvantages and applications of NDs obtained through different synthesis methods. Adapted from [212].

	CVD method	**Laser Ablation Method**	**HPHT method**	**Detonation method**
Advantages	Possibility to obtain MDs (>100 nm), NDs (10-100 nm) or ultrasmall NDs (<10 nm) by varying the relative concentration of the gas mixture (CH_4/H_2) used [195]	More environmentally friendly and less hazardous compared with detonation and HPHT methods [195]	NDs present uniform structure and minimum lattice defects [197]	DNDs present a narrow size distribution (typically 5 ± 2 nm) [196]
Disadvantages	Time-consuming High production cost [195]	Difficult to control size distribution, agglomeration, and crystal structure [217]	Broad size distribution [196]	Aggregation due to the amount of sp^2 surface carbons [195]
Dimension	3-100 nm	25–250 nm [218]	>20 nm	4-5 nm 3-5nm [219]
Applications	Biosensors Coating		Bioimaging	Drug delivery Bioimaging

1.5.5.3. Classification

NDs can be classified using different criteria, such as synthesis method and spatial size [200, 220], as depicted by Figure 10.

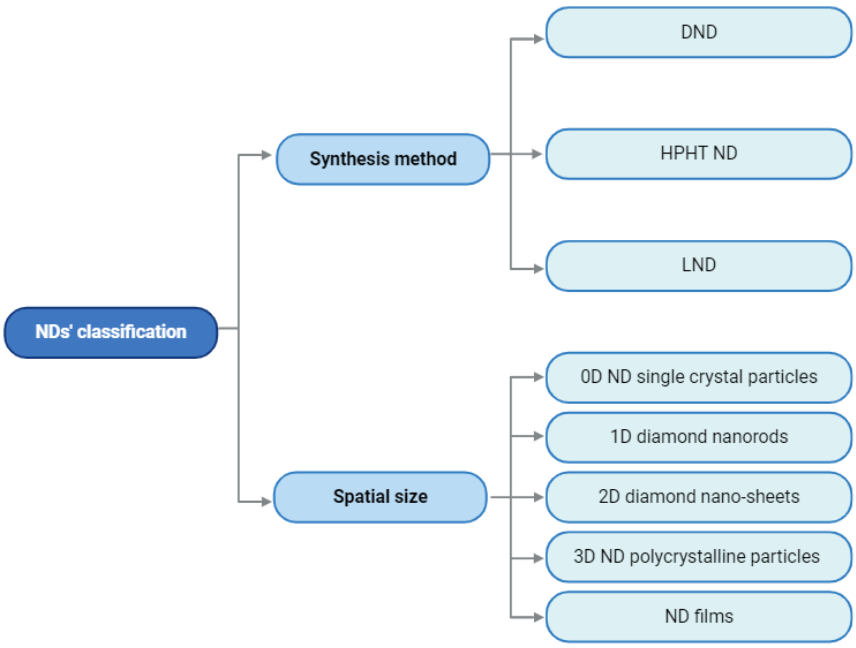

Figure 10. Classification of NDs according to the synthesis method and spatial size.

DNDs are 3-5 nm NDs synthesized through a detonation process accomplished by the use of nitrogen-rich explosives and purification processes after detonation synthesis. As a result of the process, these materials exhibit a heterogeneous surface [221, 222].

HPHT NDs are synthesized using the high-pressure high-temperature method. NDs obtained through this method typically have nitrogen-vacancy (NV) centres, which render them fluorescent. For that reason, they are also known as fluorescent nanodiamonds (FNDs) [200].

NDs can also be obtained by pulsed-laser ablation (PLA) of graphite and diamond-like carbon in water [223]. These are called laser nanodiamonds (LND) [219].

1.5.5.4. Functionalization

At a conceptual level, functionalization consists of the introduction of chemical moieties (functional groups or other ligands such as polymers or biomolecules) [224] on the materials backbone and is a strategy used to control NDs mechanical, chemical, electronic, and optical properties [225].

It has already been proven that the surface functionalization of NDs improves their biocompatibility [226], especially when it is carried out with biological molecules [205]. Their functionalization with antibodies is used as a strategy for site-specific delivery [227].

Surface functionalization has also emerged as a technique to enhance the circulation time of NDs after their administration into the human body. Due to their size, NDs have a short

circulation half-life and are rapidly eliminated via opsonization by phagocytes. Thus, surface functionalization enhances the circulation time of NDs, making them best suited for prolonged drug delivery [205].

Due to their surface inhomogeneity after synthesis [228], surface functionalization of NDs can be divided into two steps: initial surface homogenization and immobilization of functional groups onto previously homogenized NDs [224].

The most common reactions performed for surface functionalization of NDs are the following:

- **Oxidation**, which increases the amount of oxygen containing functional groups such as carboxyl, carbonyl, and hydroxyl [229]. It can be accomplished using highly oxidizing agents, such as strong acids, ozone or water at 350-450 ºC. In turn, surface oxidation facilitates the introduction of other functional groups. Once oxidized, non-covalent functionalization can be performed using hydrophobic or electrostatic interactions [137].
- **Hydrogenation** which aims to generate homogeneous C-H bonds at NDs' surface and can be accomplished via thermal annealing [211] performed under H_2 flow at 850–900 °C [230] or by microwave plasma treatment in a hydrogen atmosphere [211]. Hydrogenated diamond surface has unique properties, such as negative electron affinity and surface conductivity [231].
- **Amination** is a chemical reaction that results in the introduction of amino groups. Amino-functionalization of carbon nanomaterials can largely enhance its hydrophilicity and biological affinity [232]. The amination of NDs can be accomplished through ultraviolet (UV) irradiation of hydrogenated NDs in the presence of chlorine gas, and treatment of chlorinated NDs with ammonia at high temperature [137, 211].

Figure 11 schematically represents the three chemical reactions performed to obtain hydrogenated, oxidized, and aminated NDs.

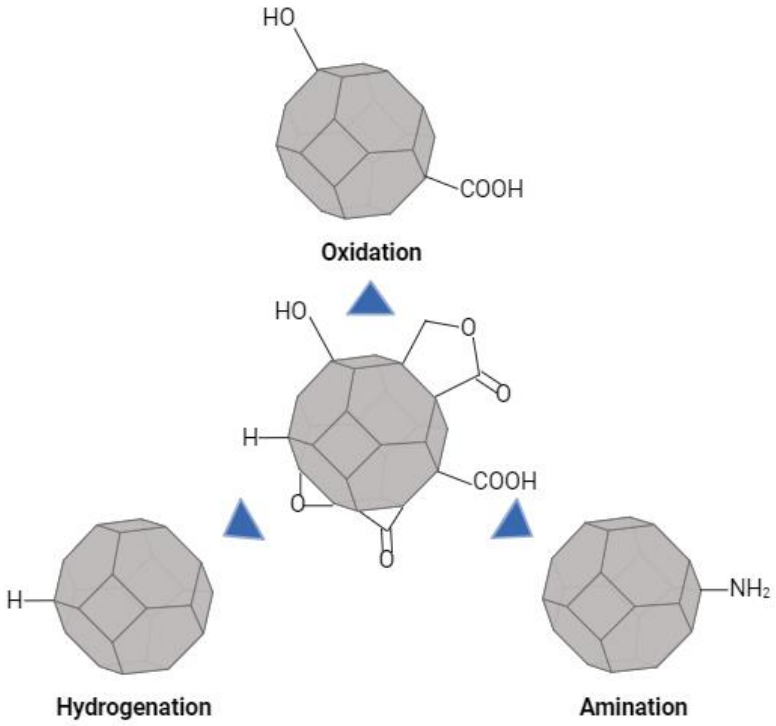

Figure 11. Reactions performed to obtain oxidized, hydrogenated, and aminated NDs.

1.5.5.5. Properties and applications

As mentioned before, NDs possess excellent physical, chemical, and optical properties that result from the combination of the properties of diamonds and nanoparticles [202, 205], such as high chemical stability (due to their sp^3 core structure [199]), crystallinity (due to the structural stability of their core [233]), stiffness (owing to the high Youngs' Modulus), hardness along with tuneable surface structures [202, 225, 234].

ND's chemical, mechanical, optical and electronic properties can be tuned through strategies like doping and functionalization [200]. The main characteristics that turn NDs attractive from a biomedical point of view are their large specific surface area (300-500 m^2/g) (although small when compared with that of SWCNTs (1315 m^2g^{-1}) [235], rendering high loading capacity, and in some cases, the possibility of stable fluorescence. By exploiting these properties, NDs can be used as biomarkers, drug, or gene carriers, and as immobilization substrates for biomolecules purification or bioanalytical purposes [156].

The high stability of NDs makes them suitable for drug delivery [205] to avoid premature drug release [236]. They also present increased drug efficacy and decreased toxicity levels [205], turning them into very prominent drug delivery systems. Xu and coworkers (2023) [200] reported that therapeutic agents, including proteins, peptides, chemotherapy drugs, imaging agents, and nucleic acids had been successfully delivered by NDs. Other characteristics contributing to this application are biocompatibility, dispersibility in water, the possibility of surface functionalization, and high adsorption capacity [197].

Research on the biocompatibility of NDs has yielded promising results, underscoring their non-toxic nature and exceptional compatibility with biological systems [205, 237].

The role of stiffness in supporting cell growth is crucial, as a rigid substrate provides superior physical support and augments cell adhesion, proliferation, and cytoskeleton construction [238]. The findings of Adel and coworkers [206] highlight the significant role of NDs in increasing cell adhesion and proliferation.

Regarding size, in 2021, Qin *et al.* described that NDs with a size of less than 10 nm are important to enter cells for biomedical applications [239]. Moreover, reducing NDs particle size down to the range of 1–3 nm may further increase their capability for penetrating cells and organelle membranes, including nucleus pores [240].

The adsorption of biomolecules on NDs is governed by different types of interactions that vary according to the functional groups present on NDs surfaces. In order to control the loading capacity and release kinetics of the adsorbed molecules, whether nucleic acids, proteins, or pharmaceuticals, it is necessary to understand the surface structure and the mechanisms of interaction of biomolecules and NDs. Electrostatic, hydrophobic interactions, hydrogen bonding, and van der Waals forces are mechanisms through which biomolecules bind to NDs [197].

In 2023 , Xu and Chow verified that when dispersed in an aqueous solution, NDs tend to form aggregates with a size around 50-200 nm [200]. Aprà and coworkers explained that high aggregation levels are correlated with low hydrophilicity, and since proper dispersibility is of paramount importance for biomedical applications, many modifications and functionalization protocols have been developed to address this [237].

1.5.6. NDs as adsorbents in SPE

Diamond-containing stationary phases have been intensively studied over recent years due to their adsorption properties. Their use has been reported in normal-phase, reversed-phase, hydrophilic interaction, and ion chromatographic modes of HPLC, as well as sorbents for SPE. Due to their multi-functional surface chemistry, it is possible for NDs to adsorb biomolecules through different types of interactions, such as electrostatic and hydrophobic interactions, hydrogen bonding, and chelation [229].

NDs, more specifically oxidized NDs, were reported by Molavi and coworkers [202] as adsorbents of dyes present in aqueous solutions.

This work intends to study microdiamonds (MD), nanodiamonds (ND), and functionalized nanodiamonds (hydrogenated ND (ND-H), oxidized ND (ND-ox) and aminated ND (ND-NH_2)) as RNA adsorbents. An initial screening is required to select the most promising

material under study. Further experiments will enable the achievement of high recovery of the molecule of interest (RNA), and, at the same time, explore their selectivity towards RNA when it is present in complex lysate samples.

Chapter 2

Objectives

This study intends to optimize an adsorption method for the pre-purification of RNA when it is present in complex *E. coli* lysate samples, using MDs, NDs and functionalized NDs as adsorbents. The main purpose is to develop a simple and efficient method, using CMs, for the capture and recovery of RNA, and explore the selectivity of the chosen CM towards RNA when it is present in complex mixtures of *E. coli* lysates containing impurities such as pDNA and proteins.

To advance on the current state-of-art for RNA purification, studies on adsorption and desorption were carried out and the best conditions regarding RNA recovery and purity were chosen. The higher the RNA purity level achieved in the low-resolution purification phase, the higher the probability of minimizing high-resolution purification steps, thus enabling cost reduction, since downstream processing accounts for nearly 80% of the total biopharmaceutical production costs. For that purpose, the following tasks were outlined:

1- Screening of RNA adsorption and desorption conditions while using different carbon materials;

2- Analysis of the selectivity of the selected materials towards RNA in the presence of pDNA or in complex *E. coli* lysate samples;

3- Characterisation of the contaminating protein levels;

4- Assessment of the nucleic acids recovery efficiency and comparison with other strategies.

Chapter 3

Materials and Methods

3.1. Materials

3.1.1. Bacterial Culture

For *E. coli* NZYStar growth, culture medium "Luria-Bertani" (LB) agar and kanamycin antibiotic, both from Grisp Research Solutions (Porto, Portugal), were used.

For pre-culture and *E. coli* culture, the reagents used were "Terrific Broth" (TB) medium, which is a complex medium composed of yeast extract, tryptone, both from Bioakar diagnostics (Beauvais, France), glycerol from LabChem (Zelienople, USA), potassium dihydrogen phosphate (KH_2PO_4) from CHEM-LAB ANALYTICAL (Zedelgem, Belgium), dipotassium hydrogen phosphate (K_2HPO_4) from Honeywell Burdick & Jackson ™ (Michigan, USA) and kanamycin antibiotic from Grisp Research Solutions (Porto, Portugal).

3.1.2. Plasmid DNA Extraction

pDNA extraction was attained by using a QIAGEN Plasmid purification kit from Qiagen (Hilden, Germany). To resuspend a pellet previously obtained from *E. coli* growth, solution P1 comprising Tris from Thermo Fisher Scientific Inc. (Waltham, USA), EDTA from LabKem Labbox Labware S.L. (Barcelona, Spain), and RNAse from Thermo-Fisher Scientific Inc. (Waltham, USA), was used. Solution P2 containing NaOH from LabChem (Zelienople, USA) and SDS from HIMEDIA (Einhausen, Germany) and neutralization solution P3 of potassium acetate from Carlo Erba Reagents (Milan, Italy) were used for alkaline cell lysis.

3.1.3. Low Molecular Weight RNA Extraction

For Low Molecular Weight RNA extraction, the reagents used were a denaturing solution (solution D) containing guanidium thiocyanate, sodium acetate, N-Lauroylsarcosine sodium salt and 2-mercaptoethanol all from Merk (Whitehouse Station, USA), chloroform from ThermoFisher Scientific Inc. (Waltham, USA); isoamyl alcohol from ThermoFisher

Scientific Inc. (Waltham, USA); phenol from Merk (Whitehouse Station, USA) and isopropanol from Carlo Erba Reagents (Milan, Italy).

3.1.4. Alkaline Cell Lysis

For cell lysate preparation, it was used a P1 solution containing tris(hydroxymethyl)aminomethane (Tris) from Thermo Fisher Scientific Inc. (Waltham, USA) and EDTA from LabKem Labbox Labware S.L. (Barcelona, Spain), a P2 solution comprising NaOH from LabChem (Zelienople, USA) and SDS from HIMEDIA (Einhausen, Germany), and a P3 solution composed of potassium acetate from Panreac (Barcelona, Spain). Agarose gel electrophoresis (0.8%) was carried out for nucleic acid visualization. Green-Safe from Grisp (Porto, Portugal) was used as an intercalating agent.

3.1.5. Protein Quantification

For protein quantification, it was used a dye reagent concentrate containing dye, phosphoric acid and methanol, as well as bovine serum albumin (BSA) for the calibration curve, all from Bio-Rad (California, USA).

3.1.6. Dispersive solid-phase extraction of RNA using different carbon materials

The carbon materials used as resins for dispersive solid-phase extraction of RNA were provided by Prof. Cláudia Gomes Silva from the Faculty of Engineering of the University of Porto. D-SPE was applied to capture RNA previously extracted from *E. coli* using five different types of carbon materials: MD, ND, ND-H, ND-ox and ND-NH_2. The reagents used to prepare the solutions used for the adsorption/desorption experiments were Tris ($(CH_2OH)_3CNH_2$) and Tween-20 ($C_{58}H_{114}O_{26}$) from Fisher Scientific (Waltham, USA), hydrochloric acid (HCl) and sodium chloride (NaCl) from Merk (Whitehouse Station, USA), and ammonium sulphate ($(NH_4)_2SO_4$) from PanReac AppliChem (Darmstadt, Germany).

3.2. Methods

3.2.1. *E. coli* growth and nucleic acids production

RNA and pDNA necessary for the study were obtained from a culture of *E. coli NZY Star* strain, previously transformed with a plasmid containing the sequence of pre-miR-29. At

first, *E. coli* was inoculated in an LB-agar medium containing 50 μg/mL of kanamycin, and the growth occurred overnight at 37 ºC. To carry out the pre-culture, multiple colonies were transferred from the plate to a shake flask containing 125 mL of TB medium (24 g/L yeast extract, 12 g/L tryptone, 4 mL/L glycerol, 0.072 M K_2HPO_4, 0.017 M KH_2PO_4), and kanamycin 50 μg/mL. The inoculated medium was then incubated at 37 ºC with vigorous shaking (250 rpm), until an optical density of around 2.6 at 600 nm was reached.

The following equation was used to determine the pre-fermentation volume necessary to start the fermentation at an OD_{600} of 0.2:

$$V_{pre\text{-}fermentation} = (OD_{fermentation} \times V_{fermentation}) / (OD_{pre\text{-}fermentation} - OD_{fermentation}) \quad (1)$$

A spectrophotometer VWR UV-6300PC Double Beam Spectrophotometer (Radnor, USA) was used to measure the OD.

E. coli growth was kept for 8 h if RNA (of low molecular weight) was required or 16 h for pDNA.

At the end, the culture medium with cells was divided into 50 mL tubes and a centrifugation at 3900 g for 15 min at 4ºC was made to recover the bacterial cells. The resulting pellets were stored at -20ºC until use.

3.2.2. Nucleic acids extraction

3.2.2.1. Plasmid DNA (pDNA) extraction

The extraction of pDNA was attained using a QIAGEN Plasmid purification kit and following the protocol provided by the manufacturer. The first step was the resuspension of the bacterial pellet corresponding to 100 mL of an *E. coli* cell culture in 12 mL of Buffer P1 containing Tris, EDTA, and RNAse A by vigorous vortex. Since pDNA is an intracellular product, it is necessary to perform cell lysis. The chosen lysis method was alkaline lysis, which was achieved by adding 12 mL of Buffer P2 containing a strong base and a detergent. The tube was gently inverted to enable homogenization and was incubated at room temperature for 5 min. The next step was neutralization, carried out by adding 12 mL of pre-cooled potassium acetate 3 M to the suspension and gently homogenizing by inverting the tube. Afterwards, the tube containing the lysate was incubated on ice for 20 min. After, a centrifugation at 20000 g at 4 ºC was performed for 30 min. Since the supernatant was visibly slightly turbid, another centrifugation in the same conditions was carried out for 15 min. In the meantime, the Qiagen purification column was equilibrated with 6 mL of Equilibration Buffer composed of NaCl, MOPS, Triton X-100, and isopropanol with neutral

pH. The supernatant obtained by performing the latest centrifugation was then transferred to the equilibrated column. In this step, the DNA binds to the column, and the contaminants are eliminated through column washing with 32 mL of Washing Buffer comprising NaCl, isopropanol, and MOPs. Pure pDNA elution was achieved by adding 15 mL of Elution Buffer containing NaCl, Tris, and isopropanol. Eluted pDNA was collected in clean tubes and then precipitated by adding 10.5 mL of pre-cooled isopropanol, carefully homogenizing the tube, and incubating it in ice for 20 min. A last centrifugation at 15000 g was performed for 30 min at 4 ºC and the supernatant was carefully discarded. The DNA pellet was air-dried for 10 min at room temperature and redissolved in 1 mL of 10 mM Tris-HCl, pH 8.0 to enable pDNA redissolution.

To proceed with the quantification of the pDNA sample, a Thermo Scientific™ NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Waltham, USA) was used. An agarose gel electrophoresis was prepared to evaluate the purity and the integrity of the pDNA. At the end of the procedure, the pDNA samples were stored at -20 ºC.

3.2.2.2. Low molecular weight RNA extraction

RNA extraction and isolation were carried out using the acid guanidium thiocyanate-phenol-chloroform method. Firstly, an *E. coli* cells pellet previously thawed was resuspended in a solution of 0.8% NaCl and centrifuged at 6000g for 10 min at 4 ºC. The supernatant was discarded, and the resultant pellet was resuspended using 5 mL of solution D (4 M guanidium thiocyanate, 0.025 M sodium citrate, 0.5 % (wt/vol) N-lauroylsarcosine and 0.1 M β-mercaptoethanol), followed by incubation in ice for 10 min. By doing this, the DNA is fragmented, which minimizes its presence in the aqueous phase. Then, 0.5 mL of 2 M sodium acetate pH 4.0 and 5 mL of phenol were added to each suspension and carefully homogenized in each step. Afterwards, 1 mL of a mix of chloroform and isoamyl alcohol on a proportion of 49:1 was added to each suspension, followed by vigorous shaking by hand for approximately 10 seconds and incubation on ice for 15 min. The next step was to centrifuge the suspensions for 20 min at 10000 g at 4 ºC. In this phase of the procedure, the extraction occurs, and it is possible to notice two phases. The upper phase contains mainly RNA, while the bottom phase is enriched in DNA. To carry out the first precipitation, it is necessary to transfer the upper aqueous phase to clean tubes through careful pipetting, to avoid DNA contamination, since it is present in the phase right below. The RNA precipitation occurs when 5 mL of cold isopropanol are added to the suspensions, and these are incubated in ice. After, a centrifugation for 20 min at 10000 g at 4 ºC was performed, and the supernatant was discarded, thus enabling the formation of a gel-like pellet which is the RNA precipitate. A second precipitation is done by adding 1.5 mL of solution D, 1.5 mL

of isopropanol and centrifuging the tubes for 10 min at 10000 g at 4 ºC. After discarding the supernatant, the RNA pellets were washed by resuspension in 2.5 mL of 75% ethanol in DEPC water and incubated for 15 min at room temperature to dissolve possible residual traces of guanidium. A last centrifugation was performed for 5 min at 10000 g at 4 ºC; the supernatant was discarded, and the RNA pellet was left air-dry at room temperature for 5-10 min. The last step consisted of RNA solubilization by dissolving the pellet in 1 mL of DEPC-treated water and incubating it at room temperature for 15 min to ensure complete solubilization. All procedures were performed inside an extraction cabinet. The Thermo Scientific™ NanoDrop™ One UV-Vis Spectrophotometer (Waltham, USA) was used to measure the absorbance of the RNA samples, whereas an agarose gel electrophoresis was performed to verify the RNA integrity. After that, the samples were stored at -80 ºC for posterior use [241].

3.2.3. Agarose gel electrophoresis

To evaluate the integrity of RNA samples, 0.8% agarose gel electrophoresis was performed. A concentration of 0.012 µL/mL of Green Safe was used to stain the nucleic acids in the gel, and the electrophoresis was run in TAE (Tris-acetate-EDTA) buffer (4 mM Tris, 20 mM acetic acid, 1 mM EDTA pH 8.0) at 120 V for 30 min. The gels were visualized under ultraviolet light exposure in a UV chamber from UVITEC (Cambridge, United Kingdom).

3.2.4. Dispersive solid-phase extraction (DSPE) of RNA using different carbon materials

In the present work, DSPE was applied to capture RNA previously extracted from *E. coli* using the following CMs: MD, ND, ND-H, ND-ox and ND-NH_2. This method is based on a previously described method in which other CMs, more precisely as-grown MWCNTs, were used to capture RNA from complex *E. coli* cell lysates [242]. In that work, it was not possible to efficiently desorb and recover the RNA adsorbed to MWCNTs. For that reason, another strategy was adopted. Instead of trying to capture and recover RNA for further use, the RNA capture by MWCNTs was studied as a strategy for pDNA clarification, removing RNA (a contaminant in pDNA samples) and other contaminants from a complex lysate sample (containing RNA).

A schematic representation of the three steps of this method is shown in Figure 12.

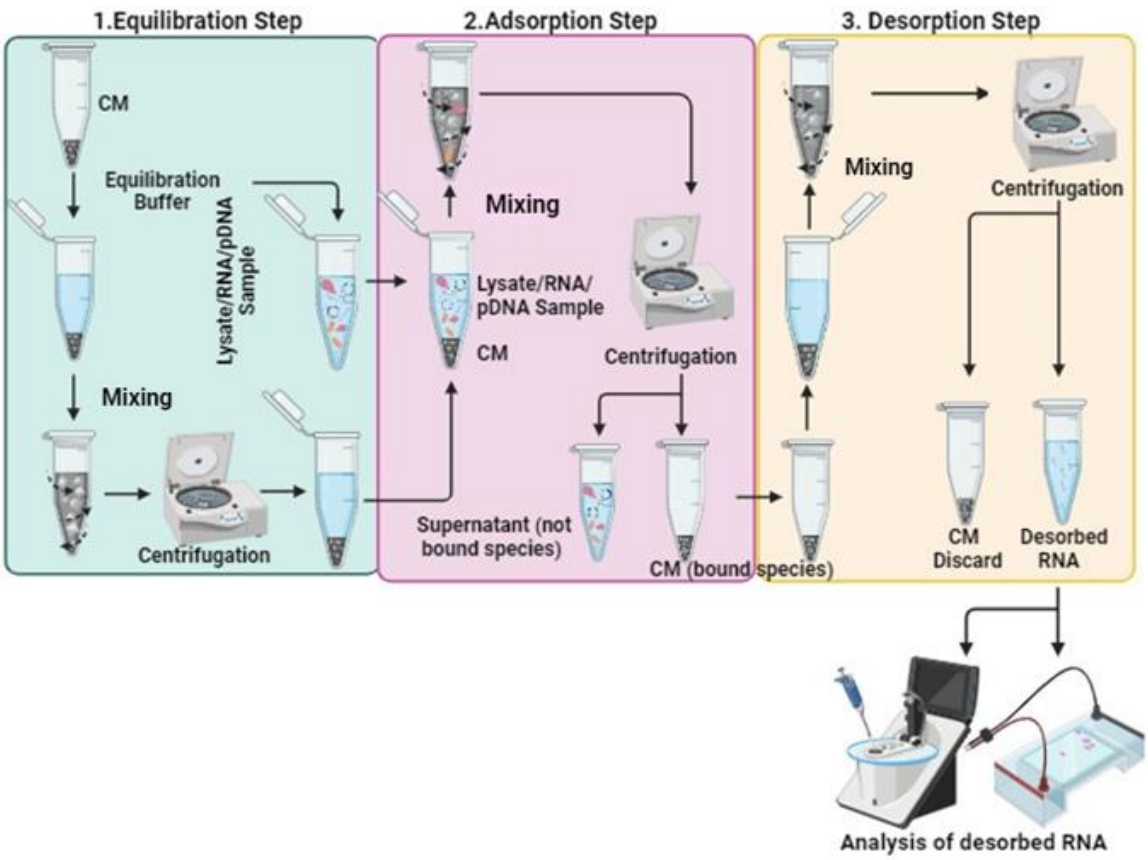

Figure 12. Schematic representation of the DSPE method.

The method consists of three fundamental steps:

- In the equilibration step, 1 mg of each CM was equilibrated using an equilibration buffer that differs between assays depending on the type of interactions intended to be promoted. Afterward, the samples were incubated and mixed for 40 min at room temperature, followed by centrifugation at 9000 g for 2 min to separate the aqueous phase and obtain a CM pellet. The sample to be extracted was diluted with the same equilibration buffer.
- In the adsorption step, RNA/pDNA/artificial mixtures of RNA and pDNA /complex *E. coli* lysates were applied to the CM pellet and kept in agitation for 40 min, at room temperature, to allow RNA/pDNA adsorption onto the CM. Afterwards, a second centrifugation at 9000 g for 2 min was performed to separate the solid phase from the aqueous one (supernatant). The supernatant was then recovered and stored for spectrophotometric and electrophoretic analysis. By measuring the absorbance of the supernatant, corresponding to non-adsorbed species, it is already possible to infer about the RNA/pDNA adsorption capacity of each CM.

- In the desorption step, a desorption solution was applied to allow the recovery of RNA/pDNA adsorbed to the CM. Different elution solutions were screened, varying the pH, salt type, and concentration and using surfactant agents.

  After adsorption and desorption steps, the absorbance of the supernatants recovered in each step was measured using a Thermo Scientific™ NanoDrop™ One UV-Vis Spectrophotometer (Waltham, USA), to assess the RNA concentration and an agarose gel electrophoresis was performed.

  All centrifugations were performed at room temperature.

3.2.5. Cell lysate extraction

An alkaline lysis method was used to perform cell lysis and recover a more complex lysate sample composed of RNA, pDNA, gDNA, and proteins. Firstly, *E. coli* cell pellets were resuspended in 12 mL of solution P1 containing 50 mM Tris-HCl and 10 mM EDTA at pH 8.0. Afterward, 12 mL of solution P2 comprising 50 mM NaOH and SDS 10% (w/v) were added, followed by homogenization and incubation at room temperature for 5 min to promote cell lysis. For sample neutralization, 12 mL of solution P3 composed of 3 M potassium acetate at pH 5.5 were added and the tubes were gently homogenized and incubated on ice for 30 min. Next, to eliminate cell debris, some proteins, and gDNA, the tubes were centrifuged at 20000g for 30 min at 4ºC, the supernatant was transferred to clean tubes, and another centrifugation at 20000g for 15 min at 4ºC was performed. If nucleic acid and protein precipitation were intended, 10.5 mL of isopropanol at room temperature were added. At the end of the procedure, the supernatant was stored at -80 ºC to be posteriorly used.

3.2.6. Total protein quantification

The Bio-Rad Protein Assay, based on the method of Bradford, was used for determining the concentration of proteins present in complex lysate samples applied to oxidised NDs, as well as in the supernatants recovered from the adsorption and desorption steps of DSPE. Prior to the assay, a calibration curve was designed using a linear concentration range from 0.05 mg/mL to 0.5 mg/mL of BSA, used as standard protein. Ten dilutions of the protein standard were performed. Afterwards, the Dye Reagent was prepared by diluting 1 part of Dye Reagent Concentrate with 4 parts of distilled, deionized (DDI) water and filtering to remove particulates.

To 10 µL of each standard solution and to 10 µL of each sample, 200 µL of Dye Reagent were added. The plate was then incubated at 37 ºC for 15 min, and its absorbance was

measured at 595 nm using a xMark™ Microplate Absorbance Spectrophotometer from Bio-Rad (California, USA).

The concentration of solubilized protein was calculated using the calibration curve represented in Figure 13.

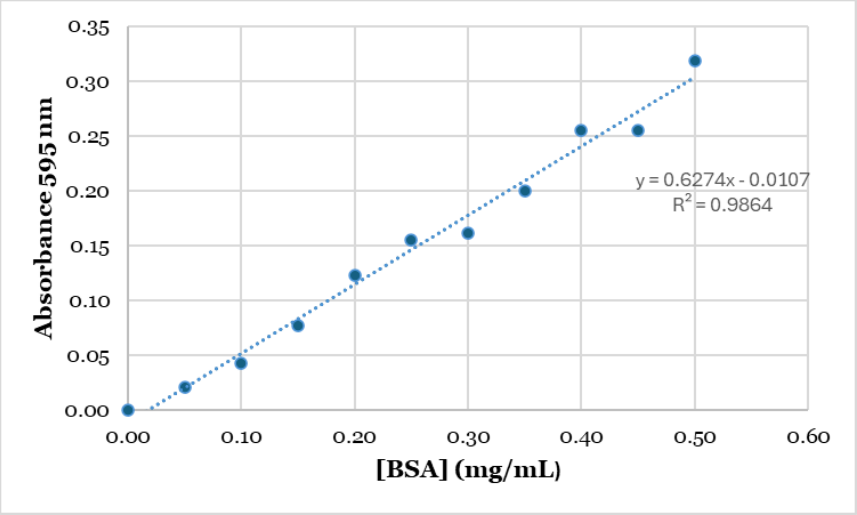

Figure 13. Calibration curve for protein quantification by Bradford assay, using BSA standards in a range of concentrations from 0.05 to 0.5 mg/mL.

Chapter 4

Results and Discussion

4.1. RNA adsorption and desorption experiments using different carbon materials

Studies using carbon materials, more precisely MWCNTs, to capture RNA were already carried out in the research group. It was described in that study that the adsorption of RNA to the MWCNTs' surface occurs mainly through π-π stacking interaction between the hydrophobic bases of RNA and the sidewall aromatic structure of MWCNTs [242]. This work intends to study other carbon materials, namely MDs, NDs, and functionalized NDs to capture and recover the adsorbed RNA.

4.1.1. Screening of RNA adsorption conditions

Some screening assays were performed to evaluate the capacity of non-functionalized MD and ND, hydrogenated ND, oxidized ND and aminated ND to adsorb RNA, previously extracted from *E. coli,* and to optimize the RNA adsorption conditions. Electrostatic and hydrophobic interactions between each CM and RNA were mainly promoted, by adjusting the experimental conditions, to understand their influence on RNA adsorption.

For this purpose, the experimental method previously described by P. Ferreira *et al.* (2023) was adapted and applied to the adsorption and desorption of RNA from the materials under study.

4.1.1.1. Conditions to promote electrostatic interactions

For the experiments where electrostatic interactions were intended to be promoted, 100 mM Tris-HCl pH 8.0 was used as equilibration/ binding buffer. After incubation with RNA, the supernatant was evaluated to measure the non-bound RNA and infer about the amount of RNA adsorbed onto the different CMs.

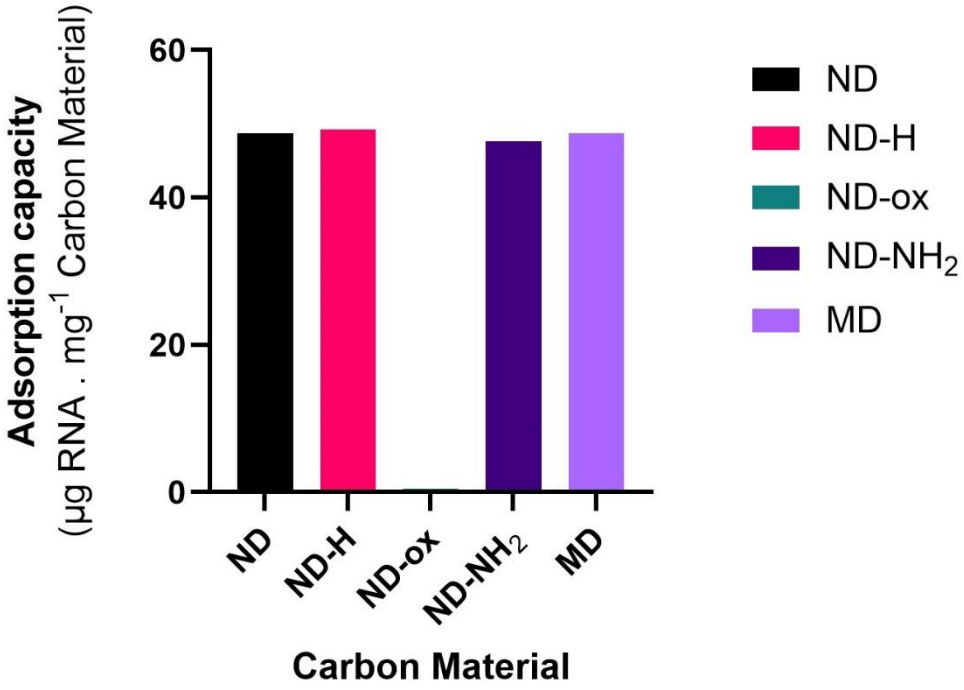

Figure 14. Study of RNA adsorption onto the following CMs: ND, ND-H, ND-ox, ND-NH_2 and MD when electrostatic interactions were promoted.

As depicted in Figure 14, all materials exhibited high RNA adsorption when promoting electrostatic interactions. In that screening, from the 50 µg of RNA in contact with the materials, the adsorption was about 48.8 µg RNA for the ND; 49.2 µg RNA for ND-H; 47.6 µg RNA for ND-NH_2, and 48.8 µg RNA for the MD. Only the ND-ox did not adsorb RNA under these experimental conditions. The lack of adsorption when using ND-ox can be explained by their negative surface charge. Indeed, ND-ox present carboxyl (-COOH) and hydroxyl (-OH) groups on their surfaces due to the oxidation process to which they are submitted during their synthesis [211]. The pKa of the carboxyl group is about 5.0-6.0, whereas the pKa of the hydroxyl group is about 9.7-10.2 [243]. This way, at pH 8.0, carboxyl groups are negatively charged, and hydroxyl groups are not ionized, resulting in a global negative charge of the materials' surface. This aligns with the fact that carboxyl groups are responsible for determining NDs' surface charge [244]. The phosphate groups of the ribose-phosphate backbones of nucleic acids are also negatively charged [245]. Since both RNA molecules and oxidized NDs' surface are negatively charged, the electrostatic interactions between them are repulsive.

Some works have already studied the possibility of using CMs as carriers of RNA for its delivery, nevertheless, research works aiming to explore NDs as RNA adsorbents are relatively new, and consequently, the literature in this area needs to be more extensive. As a comparison, in 2018, Molavi and coworkers [244] studied the adsorption selectivity of

anionic and cationic dyes from aqueous solutions using oxidized and untreated NDs [244]. ND-ox exhibited higher adsorption capacity for cationic MB, attributed to attractive electrostatic interactions between the cationic dye and the negative charges of ND-ox [244].

4.1.1.2. Conditions to promote hydrophobic interactions

Knowing that hydrophobic interactions gradually weaken when the ionic strength is decreased [246], one strategy to promote hydrophobic interactions is to increase the ionic strength in the solution. With that in mind, increasing concentrations of ammonium sulphate (1.0 M, 1.5 M, 2.0 M, and 2.5 M in 100 mM Tris-HCl pH 8.0) were used as equilibration/binding buffers, to mainly exploit hydrophobic interactions between RNA and the different CMs.

As depicted in Figure 15, the results demonstrate that the increase in ionic strength in the equilibration/binding buffer did not lead to a significant increase in RNA binding for most of the CMs, since they had already adsorbed all the RNA molecules present in the sample. However, the ND-ox were once again an exception, when establishing these conditions. The adsorption increased by 34.68 % when the concentration of ammonium sulphate increased from 1.0 M to 2.5 M. This increase was particularly significant (17.74 %) when the concentration of ammonium sulphate was increased from 1.0 M to 1.5 M, as can be observed by Figure 15.

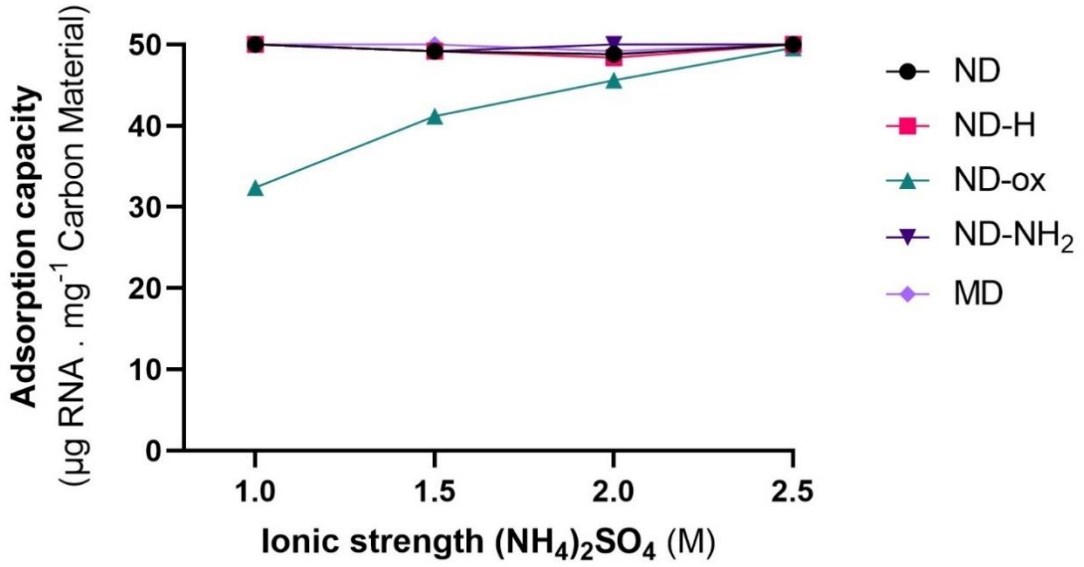

Figure 15. Study of RNA adsorption of the following CMs: ND, ND-H, ND-ox, ND-NH_2, and MD, when hydrophobic interactions were promoted.

Adding ammonium sulphate to the adsorption solution reduces the solvation of RNA molecules. As solvation decreases, RNA hydrophobic regions become exposed and they are more likely to be adsorbed. That is a possible reason for the increased RNA adsorption of ND-ox when increasing concentrations of ammonium sulphate in the adsorption solution were tested. Despite nucleic acids being intrinsically hydrophilic [247], hydrophobic effects in RNA occur at the level of the secondary structure [248]. Moreover, even though ND-ox are more hydrophilic than untreated NDs since surface oxidation turns hydrophobic surfaces into hydrophilic [249], the adsorption of hydrophobic species to ND-ox has previously been reported and exploited for the delivery of water-insoluble drugs [250]. Thus, ND-ox can establish hydrophobic interactions with RNA molecules, as confirmed by the performed experiments.

Regarding the remaining materials, ND-H (which are natively hydrophobic as bulk hydrogenated diamond [251]), were capable of adsorbing RNA in all the conditions tested without noticeable differences upon variation of the ionic strength of the adsorption buffer. The behavior of ND-NH_2 was similar to that of ND-H, although the amino-functionalisation of CMs can enhance their hydrophilicity and biological affinity [232]. Pristine NDs and MDs present hydrophobic surfaces [252, 253] and the results obtained are in line with the literature, since NDs and MDs adsorbed RNA in all conditions where hydrophobic interactions were promoted.

For environmental and economic reasons, to minimize reagent consumption, and with the possibility of scaling up this process in mind, it was decided to perform the adsorption step by applying the equilibration/binding buffer with a concentration of 1.0 M of ammonium sulphate (the lowest concentration tested), even though ND-ox had a relatively lower adsorption ability at 1.0 M than at 1.5 M, 2.0 M and 2.5 M. Thus, for the subsequent assays, when promoting hydrophobic interactions, 1.0 M of ammonium sulphate in 100 mM Tris-HCl pH 8.0 was used as adsorption condition.

4.1.2. RNA adsorption capacity

To determine the maximum adsorption capacity of each CM, solutions containing different concentrations of RNA (10 μg/mL, 20 μg/mL, 30 μg/mL, 40 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL, 150 μg/mL, 200 μg/mL, 250 μg/mL and 300 μg/mL) were added to 1 mg of each CM, as described in the previous adsorption experiments. For ND-ox, the maximum concentration of RNA applied was 100 μg/mL, as it was noticed that the maximum capacity was reached below this concentration. The absorbance of the supernatants recovered in each experiment was analyzed and each experiment was performed in triplicate under the

same conditions. The maximum amount of RNA adsorbed at equilibrium was calculated by the Langmuir isotherm equation modified for aqueous-phase adsorption:

$$q_e = q_m \times \frac{K_L C}{1+K_L C} \quad (2)$$

where q_e refers to the RNA adsorption capacity of the adsorbent (CM) at equilibrium (mg/g), q_m is the Langmuir saturation adsorption capacity (mg/g), C is the adsorbate concentration (mg/L) in solution at equilibrium, and K_L is the Langmuir equilibrium adsorption constant (L/mg) which is related to the adsorption capacity in mg/g [254]. q_e was calculated by the equation:

$$q_e = \frac{(C_i - C_e)}{W} \times V \quad (3)$$

where C_i and C_e are, respectively, the initial and equilibrium concentrations of RNA (mg/L) in solution, W is the weight of the adsorbent (CM) (g), and V is the volume of solution (L).

Adsorption isotherm models describe the interaction mechanisms between the adsorbent and the adsorbate at constant temperature [255]. Langmuir isotherm is an empirical model that proposes monolayer coverage of adsorbate molecules onto a solid surface. It assumes that no further adsorption occurs at the site once the adsorbent site is covered with molecules. In addition, it suggests that all the adsorption sites are of equivalent energy [254].

The data were fitted using the Origin 2024 64-bit software (OriginLab Corporation, Northampton, MA, USA). Figure 16 represents the RNA adsorption isotherms for each CM tested. The Langmuir parameters determined by fitting the data in Figure 16 are presented in Table 4.

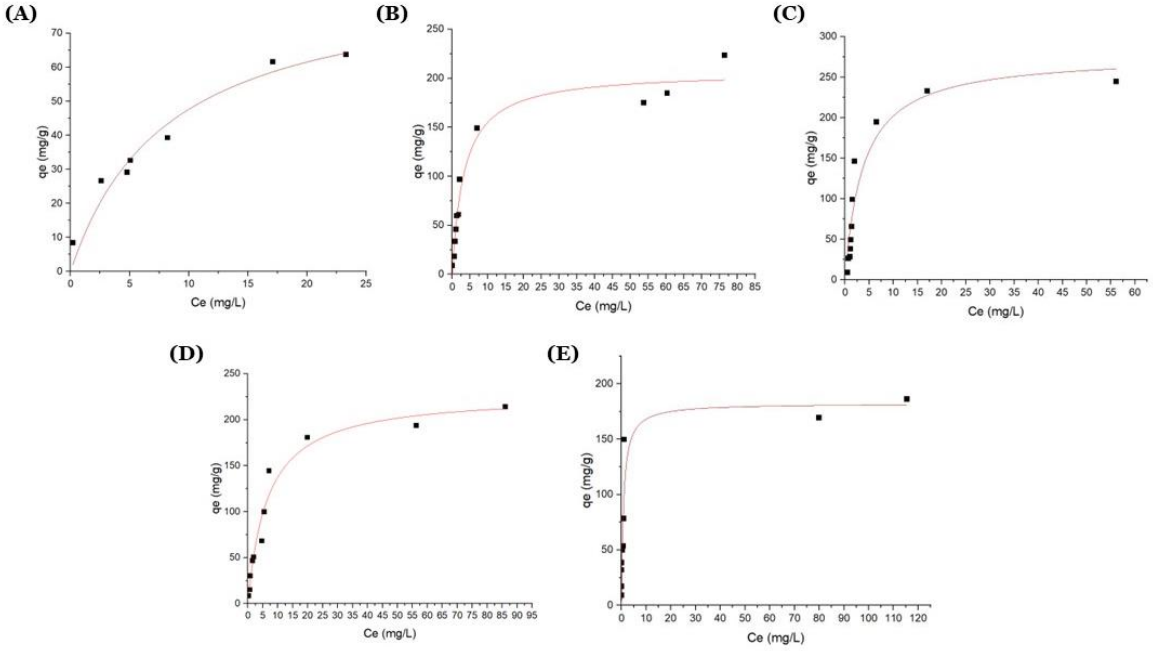

Figure 16 . RNA adsorption isotherms. The curve is representative of **(A)** ND-ox; **(B)** ND; **(C)** MD; **(D)** ND-H; and **(E)** ND-NH_2.

Table 4 . Langmuir parameters obtained by fitting the data in Figure 16.

Carbon Material	R^2	q_m (mg/g)	K_L (L/mg)
ND-ox	0.9537	86.9080	0.1214
ND	0.9647	206.9921	0.2996
MD	0.9178	277.5934	0.2677
ND-H	0.9707	228.3714	0.1515
ND-NH_2	0.9013	180.0936	0.8163

The coefficient of determination (R^2) analyzes the degree of fit of the isotherm model with the experimental data [254]. The closer the R^2 values are to 1, the more suitable the model is to correctly describe the adsorption of RNA by each CM [256]. The results show good fitting with the Langmuir isotherm model, with all R^2 values greater than 0.90. q_m represents the RNA maximum adsorption capacity at equilibrium and K_L the Langmuir equilibrium adsorption constant (L/mg).

The maximum adsorption capacity of the CMs under study, with the exception of ND-ox, surpasses that of other materials documented in the literature (Table 5).

Table 5. Nucleic acid extraction capacity using different methods, reported in the literature and obtained through this work.

Material	Molecule adsorbed	Extraction capacity (mg/g)	Detection technique	Reference
Composite UiO-66-NH_2	RNA DNA	225 75 - 100	UV-vis spectrophotometer	[257]
P (HEMA-VPBA) nanoparticles	RNA	172	UV-vis spectrophotometer	[258]
HEMA-co-VPBA membranes	RNA	16	UV-vis spectrophotometer	[259]
HEMA nanoparticles	RNA	147	UV-vis spectrophotometer	[260]
Fe_3O_4-COOH@UiO-66-NH_2@DES	RNA	246	UV-vis spectrophotometer	[257]
MWCNTs	RNA	175	UV-visible spectrophotometer	[242]
Oxidized NDs (ND-ox)	RNA	86.9	UV-visible spectrophotometer	**This work**
NDs	RNA	206.99	UV-visible spectrophotometer	**This work**
MDs	RNA	277.6	UV-visible spectrophotometer	**This work**
Hydrogenated NDs (ND-H)	RNA	228.37	UV-visible spectrophotometer	**This work**
Aminated NDs	RNA	180.09	UV-visible spectrophotometer	**This work**

The extraction capacities of MD and ND-H, of 277.6 mg of RNA / g of material and 228.37 mg of RNA / g of material, respectively, were found to be higher than that of other materials used for RNA extraction reported in the literature, such as the composite UiO-66-NH_2 that has an extraction capacity of 225 mg of RNA / g of material. The extraction capacity of the remaining materials was found to be similar to that of the materials reported in the literature. Regarding NDs, the determined extraction capacity was of about 206.99 mg RNA /g of material, which is lower than that of MD and ND-H studied in the present work, but only lower than the adsorption capacity of Fe_3O_4-COOH@UiO-66-NH_2@DES (246 mg of RNA / g of material) described in the literature. ND-ox presented the lowest adsorption

capacity of all the CMs under study (86.9 mg RNA / g of material), but when compared with other materials, the adsorption capacity of ND-ox is higher than that of HEMA-co-VPBA membranes, which present an adsorption capacity of only 16 mg of RNA / g of material. The lower adsorption capacity is not directly associated with a disadvantage, as these materials can be more prone to allow desorption of RNA or facilitate some selectivity. In general, this finding underscores the suitability of these CMs for RNA adsorption applications. Given the hydrophobic nature of the promoted interactions, the adsorption capacity can be expected to increase even more at higher concentrations of ammonium sulphate.

K_L is related to the energy of adsorption, which quantitatively reflects the affinity between the adsorbent and adsorbate. This way, lower K_L values correspond to lower affinity [254] or, in other words, weaker interactions between RNA and the CM. The weaker the interactions that enable adsorption, the easier the desorption. Thus, the strength of the interactions between RNA and ND-ox is lower than those of RNA and the other CMs. For this reason, it is expected that it will be easier to desorb and recover RNA from the surface of ND-ox (K_L is 0.1214 L/mg), than from the other CMs whose values of K_L are higher.

4.1.3. Screening of RNA desorption conditions

Since the main aim of this work is to explore the capability of CMs to capture and recover RNA, at first from artificial mixtures of RNA and pDNA and then from complex lysate samples as a strategy for pre-purification of this molecule, it was necessary to study the RNA desorption conditions that guarantee the maximum recovery of the adsorbed molecule. The desorption strategy depends on the chemical characteristics of the molecule intended to be adsorbed, the nature of the adsorbents, and the adsorption mechanisms responsible for the adsorbent–adsorbate interactions [112]. If we suppose an adsorption mechanism based on the electrostatic attraction of charged functional groups on the sorbent to oppositely charged functional groups of the analyte(s), in that case, the desorption strategy should be capable of disrupting electrostatic interactions. This disruption can be accomplished through:

- a pH modification to neutralize the compound and/or sorbent functional groups. pH manipulation can improve recovery (adjust pH opposite to load conditions).
- an increase in salt concentration (> 1.0 M) or use of a more selective counter-ion to compete for ion-exchange binding sites [117].

If the adsorption mechanism is mainly based on hydrophobic interactions, elution can be performed by a decrease in salt concentration [118]. Changes are made with a continuously decreasing salt gradient or stepwise. Most commonly, samples are eluted with a decreasing gradient of ammonium sulphate. Other elution procedures include reducing eluent polarity

(ethylene glycol gradient up to 50%) [119], adding chaotropic species (urea), or detergents [261], and changing pH [262] or temperature [263].

The selection of the desorption solutions for each experiment was based on the type of interactions previously promoted between RNA and each CM.

4.1.3.1. Desorption when the binding was driven by electrostatic interactions

For the experiments in which electrostatic interactions had been promoted in the adsorption step, four different concentrations of NaCl in the desorption solution and a variation in its pH were tested.

An initial sample of LMWRNA with a concentration of 50 μg/mL was prepared by dilution in 100 mM Tris-HCl pH 8.0 and adsorbed onto ND, MD, ND-H and ND-NH_2, previously equilibrated in 100 mM Tris-HCl pH 8.0. Different NaCl concentrations (0.25 M, 0.5 M, 1.0 M, 1.5 M) in 100 mM Tris-HCl pH 8.0 were tested for the desorption experiments.

By analyzing Figure 17, it is possible to observe that the recovery of LMWRNA previously adsorbed onto all materials, except ND-ox (that had not adsorbed RNA when electrostatic interactions were promoted), was considerably low and did not appear to depend on the ionic strength of the desorption solution used. In resemblance to ion-exchange chromatography, based on electrostatic interactions between charged patches on the surface of biomolecules and oppositely charged functional groups attached to a stationary phase [117], electrostatic interactions are stronger when the ionic strength of the surrounding buffer is low, and changes in ionic strength and pH can modulate binding. Desorption of the adsorbed molecule is commonly performed by increasing the buffer's ionic strength, thereby eluting the molecule by ionic competition [117]. Nevertheless, as depicted by Figure 17, recovery percentages were low (< 8%) for all the materials and NaCl concentrations tested.

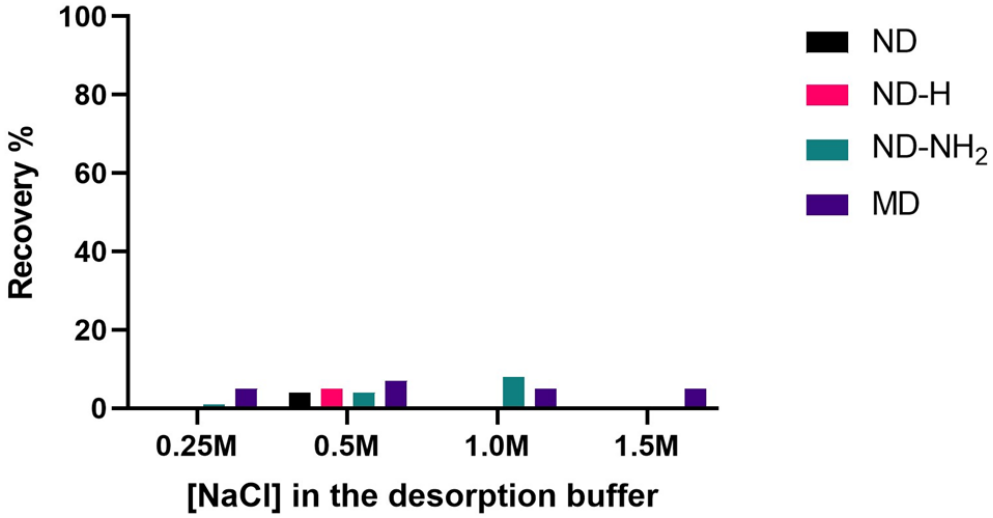

Figure 17. Study of RNA desorption from ND, ND-H, ND-NH_2, and MD when electrostatic interactions were promoted in the adsorption step, by using four increasing concentrations of NaCl (0.25 M, 0.5 M, 1.0 M and 1.5 M) in 100 mM Tris-HCl pH 8.0.

To calculate the RNA recovery percentage the following expression was used:

$$Recovery\ \% = \frac{m_{desorbed\ RNA}}{m_{adsorbed\ RNA}} \times 100 \quad (4)$$

Being the mass of adsorbed and desorbed RNA calculated by:

$$m_{desorbed\ RNA} = [desorbed\ RNA] \times V \quad (5)$$

where $m_{adsorbed\ RNA}$ and $m_{desorbed\ RNA}$ are the mass of adsorbed RNA and the mass of desorbed RNA, both in µg, $[desorbed\ RNA]$ is the concentration of desorbed RNA whose calculation is based on the measurement of the absorbance of the supernatants recovered after the adsorption and the desorption steps, and V is the volume of the supernatant recovered after the desorption step.

Low molecule recoveries could be due to the high strength of the interactions established between RNA and the surface of each CM. Although the aim of adding sodium chloride to the desorption buffer is to disrupt electrostatic interactions between RNA and the surface of CM, leading to its desorption, other types of physicochemical interactions may be occurring and promoting adsorption, thus hampering RNA desorption. Since this strategy did not work, another desorption technique was tested by changing the pH of the desorption

solution, which could change the net charge of the groups involved in the interactions [117]. Since the equilibration buffer used to promote adsorption was at pH 8.0, lower and higher pH values were tested, respectively pH 5.0 and pH 9.0. Bernhardt and colleagues, in their work, showed that acidic pH stabilizes key intra- and intermolecular RNA bonds [264]. With this in mind, the RNA molecule should be more stable at pH 5.0 than at pH 9.0. The recovery achieved for MD at pH 9.0 was slightly higher than at pH 5.0, but in both cases, it was lower than 5% (Figure 18).

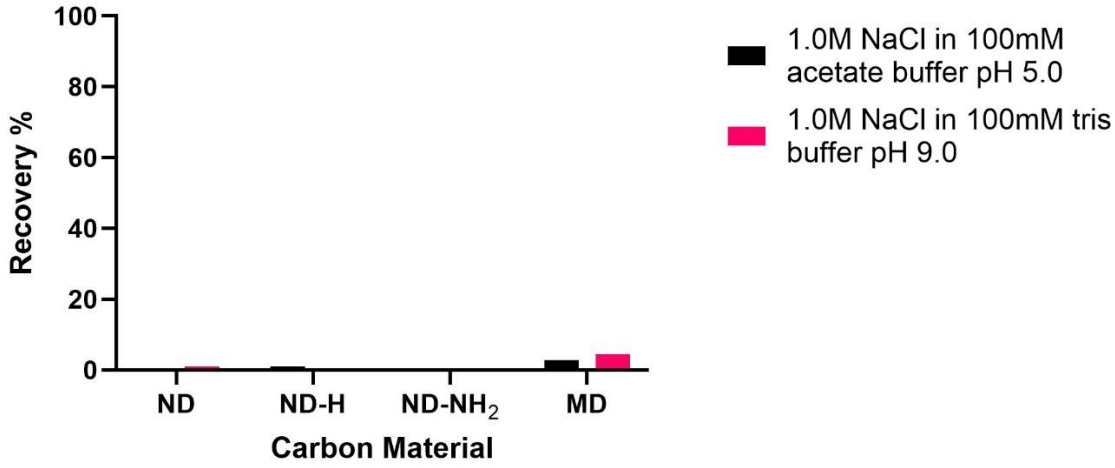

Figure 18. Study of RNA desorption from ND, ND-H, ND-NH_2 and MD when electrostatic interactions were promoted in the adsorption step, by using two desorption solutions with different pH, respectively 1.0 M NaCl in 100 mM acetate buffer pH 5.0, and 1.0 M NaCl in 100 mM Tris buffer pH 9.0.

Overall, it is verified that the RNA binding is so strong that it is not possible to recover it under these conditions.

4.1.3.2. Desorption when the binding was driven by hydrophobic interactions

Given that none of the two desorption strategies worked when electrostatic interactions were promoted in the adsorption step, it was decided to test RNA desorption when hydrophobic interactions were promoted in the adsorption step. Two different desorption solutions were tested: a solution without ammonium sulphate (100 mM Tris-HCl pH 8.0) and a solution of a surfactant agent (0.01% Tween-20).

Upon observation of the results represented in Figure 19, it was possible to verify that almost all RNA molecules previously adsorbed on the surface of ND-ox could be recovered using 100 mM Tris-HCl pH 8.0 as desorption solution, achieving a recovery percentage of

83.33%, and a total recovery was attained by using 0.01% Tween-20 as desorption condition.

Using a solution without ammonium sulphate (100 mM Tris-HCl pH 8.0), decreases the interaction strength between RNA and the surface of ND-ox, inducing its desorption [265]. Regarding the Tween-20, it was selected as it is a non-ionic surfactant agent [266]. Surfactants are amphiphilic organic molecules consisting of one or more hydrocarbon chains (hydrophobic tails) attached to a polar or ionic group (hydrophilic head) [267]. Non-ionic surfactants have polar head groups that are not electrically charged. These agents are used to promote the elution of antibodies in hydrophobic interaction chromatography, for instance. One hypothesis for Tween-20 promoting the desorption of RNA from the surface of ND-ox is that the hydrophobic interactions established between Tween-20 and CM are stronger than the hydrophobic interactions established between RNA and the surface of CM, leading to its desorption. Furthermore, the hydrophobic interactions between RNA and the surface of ND-ox may be weaker than between RNA and the surface of the other materials since, as already mentioned, oxidation increases the hydrophilicity or, in other words, decreases the hydrophobicity of CMs. Given that hydrophobic interactions are dynamic and highly adjustable [268] and the hydrophilicity of CMs is enhanced by increasing the content of surface oxygen-containing functional groups [269], oxidized NDs should be less hydrophobic than non-functionalized NDs and MDs, facilitating the desorption process.

Moreover, as mentioned before, K_L is related to the energy of adsorption, which quantitatively reflects the affinity between the adsorbent and adsorbate, and this way, lower K_L values correspond to lower affinity or, in other words, weaker interactions between RNA and CM. The weaker the interactions that enable adsorption, the easier the desorption. Thus, the strength of the interactions between RNA/ND-ox is lower than those of RNA and the other CMs, as revealed by the K_L values obtained in the Langmuir fitting (Table 4). This is in agreement with the results in Figure 19, where it can be observed that desorption was only attained for ND-ox, the CM for which the K_L value was the lowest.

The RNA recovery percentage using 0.01% Tween-20 is higher than 100% relative to the amount of RNA captured, due to interferences caused by Tween-20 during the absorbance measurement. Nevertheless, an agarose gel electrophoresis confirmed the presence of RNA in the samples recovered after the desorption step (Figure 20). All subsequent desorption assays were performed using 0.01% Tween-20 as a desorption condition.

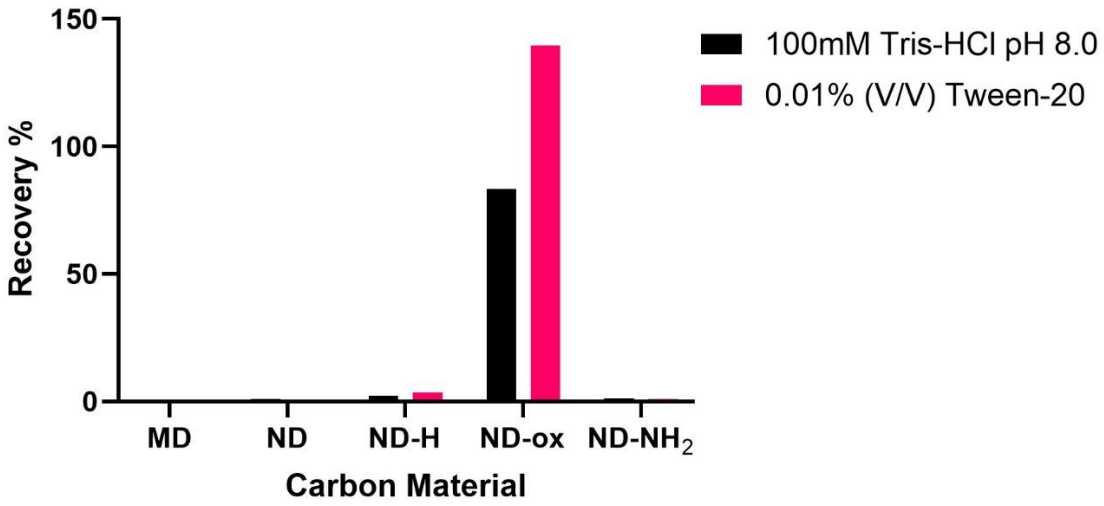

Figure 19. Study of RNA desorption from MD, ND, ND-H, ND-ox and ND-NH_2 when hydrophobic interactions were promoted in the adsorption step, by using two different desorption solutions: 100 mM Tris-HCl pH 8.0 and 0.01 % Tween-20.

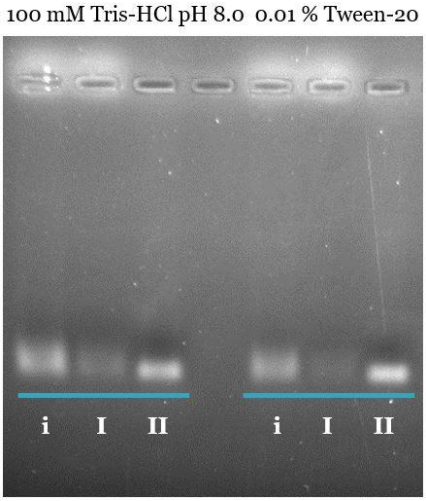

Figure 20. Agarose gel electrophoresis of RNA before and after capture and desorption: i- initial sample; I- sample recovered after adsorption comprising RNA that was not adsorbed to ND-ox; II- sample recovered after desorption comprising RNA desorbed from ND-ox.

By analyzing the agarose gel electrophoresis of both assays (Figure 20), it can be observed that there was a decrease in the intensity of the RNA band from the initial sample to the one recovered after the adsorption step, confirming the RNA adsorption. It can also be noticed that the fluorescence of the band corresponding to the RNA recovered by using 0.01% Tween-20 is more intense than that of the one corresponding to the sample recovered by using 100 mM Tris- HCl at pH 8.0. The intensity of the emitted fluorescence is proportional to the concentration of RNA present in the sample, indicating that a lower RNA concentration was present after the adsorption step, implying that ND-ox adsorbed it. After

the desorption step, a band with a similar intensity to the initial sample was obtained, corresponding to a good RNA recovery.

4.2. Plasmid DNA adsorption and desorption experiments using ND-ox

Given that pDNA is a contaminant frequently present in RNA samples, adsorption and desorption of pDNA were also evaluated for the same conditions used with RNA. With these assays, it was intended to verify if it was possible to selectively adsorb RNA when it is present in samples containing other species, such as pDNA. A pDNA sample with a concentration of 50 μg/mL was prepared with the same equilibration/binding buffer used for CM equilibration (1.0 M ammonium sulphate in 100 mM Tris-HCl pH 8.0) to promote hydrophobic interactions. A 0.01% Tween-20 solution was used as a desorption solution.

As it is possible to observe in Figure 21 (A), from the initial sample composed of approximately 50 μg of pDNA, only 7.7 μg were adsorbed. Furthermore, after the desorption step, only 3.5 μg of pDNA were present in the recovered sample, which corresponds to 7 % of the initial sample, which is a very interesting result. Since the same material, ND-ox, and the same conditions were used for the experiments performed with RNA and pDNA, the mass of RNA and pDNA adsorbed and recovered can be compared. In the case of RNA, from the initial sample with a concentration of 45.2 μg/mL, 36.4 μg/mL were adsorbed and the total RNA adsorbed was recovered. For pDNA, an initial sample with a concentration of 56 μg/mL was applied, and only 7.7 μg were adsorbed and 3.5 μg were present in the desorbed sample. An agarose gel electrophoresis was also performed to confirm the adsorption and desorption pattern of pDNA (Figure 21 (B)).

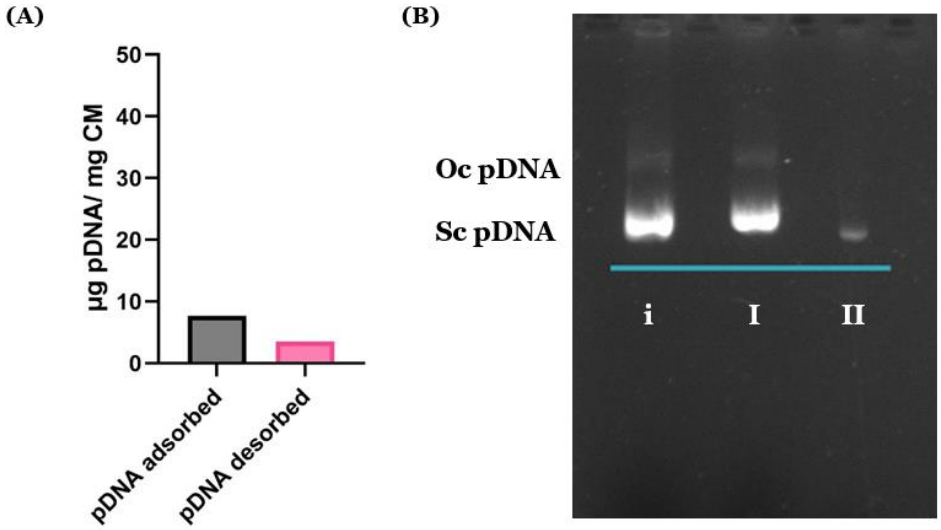

Figure 21. (A) Mass of pDNA per mg of CM adsorbed to and recovered from ND-ox; **(B)** Agarose gel electrophoresis of pDNA before and after capture and desorption: i-initial sample; I- sample recovered after adsorption comprising pDNA that was not adsorbed to ND-ox; II- sample recovered after desorption comprising pDNA desorbed from ND-ox.

By analyzing the agarose gel electrophoresis (Figure 21 (B)), it can be observed that the intensity of the pDNA bands, corresponding to the initial sample and the one recovered after the adsorption step, is similar. Thus, the initial amount of pDNA is similar to the amount of pDNA non-adsorbed, which confirms that the adsorption of pDNA by ND-ox was low. This is a promising result since this work aims to capture RNA when other molecules are present in a sample. If the same material applied in the same conditions can capture a higher amount of RNA than pDNA, it can indicate its selectivity towards RNA. The intensity of the band observed in the agarose gel, corresponding to the sample recovered after the desorption step, is low, indicating that the presence of pDNA in the recovered sample is residual.

4.3. Oxidised NDs selectivity analysis

4.3.1. Selectivity towards RNA in the presence of pDNA

To further assess the selectivity of ND-ox for RNA adsorption, some assays were performed with samples containing pDNA and RNA at different ratios. pDNA is one of the main contaminants present in RNA samples. For that purpose, artificial mixtures of RNA and pDNA were prepared and applied to 1 mg of ND-ox, previously equilibrated with 1 M of ammonium sulphate in 100 mM Tris-HCl pH 8.0. As a desorption solution, it was used

0.01% Tween-20 as previously described. Three different ratios of RNA and pDNA were prepared: 1:3 (RNA/pDNA), 1:1 (RNA/pDNA), and 3:1 (pDNA/RNA). The final concentrations of the respective samples were 25 µg/mL + 75 µg/mL (RNA/pDNA), 50 µg/mL + 50 µg/mL (RNA/pDNA), and 75 µg/mL + 25 µg/mL (RNA/pDNA). After each step of the procedure, supernatants were recovered and analysed by agarose gel electrophoresis, with the results being presented in Figure 22.

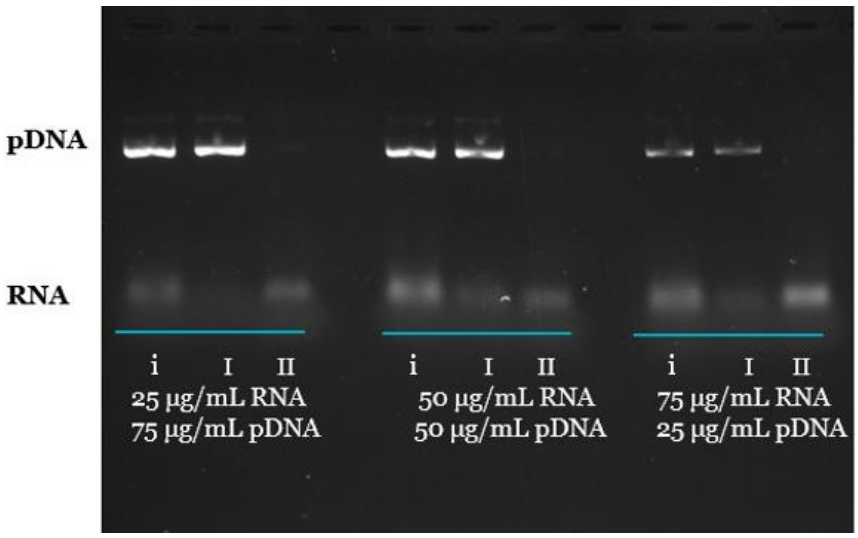

Figure 22. Agarose gel electrophoresis of RNA and pDNA mixtures in three different ratios before and after capture and desorption with ND-ox: i- initial sample; I- sample recovered after adsorption comprising the species non-adsorbing to ND-ox; II- sample recovered after desorption comprising the species desorbed from ND-ox.

Considering the representative agarose gel, it can be noted that in none of the three ratios tested, pDNA was present in the recovered sample. This is a positive outcome, indicating that RNA is selectively adsorbed onto the ND-ox surface in the presence of pDNA, even when the concentration of pDNA is 3 times higher than that of RNA. This selectivity is very important to guarantee a higher clarification of RNA samples recovered with ND-ox. Furthermore, the recovery of RNA was attained for the three concentration ratios tested. With these results, it can be concluded that ND-ox are capable of selectively capturing RNA when it is present in aqueous samples containing pDNA. Moreover, the capture of RNA did not appear to depend on the concentration of RNA nor pDNA in the sample, at least in the studied range, and if not surpassing the adsorption capacity of ND-ox.

4.3.2. Selectivity towards RNA in complex lysate samples

4.3.2.1. Clarified Lysate

After observing that it was possible to capture RNA from a sample containing pDNA and recover it without contamination with pDNA, similar assays were performed using more complex samples. For that, *E. coli* lysate samples previously clarified by isopropanol precipitation were incubated with 1 mg of ND-ox equilibrated with the adsorption solution containing 1 M ammonium sulphate in 100 mM Tris-HCl pH 8.0. Then, the desorption step was performed with 0.01% Tween-20. The aqueous phases recovered after each step were analysed by agarose gel electrophoresis, represented in Figure 23.

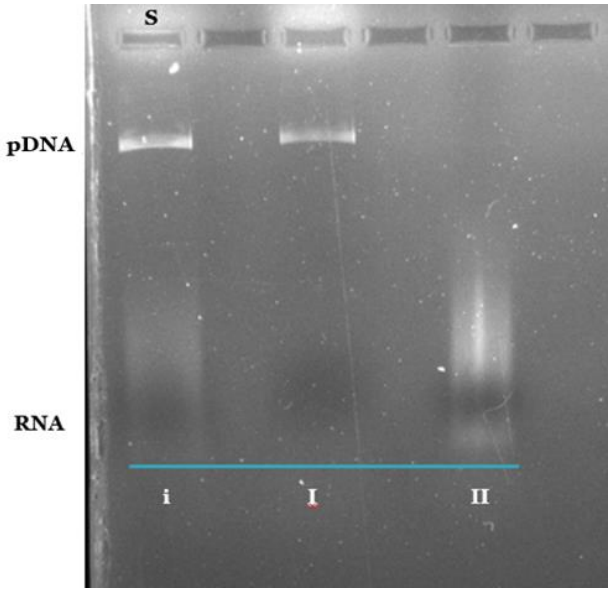

Figure 23. Agarose gel electrophoresis of the clarified *E. coli* lysate before and after capture and desorption using ND-ox: i-initial sample of the clarified lysate (comprising pDNA and RNA); I- sample recovered after adsorption, corresponding to the species not adsorbed to ND-ox; II- sample recovered after desorption comprising desorbed species from ND-ox.

By analysing the agarose gel of Figure 23, it can be observed that it was possible to capture RNA and recover it during desorption, which means that ND-ox selectively capture RNA when it is present in a clarified lysate sample.

The importance of this step relates to the fact that only 20.5% of the total dry weight of *E. coli* corresponds to RNA, while the remaining 79.5% consists of other contaminant species (gDNA, LPS, metabolites…) [270], that must be eliminated when RNA is the target. The study demonstrates that the procedure effectively captures and recovers RNA from such extracts, maintaining its selectivity towards RNA, which is a promising result.

4.3.2.2. Non-clarified Lysate

Precipitation with isopropanol is an intermediate step that eliminates a significant part of the contaminants. To test if the precipitation step with isopropanol during the lysate extraction influenced the capture and recovery of RNA, ND-ox were incubated with a lysate sample directly extracted, and without performing the precipitation step, which results in a more complex sample. The same procedure was used, and the samples recovered after each step of the procedure were analysed by agarose gel electrophoresis, as shown in Figure 24.

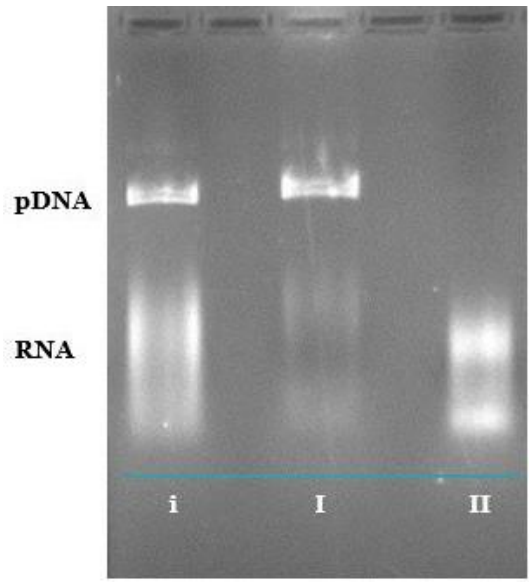

Figure 24. Agarose gel electrophoresis of the lysate sample before and after capture and desorption: i-initial complex lysate sample; I- sample recovered after adsorption, showing the non-adsorbed species to ND-ox; II- sample recovered after desorption from ND-ox.

As observed in Figure 24, from the initial complex lysate, only RNA was adsorbed, confirmed by the decrease in the intensity of the bands from the initial sample to the one recovered after the adsorption step. The bands corresponding to pDNA present a similar intensity both in the initial sample and after the adsorption step, indicating that pDNA was not adsorbed onto the ND-ox, which means that ND-ox are selective towards RNA when it is present in complex lysate samples. Furthermore, after the desorption step, only RNA was present in the sample. Thus, it is possible to say that ND-ox enable the selective capture of RNA when it is present in a complex *E. coli* lysate sample, and allows its recovery without pDNA contamination.

In both cases, for clarified and non-clarified lysates, the capture of RNA was attained. Despite the higher complexity and increased amount of RNAs in non-clarified lysates, the ND-ox were totally effective in selectively adsorbing RNA.

4.4.Total Protein Quantification

It has been reported that protein interactions with NPs are influenced by variations in the surface chemistry and size of the NPs. Proteins can be attached to a surface in different quantities depending on the physical and chemical properties of the material. Protein adsorption is a complex process involving different types of interactions, namely hydrophobic, electrostatic, van der Waals interactions and hydrogen bonding [271].

Complex *E. coli* lysate samples comprise different species, which include RNA, pDNA, gDNA, and proteins (host cell proteins (HCPs)). Proteins usually correspond to 55.0 % of the total dry weight of an *E. coli* lysate sample [270]. For this reason, it is important to verify if this contaminant can also be removed with this method.

HCPs are process-related impurities produced by the host organism during biopharmaceutical manufacturing [272] and their presence can directly affect the quality of RNA. As mentioned in USP, the measurement and control of these HCPs are important to achieve the desired efficacy and shelf-life [273]. Based on risk assessments, clinical experience, and manufacturing capability, typically accepted levels are <100 ng HCP/mg of biopharmaceutical product [274].

To determine the concentration of proteins present in complex *E. coli* lysate samples applied to ND-ox, as well as in the supernatants recovered after the adsorption and desorption steps, the Bio-Rad Protein Assay, based on the method of Bradford was used.

Preferentially, and following the goal of this work, proteins should not adsorb to ND-ox. Nevertheless, whether ND-ox adsorb the proteins or not, an outcome where no proteins would be present after the desorption step, would still be a promising prospect. The protein concentration present in each sample was calculated by measuring the absorbance and using the equation of the calibration curve previously obtained. In this work (Figure 25), it is possible to observe that just a low amount of protein was adsorbed to the CM.

As previously mentioned, the concentration of solubilized protein was calculated by measuring the absorbance of each sample using a microplate reader and using the calibration curve previously obtained (Figure 13).

$$\text{Abs } 595 \text{ nm} = 0.6274\,[\text{Proteins}] - 0.0107 \quad (6)$$

Table 6. Concentrations of proteins present in each sample.

Samples	**[Proteins]/ mg.mL^{-1}**
E. coli lysate sample (initial sample)	0.608
Sample not adsorbed to ND-ox	0.565

Sample desorbed	0.053

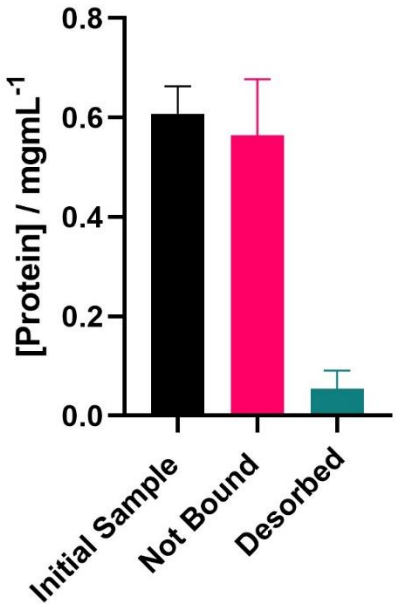

Figure 25. Protein adsorption and desorption from ND-ox. Values were calculated with the data obtained from three independent measurements (n = 3 ± sd).

It is possible to visualize that the initial lysate sample contained a protein concentration of 0.608 mg/mL. After the adsorption step, the concentration of proteins in the supernatant was 0.565 mg/mL, which means that only a small amount of proteins were adsorbed by ND-ox when 1 mL of lysate was applied. After the desorption step, a concentration of 0.053 mg of protein/mL was present in the recovered sample. This implies that the remnant amount of proteins remained bound to the surface of ND-ox after the RNA had been desorbed.

The following expression was used to calculate the protein removal percentage from the initial sample to the recovered one (after the desorption step):

$$\%\ \text{Removal} = \frac{[Protein]i - [Protein]f}{[Protein]f} \times 100 \quad (7)$$

where [Protein]i is the concentration of proteins present in the initial sample and [Protein]f represents the concentration of protein in the recovered sample (after the desorption step).

A protein removal percentage of 91.28% was attained, which is higher than a 78.75% protein removal obtained by Ferreira *et. al.* (2023) by applying MWCNTs. Therefore, it can be said

that a high protein removal efficiency was achieved by using ND-ox to capture and recover RNA from complex *E. coli* lysate samples.

This significant reduction is important since it can simplify the subsequent stages of the high-resolution purification process of RNA.

Chapter 5

Conclusions and Future Perspectives

Since its discovery at the beginning of the 20th century, RNA has been the subject of extensive research aiming to understand its multiple functions. This nucleic acid has been applied for the treatment of multiple genetic diseases, and the prevention of infectious diseases, such as COVID-19. Nonetheless, studies are still needed to continue widening the horizons of gene therapy.

For RNA to be used as a biopharmaceutical, it has to present high purity, integrity and biological activity. Biopharmaceutical production is a complex process involving multiple steps that need to be strategically combined to obtain a final product with the intended characteristics.

There is an urgent need to develop efficient and economical downstream processing strategies with fewer steps, maximize product recovery, and minimize production costs. With that in mind, DSPE, a method used for low-resolution purification, was optimized to capture and recover RNA from complex *E. coli* lysates containing impurities, such as pDNA and proteins.

Due to their large surface area, high chemical stability, biocompatibility, dispersibility in water, the possibility of surface functionalization, and high adsorption capacity, NDs and MDs arise as potential CMs to be used as adsorbents in DSPE. In this work, MDs, NDs, and functionalized NDs, more specifically ND-H, ND-ox, and ND-NH_2 were studied as adsorbents of RNA.

At first, screening experiments on adsorption and desorption were carried out to choose the best conditions for selective RNA capture. These experiments enabled an understanding of the impact of different surface functionalizations on the adsorption and desorption of RNA. Different types of interactions between RNA and the surface of CMs were promoted namely electrostatic and hydrophobic interactions. It was observed that all CMs, except ND-ox, were capable of adsorbing RNA upon promotion of electrostatic interactions in the adsorption step. When promoting hydrophobic interactions in the adsorption step, it was verified that RNA was captured by all CMs under study. In addition, increasing the ionic strength in the equilibration/binding buffer did not lead to a significant increase in RNA binding for most of the CMs since those had already adsorbed all the RNA present in the sample. For oxidized NDs, the RNA adsorption capacity increased with the increase of ionic strength.

Studies on desorption were also carried out, and the best conditions regarding RNA recovery and purity were chosen. Significant recoveries of RNA were only attained for ND-ox by applying 100 mM Tris-HCl pH 8.0 and 0.01% Tween-20 as desorption solutions when hydrophobic interactions had been promoted in the adsorption step. With these studies, ND-ox was the selected material to perform the remaining experiments, when promoting hydrophobic interactions in the adsorption step and using 0.01% Tween-20 as a desorption solution.

Subsequent experiments aiming to study the selectivity of this CM towards RNA were performed. For that, artificial mixtures of RNA and pDNA, the main contaminant present in RNA samples, in three different concentration ratios were prepared and applied to ND-ox. In all cases, it was possible to selectively capture RNA and recover it without pDNA contamination.

For a more complete evaluation of the selectivity of ND-ox towards RNA, complex *E. coli* lysate samples were used as biological samples. Resembling the previous assays, ND-ox were also capable of capturing RNA, and its recovery after the desorption step was attained without pDNA contamination. It is also important to note that complex *E. coli* lysate samples comprise different species, including RNA, pDNA, gDNA, and proteins (host cell proteins (HCPs)). Since in this work it is intended to capture RNA from complex lysate samples, the recovered RNA should be free of contaminants including other nucleic acids, such as pDNA, and proteins. Actually, in the conditions established, a protein removal percentage of 91.28% was attained. Therefore, it can be said that a high protein removal efficiency was achieved by using ND-ox to capture and recover RNA from complex *E. coli* lysate samples. This significant reduction is important since it can simplify the subsequent stages of the high-resolution purification process of RNA.

This method proved to be a simple, efficient, rapid, and environmentally friendly approach for the pre-purification of RNA when it is present in complex bacterial lysate samples.

In the future, it would be interesting to evaluate the cytotoxicity of NDs and MDs to confirm their biocompatibility. In addition, performing circular dichroism experiments to assess the integrity and stability of the RNA recovered after desorption from ND-ox would be relevant. It would also be interesting to characterize other impurities that could be present in the sample, such as gDNA and endotoxins, to further validate the effectiveness of NDs to capture RNA and potentially be translated to biomedical applications.

Chapter 6

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