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Carbon Dots for Gene Therapy

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Abstract

Carbon Dots (CDs) are extremely promising fluorescent nanoparticles. These particles are frequently used in Nanomedicine as biosensors, drug carriers and imaging probes. In this project CDs are synthesized to interact with nucleic acids to promote gene delivery and therapy.
Is convenient to use nanoparticles as DNA/RNA vectors because they can be better engineered, tuned and it's possible to control collateral effects.

Folic Acid, Citric Acid, o-Phenylenediamine and Polyethylenimine 25 kDa were used as precursors. After a purification step, the particles were characterized in term of: UV-Vis spectrum, Fluorescence spectrum, AFM images, TEM images, Zeta Potential, TGA, IR spectroscopy and Mass spectrum.
Following these preliminary characterizations, made to evaluate features and chemical structure of the particles, DNA affinity tests were performed. The complex CDs-calf thymus DNA in different ratios was studied in term of UV-Vis spectrum, fluorescence spectrum, Circular Dichroism and electrophoresis.
In the end, cytotoxicity tests were performed on different cancer and healthy cell lines.
Two different healthy cell lines were used with only CDs and CDs in combination with DNA.
The viability of cell lines was evaluated using different concentrations of particles.

The formed nanoparticles showed a very narrow distribution size (around 1 nm), a positive surface charge and a good excitation independent fluorescence spectrum. Due to the large amount of PEI 25 kDa, the particles could be identified as random polymeric structures with the presence of organized benzene like domains and folic acid domains.
In addition, the particles showed a good stability in deionized water solution and in solid state.
The DNA binding tests revealed a good interaction between CDs and DNA. Furthermore, the DNA is able to quench the fluorescence of the particles.
Cellular cytotoxicity tests confirm a good biocompatibility of the complex nanoparticle-nucleic acid, in this way, the compound can safely deliver genes to the cells.
Besides passive targeting of cancer cells, active targeting can be achieved due to the presence of Folic Acid in the particles.

Next steps in this research may include linking a particular DNA or RNA sequence to treat a specific type of cancer. More importantly, cellular and nucleus internalization studies
must be performed. The aim of the studies would be to ensure to have a good and efficient nano-carrier that is able to efficiently deliver nucleic acids to tumor cells.

This research project was aimed at the creation of Carbon nanoparticles able to deliver genetic fragments into the cells.
The first stage of the work consisted in the synthesis of a positively charged nano-dots to promote electrostatic affinity with nucleic acid fragments. To reach this objective many approaches had been investigated, tuning different reagents, synthesis protocols, purification methods.
After this step the particles were characterized in terms of : UV-Vis spectrum, Fluorescence spectrum, AFM and TEM images, Zeta Potential, TGA, IR spectrum and Mass spectrum.
Subsequently the conjugation with calf thymus DNA was evaluated, following literature's methods.
The last part of the project was focused on biological Cytotoxicity tests, which were performed on both cancer and healthy cells. Results were analyzed, processed and discussed to make work's conclusions and to plan future developments.

Summary

1 Introduction to Gene therapy

1.1 Gene therapy overview

The definition of gene therapy according to The European Medicines Agency (EMA) explains "Gene therapy is a gene therapy medicinal product is a biological medicinal product which fulfils the following two characteristics: (a) it contains an active substance which contains or consists of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, replacing, adding or deleting a genetic sequence; (b) its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence. Gene therapy medicinal products shall not include vaccines against infectious diseases"[i].

The US Food and Drug Administration (FDA) defines gene therapy as products "that mediate their effects by transcription and/or translation of transferred genetic material and/or by integrating into the host genome and that are administered as nucleic acids, viruses, or genetically engineered microorganisms".

Gene therapy is a new way to modify the genome or the expression of a certain gene, in order to cure or prevent different kind of pathologies.

The main steps for a generic Gene therapy are illustrated in figure 1. From the selection of the nucleic acid sequence, which is determined by the therapy's goal, to the real expression or genome modification.

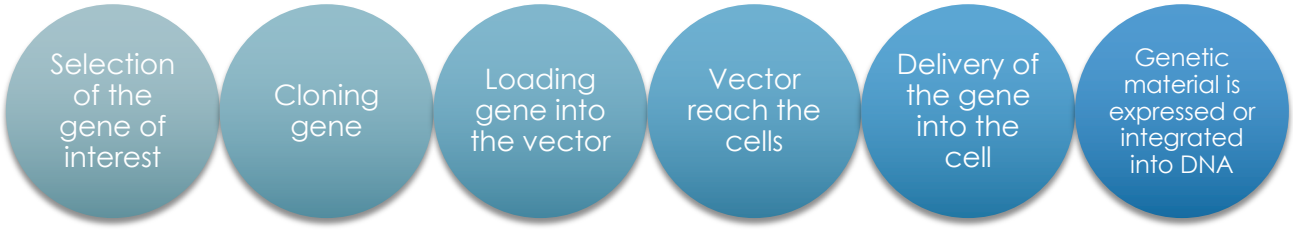


Figure 1: Illustration of gene therapy's key points

Initially, gene therapy was developed as a treatment for primary immunodeficiencies, as the only therapeutic option.

The first gene therapy treatment was Glybera in 2012, approved by the European Medicines Agency. Glybera consists in Adeno Associated Viral Vector that is capable of delivering a specific gene for the production of Lipoprotein Lipase in patients with LL-protein deficiency[ii].

In 2017 FDA approved the first gene therapy treatment named Kymriah. Kymriah (CAR-T Therapy) consists in a genetically modified autologous T-cell immunotherapy for the Treatment of Acute Lymphoblastic Leukemia[iii].

Considering the good results reached, gene therapy has been studied as a method to treat other types of malignancies such as cancer[iv], neurodegenerative diseases[v], chronic diseases[vi], and metabolic disorders[vii].

1.1.1 Germinal and somatic gene therapy

It's possible to separate gene therapy into two branches referring to the type of cell that undergo the treatment:

Germinal Gene therapy in which Germinal cells are transfected[viii]. In this case, embryonal and totipotent stem cells, are directly involved. This type of treatment modifies both the cells and but also all the next generations.

Somatic Gene therapy is applied to normal, non-germinal cells. It can be implemented in two ways: in vivo and ex vivo[ix].

After the transfection of the gene, there are two different evolutions:

- The gene is not integrated in the genome, in other words is not transferred to the cells' new generations. It remains present as episome.
- The gene is integrated in the genome, therefore replicated and transferred to the new cells.

1.1.2 'in vivo' and 'ex vivo' gene therapy

The therapeutic objective determines:

1) the type of gene to be used
2) The method to transfer the gene to the target cells' nucleus
3) In vivo or ex vivo gene therapy strategy

In vivo gene therapy technique works by inserting the therapeutic gene directly into the biological environment of the patient. In the majority of times, the genetic material is carried through a vector injected in loco or in the bloodstream[x].

This method is easier and cheaper but is not possible to control the transfection and select specific cells.

The **ex vivo** procedure consists in collecting somatic cells from the patient, followed by transfection in vitro. After these steps, treated cells are selected, expanded and inserted into the patient's body[xi].

This method is extremely efficient and permits the selection of useful cells, on the contrary, is both very time consuming and expensive.

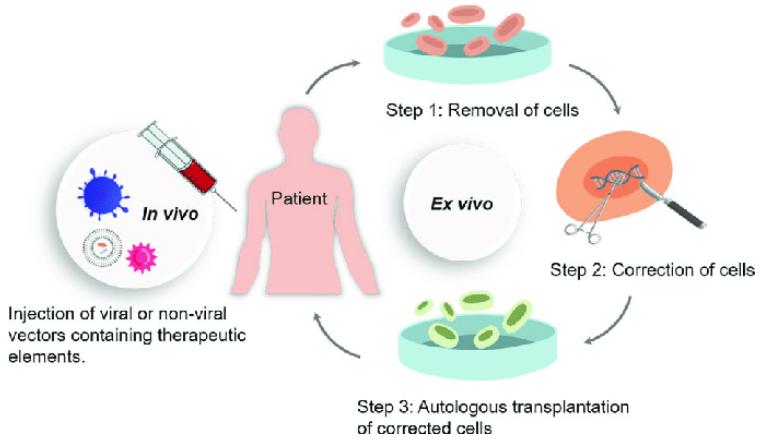


Figure 2: Graphic description of In vivo and ex vivo techniques [xii]

Nowadays this field is still in the research phase because of multiple reasons such as ethical problems and the lack of specific protocols and regulations.

Is convenient and necessary to deliver the genetic material into the cells and eventually into the nucleus through a carrier [xiii]. It's also possible to deliver

genes into the cells without carriers using different Physical Methods such as: Needle Injection[xiv], Electroporation[xv], Sonoporation[xvi], Photoporation[xvii], Magnetofection[xviii], Hydroporation[xix] etc.

Unfortunately, the uptake of naked DNA is less controlled, inefficient. It's even made more difficult by the degradation of the free genetic fragments in biological environments.

1.1.3 Regulated gene expression

After the delivery of genetic material to the cells, is necessary to regulate the expression of the newly inserted genes. It may be dangerous if the gene is over-expressed or under-expressed. For this reason, is convenient to develop a system to regulate expression. These systems that usually consist in regulatory sequences, can be incorporated into the carriers.

1.2 Type of carriers

Experiments on delivery of naked nucleic acids were performed from Kobayashi, N., in 2002[xx]. Unfortunately, is not possible to deliver naked DNA to the cells because of the phosphodiester groups. Being negatively charged, they don't allow a good cellular attachment and uptake. Moreover, the genetic fragments can barely survive to nuclease degradation.

It's convenient to use delivery vector that can easily enter in the cell or in the nucleus and release the genetic material.

Carriers for gene delivery can be divided in two main families: viral vectors and non-viral vectors.

1.2.1 Viral carriers

Viruses are microscopic infectious agents, formed by genetic material, either DNA or RNA, and a protein (CAPSID) that protects the carried genes and allows the transfection of this material into the cells. Capsid can be covered by a lipid

membrane. Viruses don't have their own metabolism and therefore they can't reproduce. They need to infect an eucaryotic or procaryotic cell.

Viral vectors are the widely used type of carrier[xxi]. Because of their modulability they can easily substitute the genetic material with the genes of interest. Also, the gene delivery in cells is highly efficient.

Moreover, they have low toxicity, they are extremely stable in biological environments. They can target specific cell types and they can carry a high capacity of genetic material.

1.2.1.1 Retroviruses

Retroviruses is a compact RNA virus. Virus' RNA is enclosed in an envelope protein capsule that specifically interact with cell membranes' receptors. For this reason, the virus is efficiently internalized into the cell. When the RNA remains uncoated, there is the reverse transcription process in which the virus RNA is transcribed in a virus DNA. The virus DNA enters in the nucleus and is stably integrated in the cell's genome.

For some types of retroviruses, mitosis or breakdown of nuclear membrane are necessary for the DNA to reach the nucleus.

In the end the infected cell will produce retroviruses thanks to the transcription process of the newly integrated DNA.

Retroviruses are hence able to insert genetic material permanently into the host cells.

The infected cell generates viruses steadily without noticeably changing the cell's development and behaviour.

Retroviral vectors are excellent candidates for ex-vivo gene therapy. Cells can be collected, isolated, propagated in culture, transfected with a modified retroviruses and implanted into the patient.

Retroviral vectors may also be utilized in vivo to infect some quickly dividing cells such as: proliferating synovial cells (covering inflammatory joints), tumoral cells and, hepatocytes after partial hepatectomy.

Guild *et al.*[xxii] investigated and characterized Monoley Murine Leukemia Virus (MMLV) which is now one of the most used retrovirus in clinical treatments.

The virus' genome decodes three proteins that have to be removed and substituted by regulatory sequences and heterologous genes.

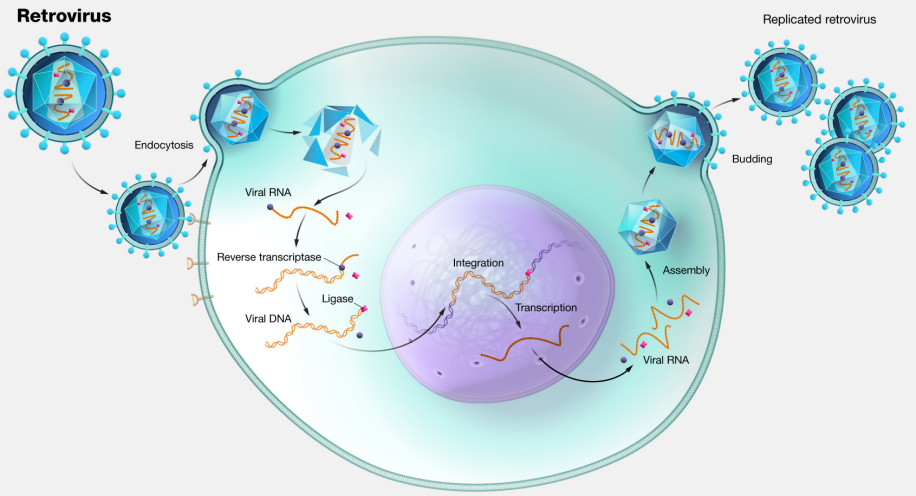


Figure 3: Mechanism of infection and replication of Retrovirus [xxiii]

1.2.1.2 Adenoviral vectors

Adenoviruses present an outer spheroidal envelope in which is enclosed a double stranded DNA of about 35 kb length[xxiv].

This family of viruses is widely used for in vivo gene therapy but also for other purposes (recombinant vaccines[xxv]). They are relatively stable, and the insertions of a heterologous gene is simple and it remains unaltered through successive rounds of viral replication[xxvi].

Adenoviruses can infect a variety of different cells thanks to a good interaction between viral capsule proteins and cell membranes' receptors.

The mechanism of infection is described in detail in figure 4.

For what concerns gene delivery, this type of virus is extremely efficient in infecting non-dividing cells, both in vivo and in culture. The result consists in a high level of short-term gene expression. For this reason, adenoviral vectors are

more suitable to treat transient gene expression disorders. For example, they can be applied in cancer treatment, cardiovascular disease, restenosis .

In this case, it is necessary to improve and develop long term therapies, principally investigating on the body's immune response.

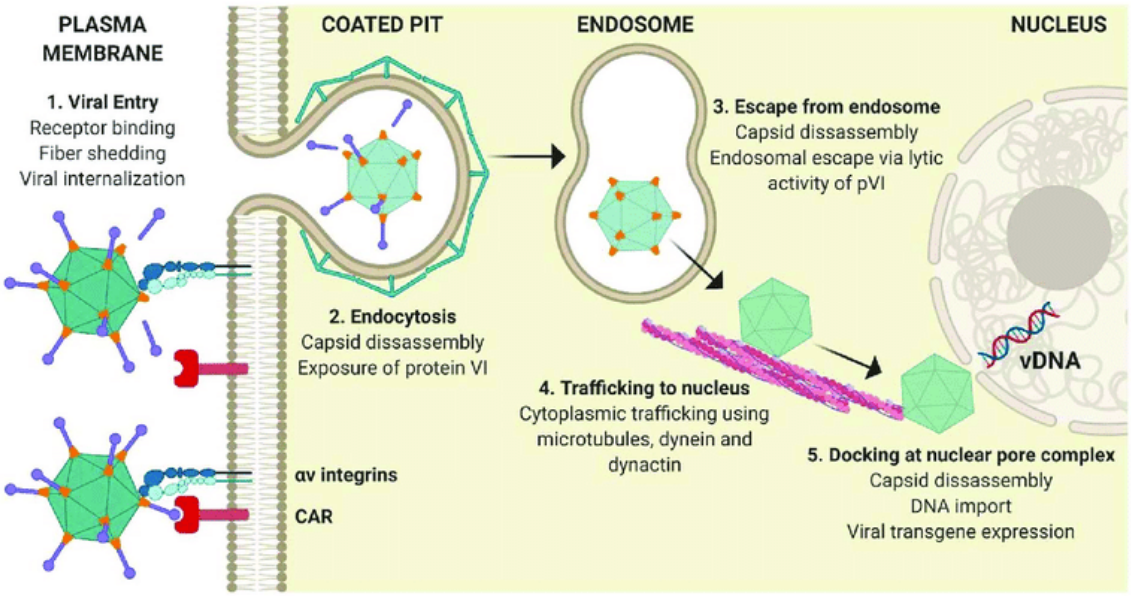


Figure 4: Adenoviral's transfection pathway detailed description[xxvii].

1.2.1.3 Adeno Associated Virus

Adeno Associated Virus is one of the most studied and utilized gene therapy carrier[xxviii],[xxix]. This virus contains a single strand DNA covered by a protein capsule. The capsule has an icosahedral structure and is covered by many capsid proteins. Unfortunately, it can only contain up to about 4.8 kilobases, a limited capacity.

AAV can't replicate itself alone, it needs another virus like herpesvirus or adenovirus (dependant on co-infection).

It promotes a long-term and safe gene treatment, in fact it's possible to administer high doses without relevant inflammatory responses and cytotoxicity effects. Furthermore, there aren't any associated diseases.

Being one of the most promising viral vectors, it can be exploited to treat dividing and non-dividing cells. The epsome remains stable and it is not integrated in post-mitotic daughter cells.

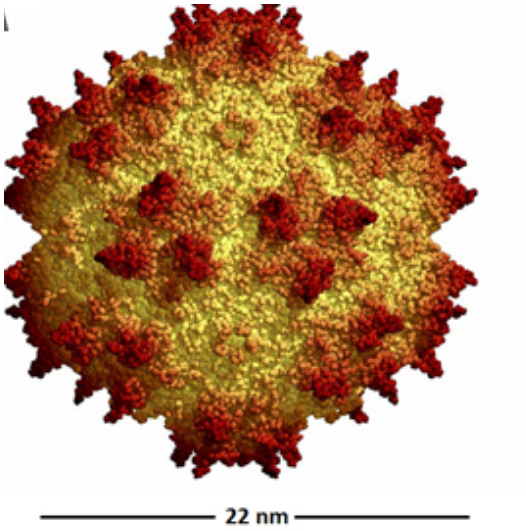


Figure 5: Simple external structure of Adeno Associated Virus [xxx]

The majority of AAV vectors used in gene therapy are based on AAV-2, AAV-2-based and rAAV vectors. The best expression takes several weeks and can be achieved by transducing muscle, liver, brain, retina, and lungs.

1.2.1.4 Issues

The main challenge that needs to be faced using Viral gene carriers is the immunoreaction. The worst-case scenario is a strong and inappropriate activation of the inflammatory response that can cause the death of the patient (Raper et al, 2003)[xxxi]. Immunoreaction consists in the immunological response against a foreign entity infection (in this case viruses) [xxxii],[xxxiii].

Adenoviruses are the most immunogenic; T-lymphocyte (CTL) and T-cell responses against foreign gene products, expressed by infected cells, are very common.

Also, the capsid proteins induce a strong humoral response with a consequent production and release of antibodies. Moreover, inflammatory response cytokine-mediated is also induced by virus' extern envelope.

A solution to decrease the unwanted immunogenic process is to delete all the viral genome and to modify capsid proteins which can also increase the treatment's duration. (Büning et al, 2008)[xxxiv] developed Capsid Engineering to face this challenge. His solution is suitable for subjects having a pre-existing anti AAV humoral immunity.

Another secondary problem is that native viruses are more subjected to infect a specific tissue. This issue could evolve in two different adverse situations:

a) An unwanted uptake that makes the viruses transfect and deposit gene in different cells, not the targeted ones.
b) Leak, followed by inefficient uptake of therapy in target cells.

Some possible solutions to this problem could be for example injecting the carrier as close as possible to the region of interest. In combination with the previous solution, engineering a virus' protein envelope would further improve the interaction with the targeted cells.

1.2.2 Non-viral carriers

1.2.2.1 Overview

An alternative to viruses' usage is non-viral vectors such as: liposomes, DNA complexes, nanoparticles, etc… They all present low toxicity and low immunogenicity risks in comparison to viruses. Their production is simple and low-cost with a high cargo loading.

Non-viral carriers usually can only perform short-term treatments; for this reason, they are suited for regenerative medicine.

On the other hand, the disadvantages consist in some seldom toxicity and limited transfection efficiency. Using cationic non-viral vectors may sometimes incur in removals of DNA by charged plasma proteins.

1.2.2.2 Liposomes

Liposomes are the most widely used Gene Therapy non-viral carriers. A Liposome consists in a particle delimited by a double phospholipidic membrane (like the cell's cytoplasm membrane). Inside the liposomes (hydrophilic domain) can be incapsulated DNA, RNA, hydrophilic drugs[xxxv]. In addiction the inside of the double fold of phospholipids, hydrophobic drugs can be loaded.

These lipidic-based particles are extremely versatile since they are non-pathogenic, cheap, easy to produce and suitable for multiple treatments. However, they are less stable in physiological media and present a low efficiency concerning gene expression compared to virus carriers.

To promote the active targeting and to avoid an early degradation, it's convenient to functionalize the surface of the liposome with i.e., ligands, PEG.

To increase the efficiency of transfection, Liposomes can be conjugated with viral particles or viral peptides, in order to help the DNA to reach the nucleus and to disrupt lysosomes.

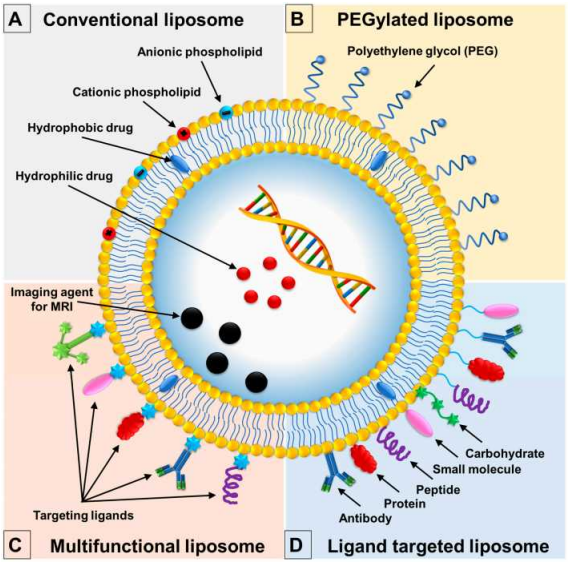


Figure 6: Liposome structure and organization of liposomes[xxxvi].

1.2.2.3 Gene gun

Another way to deliver DNA is using a gene gun. The genetic material is loaded into gold beads of about 1µm and shot inside the cells with a helium gun.

The transfection is transient, and the efficiency is not as high as the viruses' one. The gold beads are safe in biological and cell's environment but are not cleared and expelled from the body.

The gene gun may be useful for transferring genes to skin, where they might be utilized to deliver genes that help speed up wound healing or to immunize against certain viral or tumor-associated antigens (Hui and Chia, 1997)[xxxvii]. It could also be useful for ex vivo techniques, which allow tissue or tumor-explants to be "shot" in culture before being reintroduced into a patient.

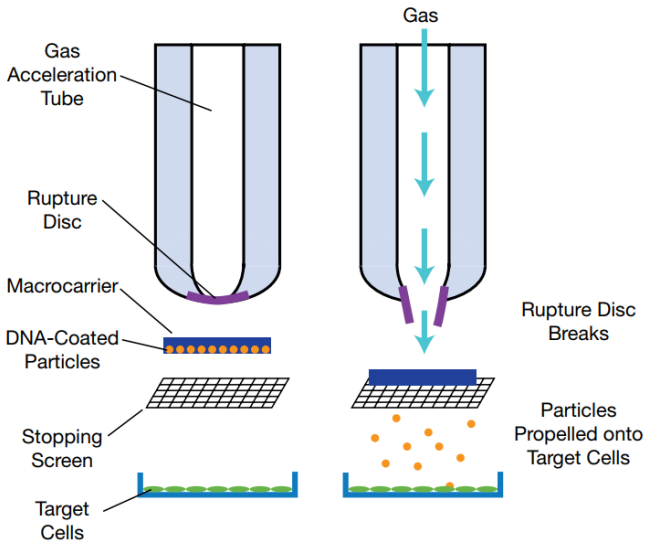


Figure 7: Gene gun working mechanism[xxxviii]

1.2.2.4 Nanoparticles[xxxix]

Nanoparticles are nanosized entities that are applicable in multiple fields like drug delivery, imaging, tissue engineering. Nanoparticles can be divided in two main families: inorganic particles and organic particles.

A. Inorganic particles are generally made by inorganic materials such as calcium sulphate, silica, gold and magnetic compounds. Quantum dots are the most used type of inorganic nanoparticles, they are made of metallic materials, and are used as a luminescent carrier for drugs and genetic material. Shao, D et al. developed a cancer suicide gene therapy using Cadmium based Quantum Dots[xl].

B. Organic particles are made of organic structures, such as lipids, graphene structures, polymers.

They can be engineered with different shapes, sizes and porosities either to safeguard their cargo from deteriorating or to escape the cells' reticulo endothelial system.

They can also be covered, functionalized, and complexed with other molecules in order to better suit the therapy goals.

1.2.2.5 Polymer based vectors

Polyplexes are nanoscale complexes made of DNA and cationic polymers.

At physiological pH, DNA is a polyanion which allows to engage with a positively charged polymer and to create a polyplex that protects DNA from deterioration.

There are two types of polymers: natural and artificial. The most used natural ones are polysaccharides, peptides, or proteins. On the other hand, synthetic substances include polyphosphoesters, dendrimers[xli], and polyethyleneimine (PEI)[xlii].

1.3 DNA and RNA

DNA is a one of the most relevant biomolecules, because it contains all the information that a cell needs to survive and to replicate. It presents a double helix structure, composed by two complementary strands. Every strand is formed by a multitude of nucleotides linked together. A Nucleotide is consists in: a phosphate group, a pentose sugar group and a nitrogen base (figure 11).

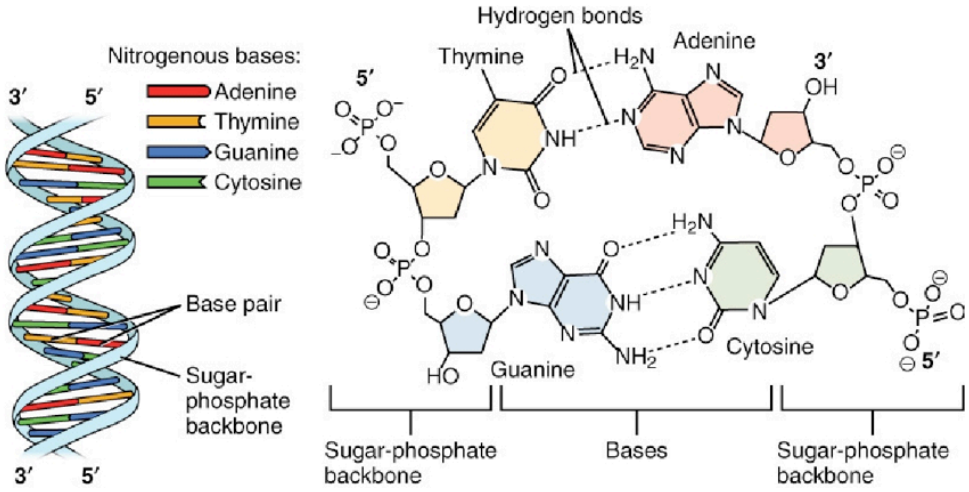


Figure 8: DNA's Chemical structure and special configuration.

There are 4 types of nitrogen bases: adenine (A), thymine (T), guanine (G) and cytosine (C).

The order of the bases is the genetic information; some of DNA's regions are called genes, every gene codified a specific protein.

RNA is a molecule that is responsible to transport the genetic information from the nucleus to the cytoplasm in order to produce the protein codified in the gene (DNA).

RNA is the complimentary copy of one of the DNA's strands, so it's formed by a single chain of nucleotides. RNA is synthesized in the nucleus, after this step the new RNA molecule is able to exit the nucleus and to bring the genetic information to the cytoplasm to promote the production of the corrispettive protein.

1.4 Gene therapy with plasmid DNA

Plasmids are circular DNA molecules that are different from a cell's chromosomal DNA since they autonomously replicate in a host cell[xliii].

They are naturally present in bacteria cells but sometimes they are also found in eucaryotic cells. Most of the time, genes carried in plasmids provide genetic advantages and features that can't be found in chromosomal DNA. A popular example is the antibiotic resistance.

The amount of deoxyribonucleic acid contained in plasmids ranges from few kilobase pairs to several hundreds. Plasmids for gene therapy can contain both human and non-human genes, and they can be delivered by both viral and non-viral carriers. Moreover, the injection of Plasmid DNA containing selected genes from pathogens, can promote a specific protective immune response. For this reason, plasmids are investigated to be used as a vaccine active principle[xliv].

Another filed in which they are applied is regenerative medicine, in fact plasmids encoding Vascular endothelial growth factor (VEGF) helps and increase the revascularization[xlv].

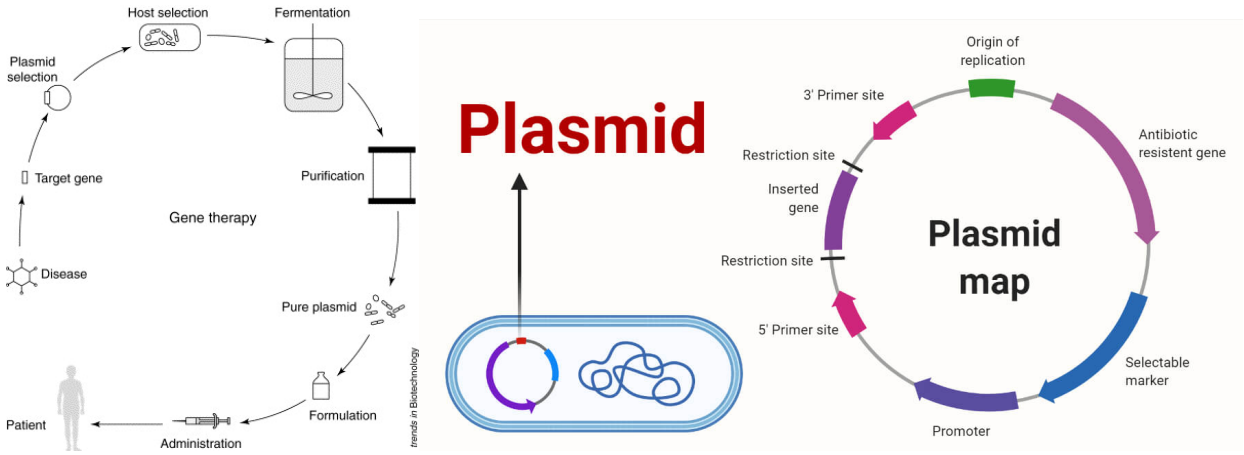


Figure9: On the left part of the image are illustrated the main steps of a gene therapy treatment using plasmids. On the right part are schematized all genetic information of a generic plasmid

Nowadays Plasmids are mainly produced by Escherichia Coli through the fermentation, followed by purification. Other new strategies should be implemented in order to promote a large-scale production, satisfying standards and requirements set by regulatory agencies[xlvi].

1.5 Gene therapy with miRNA/siRNA

1.4.1 siRNA

SiRNA is the abbreviation of Small interfering RNA, the average length of this RNA fragment is 20-25 nucleotides.

Gene silencing through small interfering RNA, is a promising technique that is responsible of inhibiting the expression of a certain gene[xlvii].

The silencing process starts with dsRNA that, after a specific trigger, is cleaved by a Dicer (RNase protein). The resulted DNA fragments (SiRNA) are the key point of the RNA induced silencing complex (RISC). This complex oversees the attachment itself to a mRNA of interest in order to promote its degradation.

Gene silencing via SiRNA has a huge potential to treat either gene overexpression or gene mutations. This approach can be exploited to fix genetic or autoimmune disorders, cancer and other types of syndromes[xlviiixlix]. They can interact with diseases' biological pathways. SiRNA can interfere and suppress oncogenes. Thanks to its specificity and its adaptability, siRNA can be synthetized "ad hoc" for specific patients and diseases. It will be a useful tool for future personalized medicine[l].

1.4.2 miRNA

On the other hand, we have microRNA (**miRNA**) which are fragments of non-coding RNA that interact with cellular pathways to regulate some biological processes. They also take part in the natural RNAi's mechanism[li].

They are synthetized in the nucleus, and, after a post-transcriptional modification, which occurs both in the nucleus and inside the cytoplasm, they become mature miRNA.

siRNA and miRNA are very similar, but miRNA is able to suppress and degradate mRNA despite having a partial or imperfect base complementarity. This characteristic gives miRNAs the possibility to perform a multi target effect. For example, one miRNA could silence about one hundred of mRNA.

Tumor growth, proliferation and migration, involve a large quantity of miRNAs.

The main goal coincides to upregulate tumor suppressor miRs, in order to suppress the formation of protein in tumor cells.

The supplementary goal is to inhibit the oncomiRs using a complementary miRNA sequence. Deepak Bhere's article deeply explains this strategy using mir21[lii].

The main problem concerns the regulation and the balance of the therapy. The delivery efficiency is very important, and the optimal quantity of the required interfering RNA must be deeply investigated to promote an effective outcome.

Another problem that all the genetic cargos have in common, is to find a suitable carrier for their delivery. This carrier should be stable, non-toxic, and should promote an active targeting against the unhealthy cells.

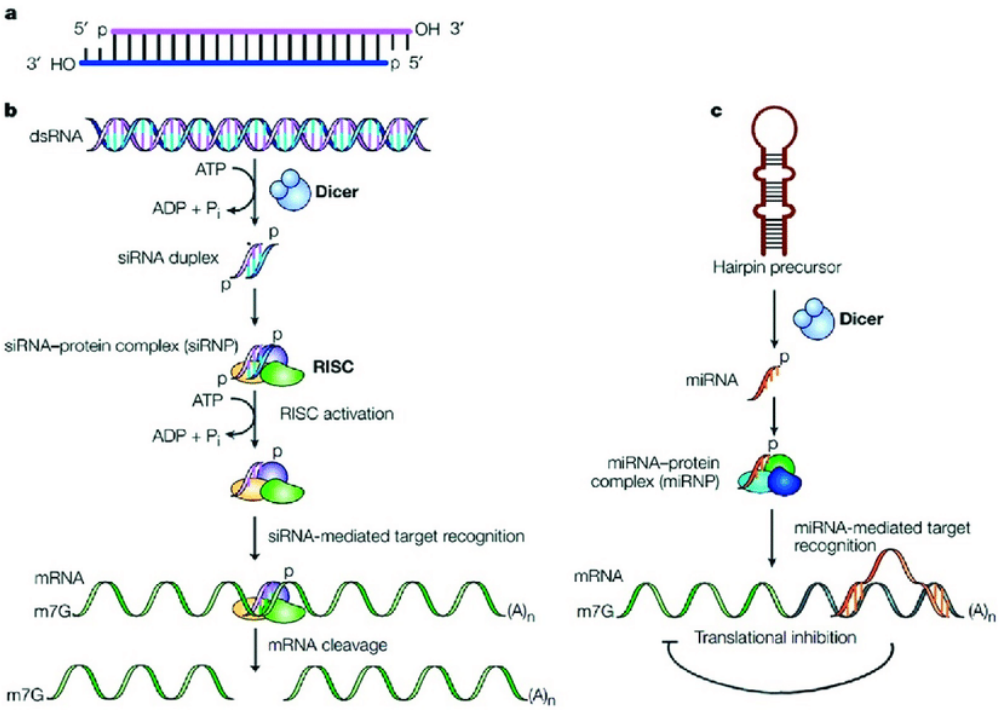


Figure 10: Differences between siRNA and miRNA biological pathways [liii]

1.5 Carbon dots

1.5.1 Carbon dots overview

Carbon Dots are a subspecies of nanoparticles that range from 1 to 10/12 nm in size. The significant presence of carbon atoms and carbon structures in these nanoparticles is the main feature that distinguishes them from other nanomaterials.

CDs were discovered by Xu et al.[liv] that synthetized single-walled carbon nanotubes. After the purification they isolated a fluorescent carbon compound and a tubular compound. The first one is CDs.

1.5.2 Types of carbon dots

There are three main types of carbon dots which are classified according to their internal structure[lv].

1 Carbon Nitride Dots: they present a structure in layers of benzene plans. These flats are not graphene like, but there are nitrogen's impurities inside the benzene rings. They are Synthetize by a bottom-up approach, using organic precursors having a certain number of amino groups like urea, Citric Acid, amino benzene rings.

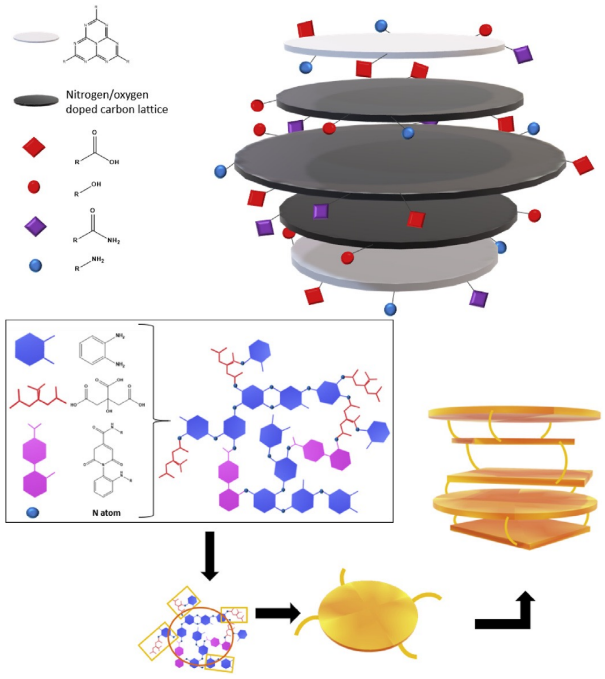


Figure 11: Scheme of Carbon Nitride Dots' inner structure

2 Graphene Carbon Dots: they are formed by pure graphene layers. They are formed by a top-down approach, from a graphitic bulk material that is broken off. The extremely small particles are formed by an oxidation process, that's why the surface of every plan is full of functional groups such as alcohols and carboxylic acids.

3 Polymer Carbon Dots: has a polymeric random structure. the properties of this last group are different from the previous ones, due to the organization of the internal structure.

The feature that every category has in common is the core shell organization. The core is represented by the inner structure of the particle. It contains the carbon atoms hybridized sp2 or sp3 in graphitic or benzene like structures. In the case of polymer CDs, the core is amorphous.

The shell is represented by the external functional groups. Those organic groups determine the reactivity of the particle and can be used to further functionalization.

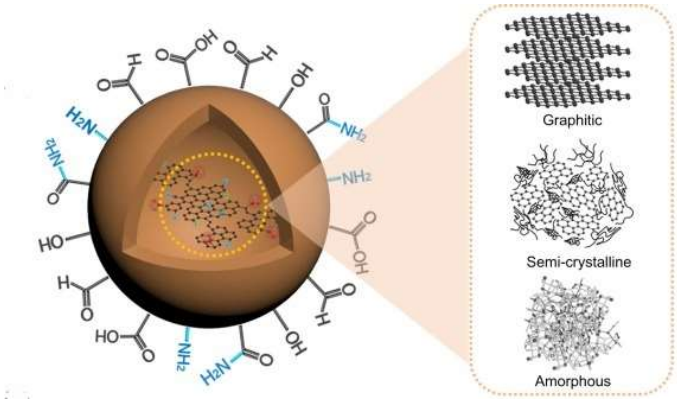


Figure 12: Core-Shell organization of a generic carbon dot particle[lvi]

1.5.3 Synthetic strategies

There are two ways to form Carbon Dots: the top-down approach that consist in the fragmentation of a bulky material or graphene-like sheets like Tayyebi et al did[lvii]. Electrochemical synthesis, Chemical oxidation, Solvothermal synthesis are some of the techniques that can be used to form CDs using top-down approach.

On the other hand, we have the bottom-up approach that condense, polymerize, carbonize or passivate small organic molecules due to the creation of the final particle. Zhou et al [lviii] used Citric Acid and Urea to create Carbon Dots able to inhibit the aggregation of Microtubule Associated Protein Tau, to potentially treat Alzheimer's disease. The most common methods to synthetize carbon dots

from small precursors are Hydrothermal method, Microwave-assisted synthesis, Pyrolysis or carbonization of precursors.

All these techniques are very easy to implement, most of them are very cheap and low time consuming (e.g., Microwave). Also, the Carbon source are very easy to find and purchase and furthermore it's possible to synthetize these nanoparticles using biomasses or recycling sources (Green Chemistry)[lix].

For example, small molecules or biomolecules can be combined to CDs to modulate the specificity of analytical detection. Also is possible to dope CDs to increase Photoluminescence properties or to modulate the energy gap and electronic characteristics. Examples of doping heteroatoms are N, S, Si, P, B, Ga, halogen (Cl, Br, I) Se, Ge, Mg, Cu, Zn.

In order to increase the photoelectric properties or to expand the range of application of CDs, composites or hybrids could be formed with Ag/Au nanoparticles, aerogels, nanofibers, MWCNTs, Pt, Fe3O4 and other materials.

1.5.4 Physical and chemical properties

These carbon nanoparticles present peculiar characteristics, such as water solubility, stability, biocompatibility, and relevant UV-visible absorption.

The most relevant properties are the optical ones, in particular the photoluminescence effect is a good feature that can be useful for bioimaging.

Usually, Carbon Dots are fluorescent particles when excited by a laser, because of their small dimension they tend to emit at smaller wavelengths of the visible spectrum (450-500 nm). This blue-violet emission range is not very useful because it can interfere with organs' autofluorescence, and photons can't pass through the human/animal skin. It's preferred to create carbon nanoparticles that can emit photons having lower energy, for example in the region of yellow-red visible spectrum.

Photoluminescence effect can be excitation dependent, in which the wavelength of the emission photons changes varying the energy of excitation. CDs can also have an excitation independent emission spectrum.

In addiction is possible to tune the fluorescence emission spectrum, varying structure and the size of the particles. Also, photoluminescence is affected by different reasons like used solvent or changes of rection temperature.

Another interesting characteristic is the high amount of external polar groups. These groups make the particle hydrophilic and water soluble.

Like all the engineered particles, there is the possibility to functionalize their surface, to improve their characteristics and their interaction in physiological environment. Is very easy to functionalize these particles because they expose a lot of reactive functional groups on their surface. For that reason, they can be bonded to molecules, Drugs, polymers[lx], etc.

1.5.5 Biological applications

Thanks to the Photoluminescent effect, and the high percentage of quantum yield Carbon Dots can be used as probes for bioimaging. As an alternative to QDs in optical imaging applications, CDs with improved photostability and reduced cytotoxicity have received extensive study. CDs are good candidates for bio applications, according to both in vitro and in vivo studies.

Having a very narrow size and an enhanced biocompatibility Carbon Dots can enter the cells and can also be exploited for therapy. In this case we are talking about theragnostic, the field that combines diagnostic images with specific treatments.

This type of approach is widely researched for treating tumours to substitute or improve the efficacy of the traditional treatments. By using nanoparticles, it is feasible to improve tumour's radiosensitivity, reduce adverse effects from the confined therapy, and utilize imaging characteristics for real-time monitoring.

A useful chemical feature is the ability to be electron donor or electron acceptors, this peculiarity makes CDs usable as Biosensors.

In fact, they can detect various types of ions such as Cu_2, Hg_2, Ag_2, Cr_3, Fe_3, Cl^-, and H^+. Hou et al. produced a new functionalized particle sensitive to mercury ions[lxi]. This can be possible because of the presence of the functional groups (on CD's surface) which can target different ions. The detection's result can be a

quenching of the fluorescence intensity. This process usually involves an electron or energy transfer process[lxii].

They are sensitive to biological variations of pH, and they can also detect the presence of nucleic acids, proteins or enzymes.

1.7 Interaction between carbon dots and DNA

For what concern the interaction between Carbon Dots and DNA or more in general nucleic acids there are many hypotheses:

Liang, et al. suggest that their CDs and CtDNA interact between through intercalation[lxiii]. Intercalation is a non-covalent interaction usually involving flat type of nanoparticles that are able to insert themselves between nucleotides, inside the DNA's double helix. This interaction is possible thanks to the narrow size of the carbon dots.

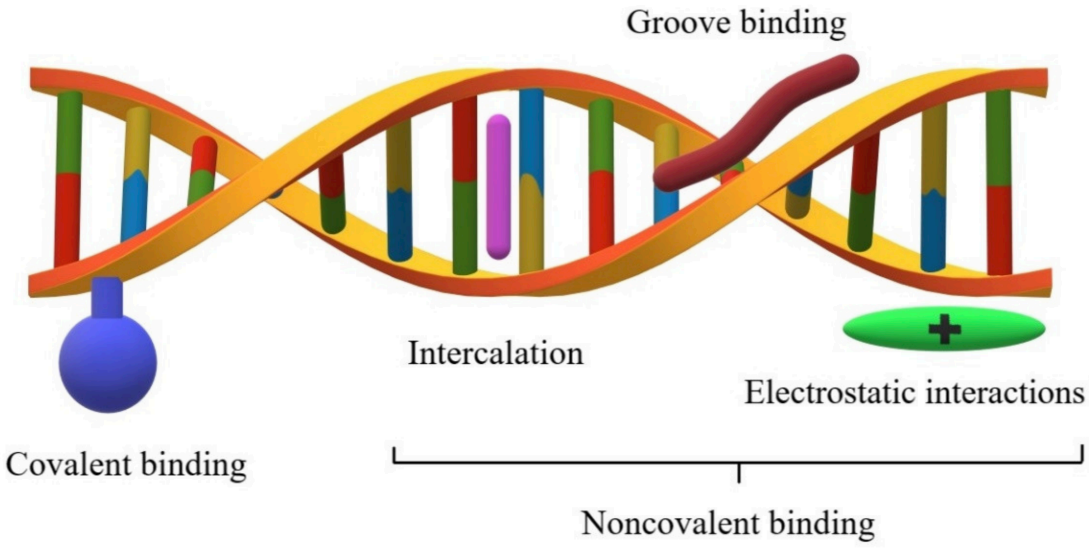


Figure 13: Graphic rappresentation of all possible interactions between DNA and nanoparticles[lxiv]

Another mechanism of interaction is Groove binding, It is also a non-covalent interaction (usually hydrogen bonds) between the nanoparticle's surface functional group and the base pair of DNA. It can be differentiated in major or

minor groove, depending on the size of the particles and their relative position along the DNA's chain[lxv].

Feng, et al. found thanks specific binding studies that their SC-dots can bind DNA through major groove having a particular affinity with GC pair bases[lxvi].

It's possible to link nucleic acids and Carbon Dots through an electrostatic interaction. Since the phosphate group present at the end of the chains is negatively charged, it can easily interact with positively charged particles. For this reason, it is convenient to synthetize CDs with a positive surface charge using for example positive charged polymers (Yang, X. et al.[lxvii]).

1.8 Aims and objectives

To reach the goal of using Carbon nanodots as a carrier for genetic material we need peculiar characteristics.

a) Particles need a positive surface charge to promote an electrostatic interaction with the genetic material like Liu et al did[lxviii].
b) Would be useful to use Folic Acid as a carbon source for particles, since a large number of tumour cells overexpress Folic Acid receptors[lxix]. Having the presence of this molecule inside the particles could be useful for a better cellular internalization. Furthermore, this specific interaction (ligand-receptor) can enable an active targeting treatment.
   Folic Acid is also useful because is a non-toxic molecule, biocompatible, often used as a dietary supplement. This molecule is fluorescent, big, very stable and poorly insoluble in water. Thanks to the presence of benzene structure sand carboxylic functional groups.

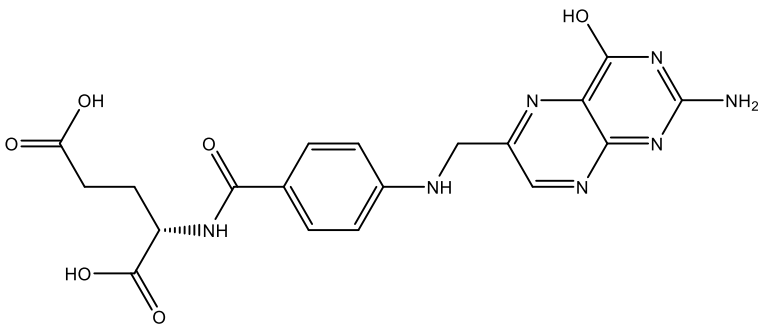


Figure 14: Folic Acid's chemical structure

c) Is preferred to have a high mass yield of the products.
d) Nanometric diameter of the particles to easily cross biological barriers as cellular membranes, blood brain barrier, nucleus membrane…
e) Carbon Dots are often used as a fluorescent probe for diagnostic and theragnostic, for this reason could be interesting to have a nanocarrier that can be used as a fluorescent probe for tumour and at the same time a monitor for treatment's follow ups.
f) Particles has to be biocompatible and non-toxic, so they must not to cause damages to healthy cells and don't have to promote stress-oxidative pathways on them.

2 Materials and methods

2.1 Materials

Deionized (DI) water was used as solvent for all the process (resistivity: 18.2 MΩ.cm; surface tension: 72.6 mN·m-1; pH: 6.6 ± 0.3 at 20.0 ± 0.5 °C). It was obtained using a MilliQ3 water purification system obtained from Millipore Sigma (Burlington, MA).

The Reagents: Folic Acid (>= 97%), Citric acid (99%), 1,2-phenylene-diamine, branched Polyehilenimine (average Mw ~ 25000, average Mn ~10000) were purchased from Sigma Aldrich.

2.2 Synthesis protocol

50 mg of FA, 50 mg of CA and 370 mg of o-PD were pounded in a mortar and mixed together in solution using 32 ml of Deionized water as a solvent. After 10 minutes of sonication, 988 mg of PEI 25kDa branched, were added. Until the solution is homogeneous, both sonicator and vortex were used. The sample was divided in 4 parts (8 ml of solution) and put in Teflon Autoclave at 160°C for 3 hours. The 4 samples were filtered with filter membrane of 0.2 µm and subsequently inserted in dialysis bags of 1000 Da MWCO for 6 hours. The solution inside the bags was collected and lyophilized for 2.5 days.[lxx]

Figure 15: Four batches of the sample were inserted in dialysis bags of 1000 Da MWCO

2.2.1 Mass yield

Mass Yield is evaluated in percentage by this formula:

$$\frac{mass\ of\ reaction's\ products}{mass\ of\ the\ reagents} \cdot 100$$

The solid sample was weighted after the lyophilization and stored in a dryer chamber.

2.3 Characterizations

2.3.1 UV-Vis spectroscopy

Agilent Cary 100 UV-Vis spectrophotometer was used to measure the absorbance of the compounds from 200 to 800 nm. The solution of Carbon Dots in DI water has a concentration of 0.05 mg/ml.

2.3.2 FLUORESCENCE spectroscopy

A Fluorolog HORIBA Jobin Yvon fluorometer with a 5 nm slit width for excitation and emission was used to characterize photoluminescence (PL). All optical characterization spectra were collected using quartz cells with a 1 cm pathlength. The acquisition of emission spectra are collected exciting the cuvette with a range of wavelengths (from 350 to 500nm having 25 nm of steps). The solution CDs in DI water is 0.01 mg/ml concentrated.

2.3.3 Atomic Force Microscope

On an Agilent 5420 atomic force microscope, CD pictures were taken utilizing the tapping mode (Santa Clara, CA, USA). On a clean silica mica slide, a drop of diluted CDs aqueous solution was applied, let to air dry, and then transferred to do the screening in tapping mode for AFM measurement. The tip utilized had a

force constant of 15 N/m and was made of silicon with dimensions of 225 m in length and 5 m in thickness.

2.3.4 Transmission Electron Microscope

A JEOL 1200X TEM was used for the TEM (Peabody, MA, USA). For TEM measurements, a drop of the CD solution was applied on a copper grid that had been coated with carbon and allowed to air dry before being inspected.

2.3.5 Zeta potential

CDs' Zeta potential was measured using DLS nano series Malvern Zetasizer (Westborough, MA). Using Universal Dip Cell in conjunction with DTS0012 and PCS1115.

2.3.6 Thermogravimetric analysis

The sample was heated using a Netzsch TG 209 F3 Tarsus thermo-microbalance (Netzsch, USA) at a rate of 10 °C/min from 40 to 1000 °C while being shielded by nitrogen gas.

2.3.7 MALDI-TOF Mass spectroscopy

For mass spectroscopy analysis, MALDI-TOF spectrometer (Bruker) was used. Samples were processed using matrix-assisted laser desorption ionization time of flight.

2.3.8 IR spectroscopy

Fourier Transform mass spectroscopy was evaluated in terms of attenuated total reflection (ATR-FTIR). FT-Nicolet 5700 in combination with a Smartorbit by Thermo Scientific was used for this analysis.

2.4 DNA electrostatically interactions

2.4.1 CDs and DNA conjugation

Calf Thymus DNA was used for preliminary studies concerning the interaction with the particles.

This sonicated DNA was purchased from Merk, the strands have a range size from 200 to 6000 nucleotides and the average length is 400 base pair.

The DNA was characterized both alone and in combination with the CDs in different ratios. To promote the interaction between CDs and DNA, the two solutions were mixed with the help of the Vortex and sonicated. The solution was stored at 4° C.

2.4.2 UV-Vis spectroscopy

UV-Vis tests were carried out as above described for CDs analysis in subsection 2.3.1.

Different solutions of CDs in dialyzed water were tested with the concentration of:

- 0.005 mg/ml
- 0.01 mg/ml
- 0.02 mg/ml
- 0.03 mg/ml
- 0.04 mg/ml
- 0.05 mg/ml

To have a reference, DNA 0.005 mg/ml in ultrapure water was tested.

Different DNA-CDs weight ratio were also evaluated:

- 1:1 DNA-CDs
- 1:2 DNA-CDs
- 1:4 DNA-CDs
- 1:6 DNA-CDs
- 1:8 DNA-CDs
- 1:10 DNA-CDs

All these samples were replicated three times.

The concentrations were determined based on the previous CDs UV-Vis spectra.

2.4.3 Fluorescence spectroscopy

Fluorescence tests are done with the same machine and protocols as the previous study on the absorbance of the Carbon Dots alone. Specifics are reported in the section 2.3.2.

Different solutions of CDs in dialyzed water were tested:

- 0.0005 mg/ml
- 0.001 mg/ml
- 0.002 mg/ml
- 0.003 mg/ml
- 0.004 mg/ml
- 0.005 mg/ml

To have a reference, DNA 0.0005 mg/ml in ultrapure water was tested.

Different DNA-CDs weight ratios were also evaluated:

- 1:1 DNA-CDs
- 1:2 DNA-CDs
- 1:4 DNA-CDs
- 1:6 DNA-CDs
- 1:8 DNA-CDs
- 1:10 DNA-CDs

All these samples were tested three times.

The concentrations were determined based on the previous CDs' fluorescence spectra.

2.4.4 Circular dichroism

JASCO J-810 spectropolarimeter was used to collect Circular dichroism spectra (CD). The range of the measurement started from 200 to 340 nm using a 1 cm quartz cells (Starna Cells, Inc.; Atascadero, CA). The scanning speed was 200 nm/min with three accumulation points and a continuous scanning mode.

The measurements were filtered with a low band pass filter to remove the noise at highest frequencies.

DNA and CDs were tested as references together with all the mix solutions in weight ratios of:

- 1:1 DNA-CDs
- 1:2 DNA-CDs
- 1:4 DNA-CDs
- 1:6 DNA-CDs
- 1:8 DNA-CDs
- 1:10 DNA-CDs

2.5 Electrophoresis tests

Electrophoresis gel tests were done on agarose gel checking the best binding ratio of DNA and CDs using an excess of DNA investigating the loading on the particles surface.

CDs and different weight ratios of CDs-DNA were studied: (1:1,1:5,1:10,1:20).

2.6 Cytotoxicity

2.6.1 Cytotoxicity tests of Carbon dots[lxxi]

The cytotoxicity of the carbon dot towards different cell lines (4T1, HEK293, MCF-7 and AsPC-1 cells) was evaluated with MTT assay. HEK293 and ASPC-1 were both cultured in Dulbecco's Modification of Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% of penicillin/streptomycin, 4.5 g/L of glucose, L-glutamine, and sodium pyruvate. 4T1 was cultured in RPMI

1640 medium containing 10% of FBS, 1% of penicillin/streptomycin, L-glutamine and HEPES buffer. MCF-7 was cultured in Minimum Essential Medium (MEM) containing 10% fetal bovine serum, 1% of penicillin/streptomycin and Earle's salts and L-glutamine. All the cells were grown in tissue culture flasks in a humidified incubator at 37 °C and an atmosphere of 5% CO 2 . Using a 96-well plate, with 3000 cells/well, cells were incubated for 24 hours. The media were aspirated, and then the cells were treated with fresh media containing different concentrations of carbon dots (0, 10 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL, 250 μg/mL, 500 μg/mL, and 10 mg/mL) and incubated for 72 hours. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL, 20 μL/well) was added into each well and continued to be incubated for another 4 hours for the conversion of MTT to formazan by cellular reductase enzymes. The media was aspirated carefully, and cells were lysed using 100 μL of DMSO and homogenized with gentle shake at room temperature. The absorption at 570 nm of each well was measured using a Molecular Devices SpectraMax i3x plate reader, and the relative cell viability of cells were calculated.

2.6.2 Cytotoxicity tests of the complex CD-DNA

Cell viability experiment was performed using two healthy cell lines: MSCs (human mesenchymal stem cells) and VSMC (brain derived human vascular smooth muscle cells). CDs alone in different concentrations (0.1 μg/ml, 0.5 μg/ml, 1μg/ml) and CDs-DNA in different ratios (1:1,1:5,1:10,1:20) were tested.

3 Results

3.1 Preliminary considerations of the synthesis

After the lyophilization, the final product was sticky, melty and had a bronze-brown colour. In order to promote a good stability of the properties in solid state, it had been stored in a dried chamber.

The product can also be stored in a Dialysed water solution at 4° C.

When irradiated by a UV lamp, it shows a bright blue colour.

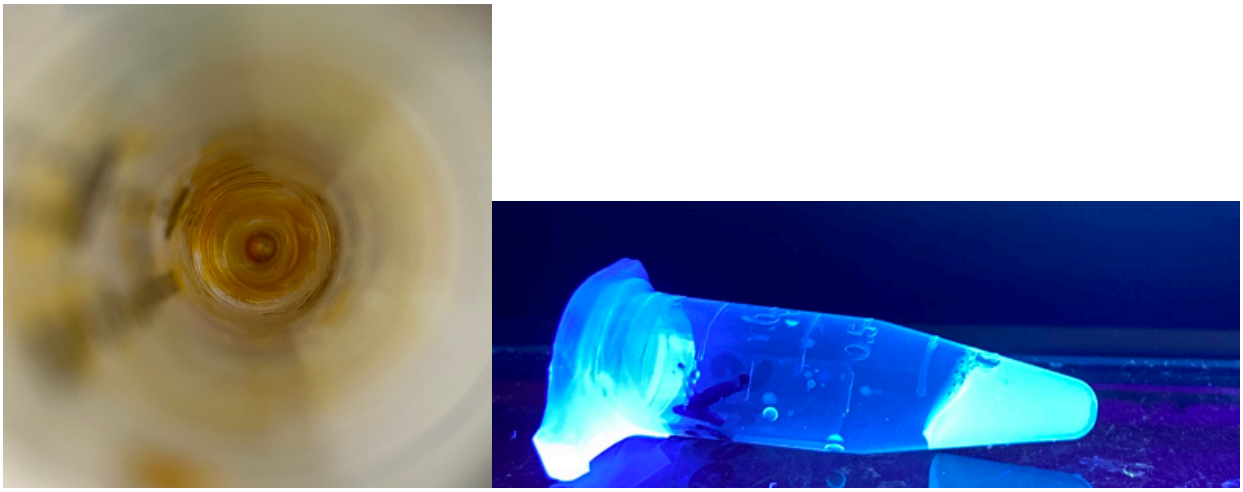


Figure 16: Sample after lyophilization (left), fluorescence emission of sample in solution under the light of a UV lamp(right)

The sample is extremely difficult to weight. All of the reacted solution was divided in 4 equal samples. The content of each tube is 53.8 mg.

The mass Yield percentage is 14.8%.

3.2 Characterizations of Carbon Dots:

3.2.1 UV-Vis spectroscopy

Preliminary characterizations of carbon dots were carried out collecting UV-Vis spectrum as reported in figure 17.

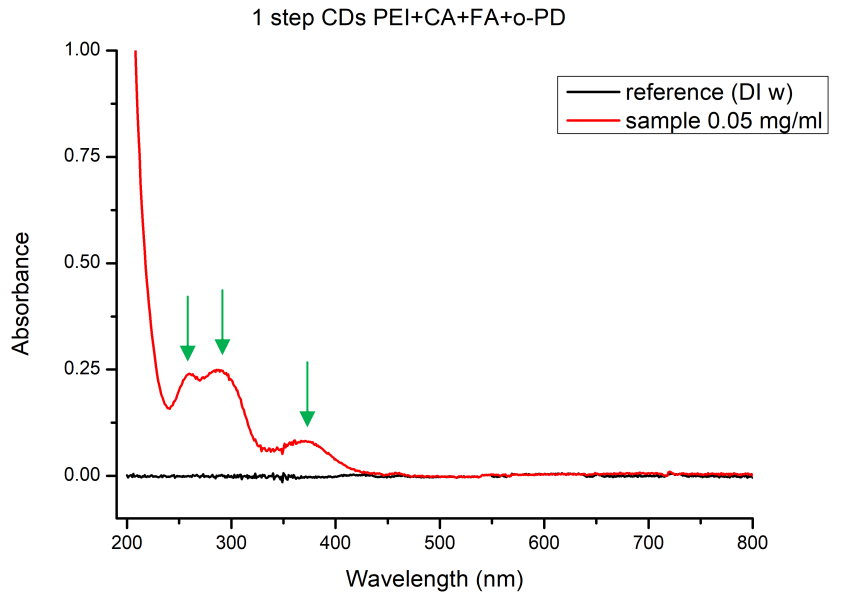


Figure 17: UV-Vis spectrum of CDs in DI water solution, concentration of 0.05 mg/ml

There are two peaks in the region between 100 and 300 nm, at 264 and at 279 nm. These peaks are due to π-π* transitions of the Carbon-Carbon bonds of sp2 networks. These bands evidence the presence of substituted benzene-like layers collocated in the CDots' core[lxxii].

The last peak, at 370 nm is correlated to the presence of unsaturated carbon-heteroatoms bond such as C=O bonds, having n-π* transitions. These bonds are due to the structures of Folic Acid and Citric Acid which can be found in nanoparticle's inner parts.

3.2.2 Fluorescence spectroscopy

Fluorescence spectroscopy was carried out to investigate the emission features of Carbon Dots, which are shown non normalized in figure 18 (a) and normalized in figure 18 (b).

The maximum emission intensity is showed at excitation wavelength of 375-400 nm of about 2 million CPS. Moreover, the fluorescence emission spectrum is excitation-independent (figure 19 a), having a broad emission band centred at 460 nm.

Having the maximum intensity of the higher emission spectra at 460 nm means that particles are emitting in the visible region (blue emission) according to figure 16. This is a demonstration of the tiny size of the particles.

As shown in figure 19 (a) and 18 (b), emission spectra maximum at higher wavelengths (lower energies) are shifted and they have also a very low intensity (figure 18 (a)) because they correspond to the tail part of the emission spectra.

a) b)

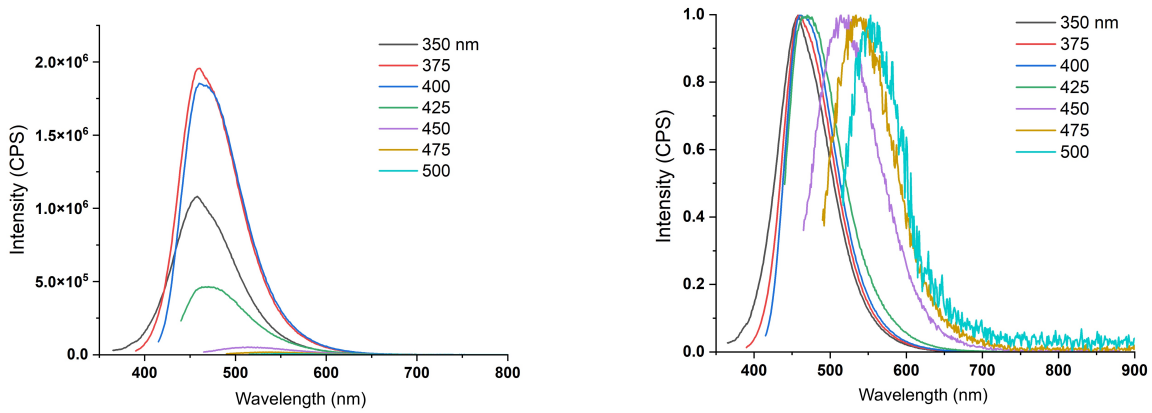


Figure 18: Fluorescence spectrum of CDs in DI water solution, concentration of 0.01 mg/ml

Below, in figure 19 (a) is shown the band corresponding to the peak position as a function of the excitation energy of the laser. In Figure 19 (b) is plotted the fluorescence spectrum as a function of the energies in eV.

a) b)

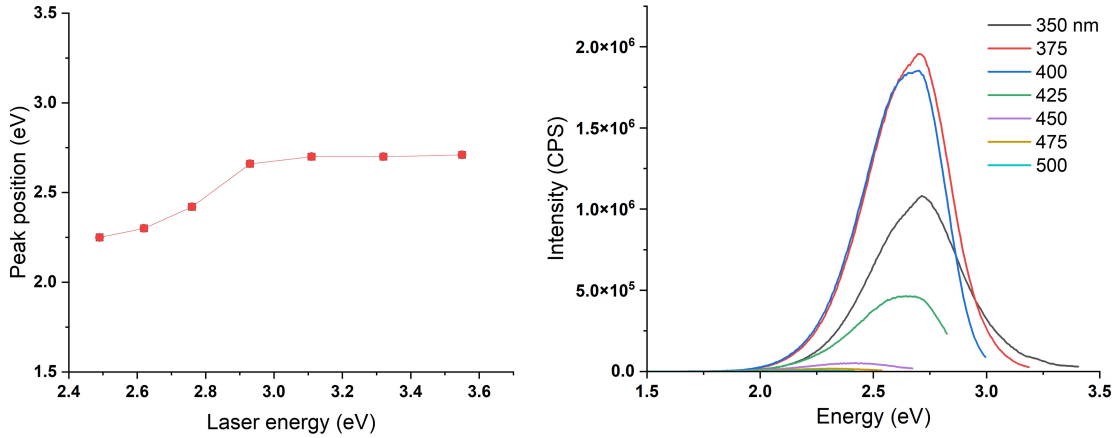
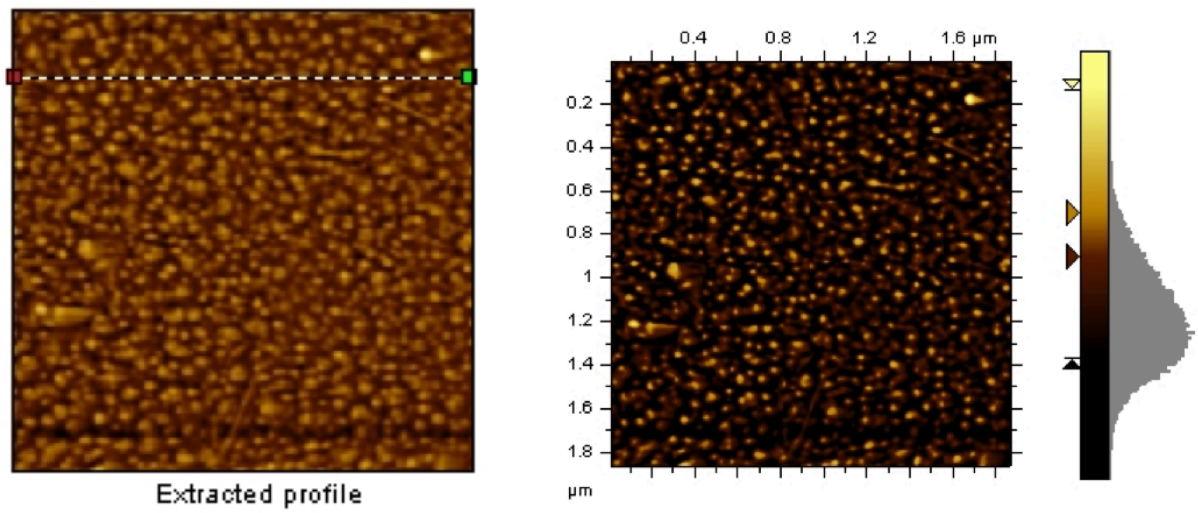


Figure 19: a) Plot of the peaks' intensity versus the energy of the excitation laser b) Fluorescence spectra at different excitation wavelengths with emission energies as x axis.

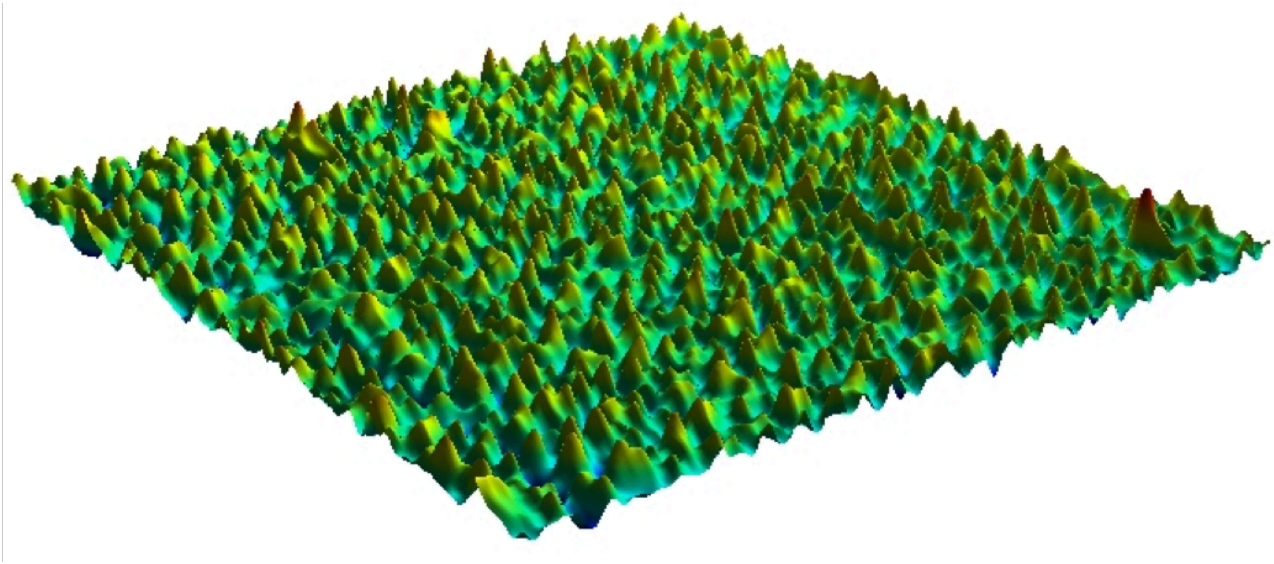
3.2.2 AFM and TEM microscopy

In figure 20 the Atomic Force Microscope outputs are shown suggesting that the nanoparticles are very homogeneous and well distributed on the plate's surface. The roughness of the particle is around 1.2 nm as the profile diagram in figure 20 (d) suggests.

a) b)



c)



d)

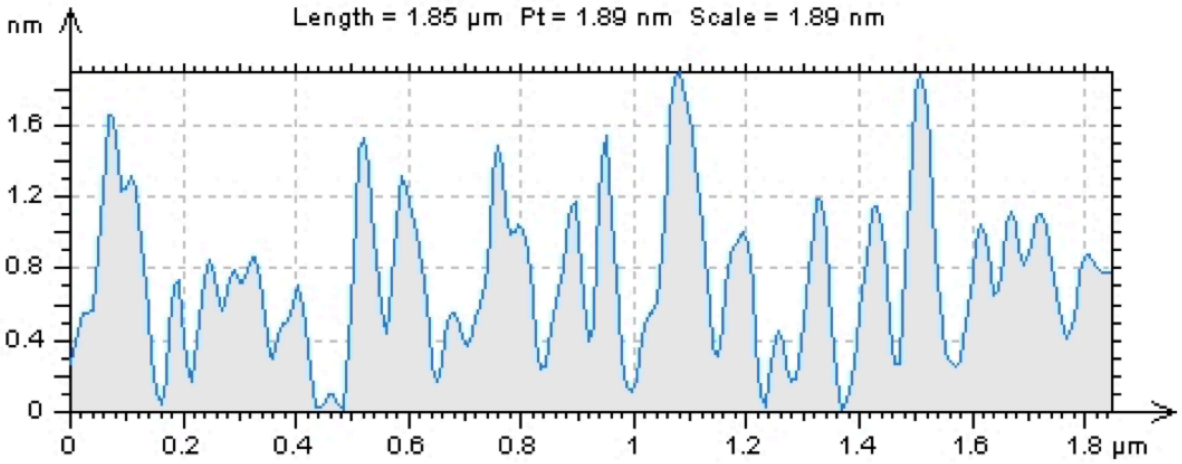


Figure 20: a,b) Analysis profile on microscope's plate with histogram of the z axis' dimensions, c) 3D image of plate's surface, d) effective roughness measured profile

After the evaluations of the z profile of CDs, TEM analysis was done to measure the particles' dimensions on the x-y plan.

Transmission Electron Microscope images (figure 21) confirm the previous size and the morphological characterizations.

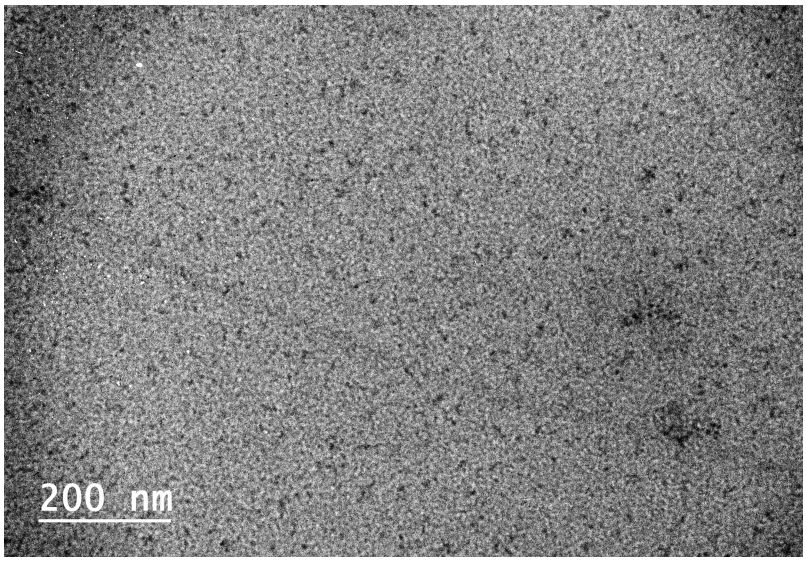


Figure 21: TEM image 200000X focus

Figure 22 shows the size distribution that is centred in 1.2 nm. This distribution presents a positive skewness, which is very common for polymeric nanoparticle's size. Nupura S. Bhise[lxxiii] et al. also found the same type of data implementing polymeric nanoparticles for gene delivery. The absence of the left tail of the distribution can also be attributed to the dialysis purification, particles smaller than the fixed cut off are removed during this step.

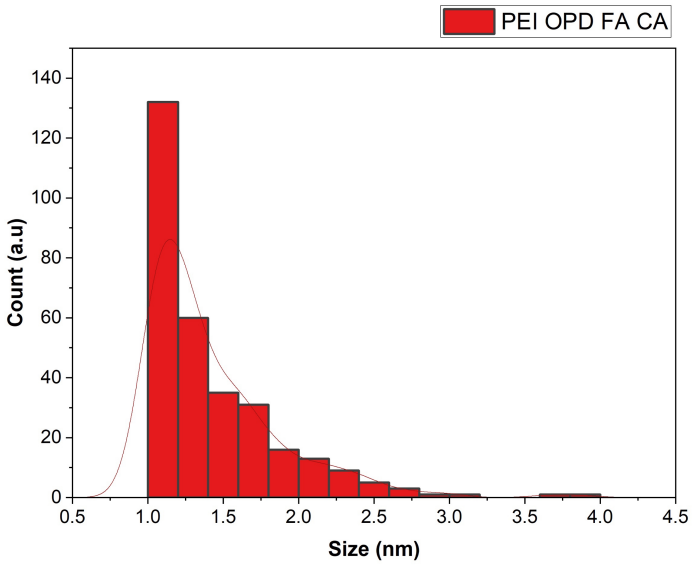


Figure 22: Size distribution istogram of particles' diameter

3.2.3 Zeta potential

This analysis is performed to study the surface's potentials of the particles as it is reported in the graph (figure 23) and in the table 1. The distribution of the surface's potential is a gaussian bell. The average of the measurements is 6.7 mV.

This sample shows a positive, stable and homogeneous surface potential.

Record	Name	T (°C)	ZP (mV)
1(red)	Num3-cds1	25	7.02
2(green)	Num3-cds2	25	6.52
3(blue)	Num3-cds3	25	6.48

TABLE 1

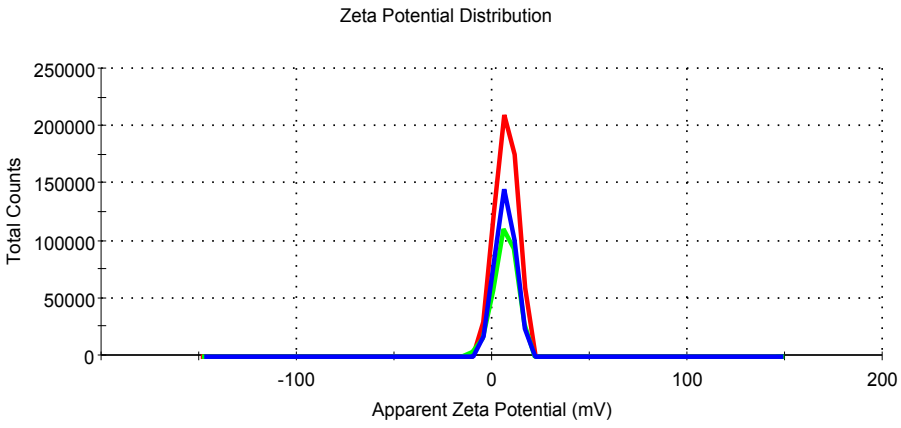


Figure 23: Zeta potential distribution curve

3.2.4 TGA analysis

Thermogravimetric analysis was carried out in order to investigate the mass degradation of the particles (figure 25). It's possible to notice that the first mass loss is due to the dehydration process. In fact, the first peak of the derivative is between 50 and 120° C. It is due to the evaporation of water molecules soaked

up by the sample. The second relevant mass reduction is at about 200°C corresponding to the loss of the external functional groups such as hydroxylic and amino groups. These species belong respectively to outer Folic Acid or benzene structures and PEI chains.

Subianto et al.[lxxiv] developed a sustainable material based on PEI, and they found a degradation of the aliphatic PEI's chains between 300 and 400° C . That means that PEI's fragments compose the random structure of the particles, inducing the most relevant decrease of the mass percentage. This phenomenon demonstrates a major amount of polyamine chains randomly arranged in the particle's structure.

The last degradation phase started around 500 °C and is due to the breakdown of the aromatic moieties as Achyut Konwar et al.[lxxv] confirm. These domains are formed by Folic Acid molecules or condensed structures of Citric acid and ortho-phenylene-diamine. The high temperature of the degradation confirms that aromatic-like domains are the most stable and organized component of the particles.

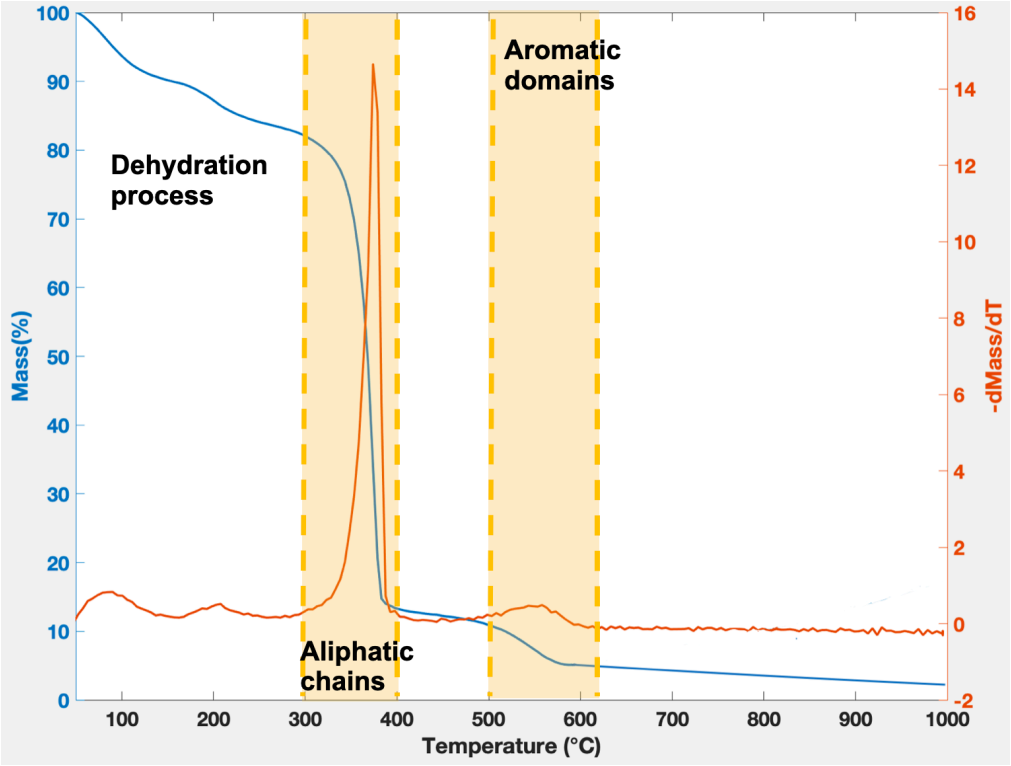


Figure 24: Plot of mass percentage loss as a function of the temperature (blu), mass loss derivative with respect to temperature

3.2.5 MALDI-TOF Mass spectroscopy

Carbon Dots were investigated on MALDI-TOF results have been normalized and plotted in figure 26.

An absence of native PEI 25kDa can be confirmed because there aren't any peaks at 25000 m/z supposing a unitary charge.

James E. Girard et al.[lxxvi] reported the characterization of the mass spectrum at different PEI molecular weights, the most relevant peak of PEI-600 corresponds to some of the peaks in the 500 m/z region of the CDs spectrum. This highlights that the random polymer coil of the nanoparticle is constituted by PEI strands of about 600 Da or less.

In the region between 440.565 and 443.609 m/z there are the highest peaks which evidence the high concentration of Folic acid in the particle. It means that Folic Acid's structure remains unaltered inside the particle. These Folic acid molecules are arranged in the outer layers of the molecule.

There are some structures (aromatic domains) having a weight of about 862.140 m/z and others with a lower molecular mass ranging between 600 and 700 m/z.

These structures have a wide weight range and are similar to functionalized flats of carbon rings linked together by poly-amino-chains.

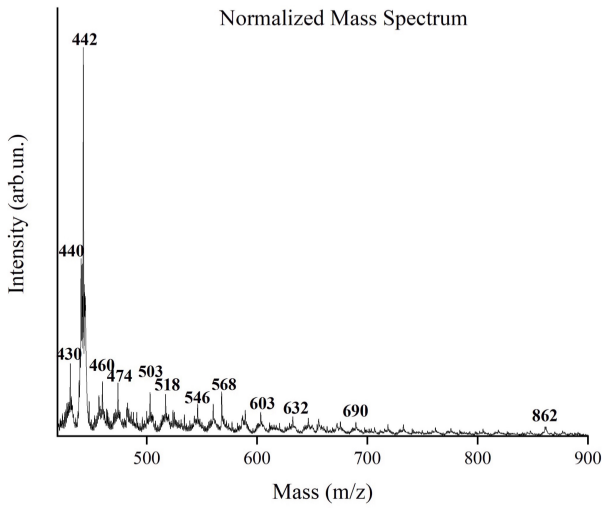


Figure 25: Normalized mass spectrum of the intensity of the measured mass-to-charge ratio depending on the time of flight of the ions.

MALDI-TOF Mass Spectroscopy demonstrates that in the outermost parts of the Carbon Dot there are Folic acid domains. According MALDI-TOF and TGA analysis Particles' hypothesized structure is modeled in figure 27.

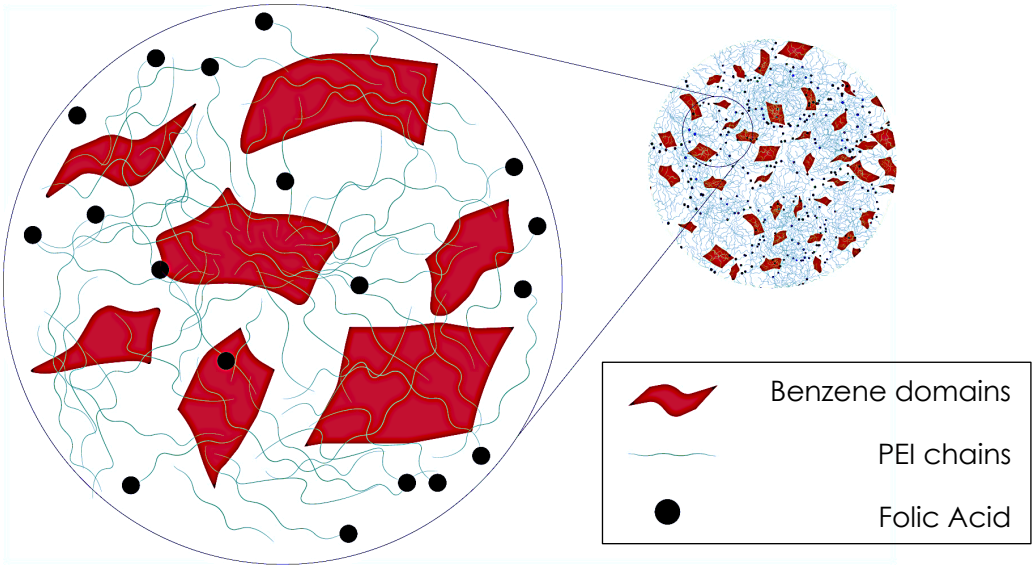


Figure 26: Proposed model of CDs, polimeric random coil having more ordered aromatic domains and Folic Acid molecules on the surface.

3.2.6 IR spectroscopy

In the figure 28 are reported the IR spectra of CDs and precursors.

Starting from the right side of the purple line in the following graph, there's a deflection in the region from 3006 to 3614 nm due to NH stretching (ν_{N-H}), and a deflection between about 2750 and 3000 that corresponds to ν_{C-H} aliphatic. These two regions are almost identical to the PEI 25 kDa IR spectrum.

Other relevant signals contain bands between 1517 and 1730 due to CN bending (δ_{C-N}), $\nu_{C=C}$ and $\nu_{C=O}$. At 1630 nm there is the ring breathing signal which proves the presence of the aromatic rings either in the most ordered domains of the particle or in the Folic Acid molecule. CN bending is the proof of the presence of nitrogen substituted aromatic rings, besides that, C=O stretching is correlated to carboxylic acids functional groups belonging to Citric Acids or Folic Acids derivate structures.

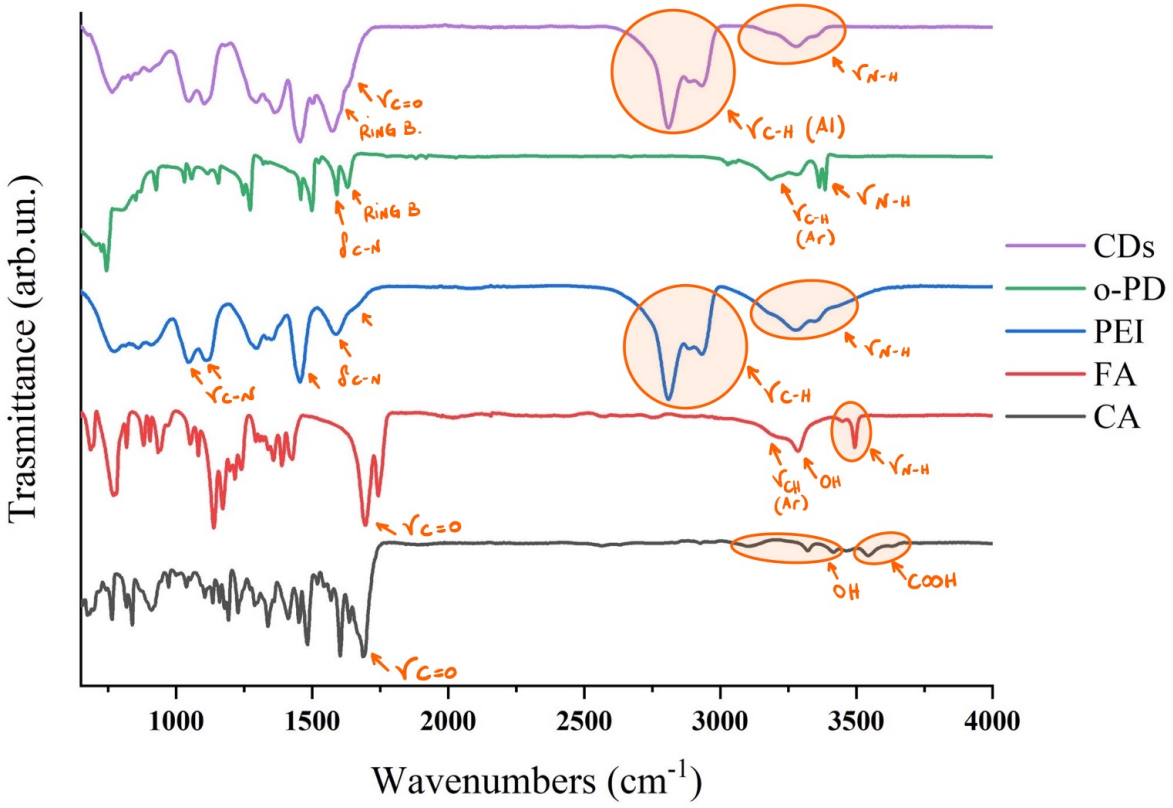


Figure 27: IR spectrum of CDs and reaction precursors

3.3 DNA binding tests

3.3.1 UV-VIS spectroscopy

Binding studies between CDs at different concentrations and DNA were performed (figure 29a). The resulted spectra are coherent with the concentrations in fact, increasing the concentration of carbon dots increases the absorbance as well keeping the same trends and peaks. The DNA spectrum has a peak at 264 nm.

The spectra of the conjugates (figure 29b) are similar but not a perfect overlap. The highest peak position is different, shifted towards lower wavelength (blue shift). This phenomenon can be associated with hyperchromism, which can be an effective proof of the electrostatic interaction between positively charged CDots and negatively charged DNA strands[lxxvii]. Not only this is a symptom of

DNA's secondary structure modifications[lxxviii] but there are also interactions π-π between azotate bases and benzene-like aromatic domains.

In figure 30 is possible to notice the differences between peak's highest intensities. Looking at the linear interpolations, the lines have very different angular coefficients meaning that the absorbance of DNA, (which is constant) is not added to the CDs spectrum but independently contributes. Furthermore, the angular coefficient of the lines in figure 30 is proportional to the molar extinction coefficient of an absorbing solution. For this reason, observed different molar extinction coefficients suggest an interaction between particles and DNA.

a) b)

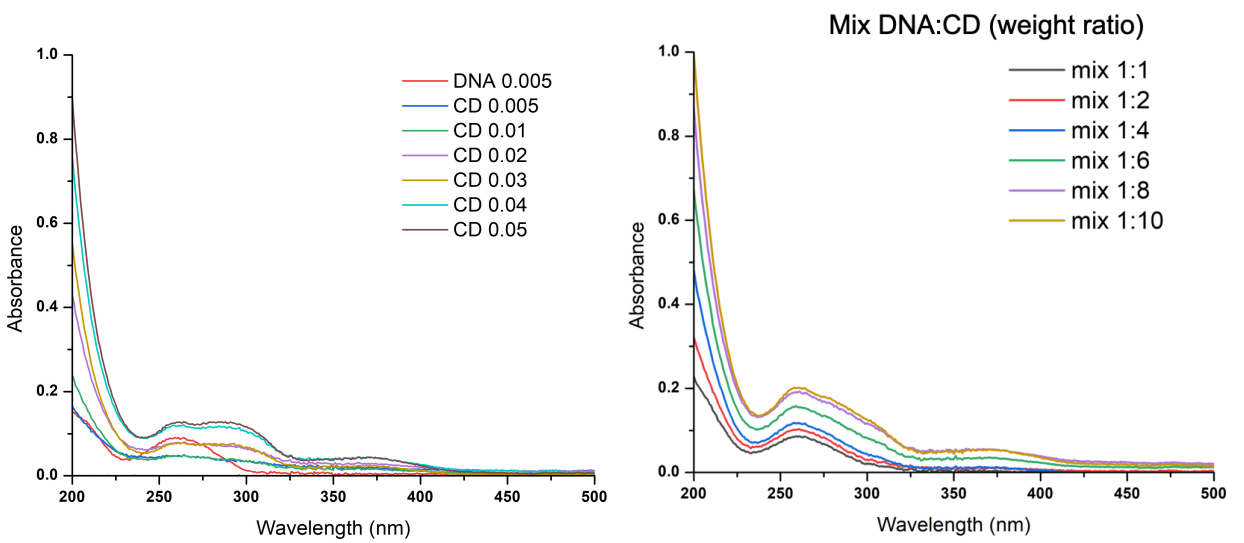


Figure 28: UV-Vis spectra of CDs in different concentrations and DNA(a), UV-Vis spectra of conjugates CD-DNA in different weight ratio with a constant amount of DNA.

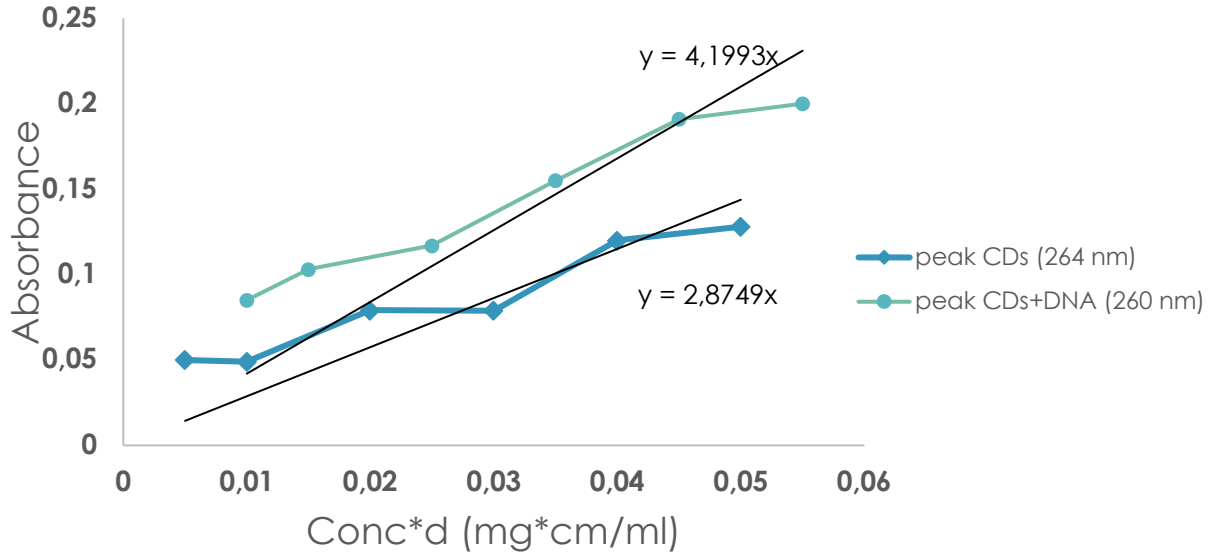


Figure 29: Absorbance versus concentration*distance (1cm) of all the spectra presented in figure 29 (a) and figure 29 (b)

3.3.2 Circular dichroism

Circular Dichroism is applied to identify conformational changes in DNA's secondary structure.

The Circular Dichroism spectrum was evaluated with different mass ratio of CDs with fixed amount of DNA

CDs alone are not supposed to have a signal (figure 31 (a)); Wei Zhang et al.[lxxix] found that naked DNA has a negative band at 245 nm which is associated to right-handed helical geometry, and a positive band at 275 nm that is correlated to DNA's B-form. This is the demonstration of the presence of these two secondary structures in the DNA's mixture.

Introducing the CDs, the spectra's form changes completely. Peaks become valleys and valleys become peaks. In 1:1 CDs-DNA solution, there is a negative valley that is correlated to Left-handed DNA's structure. The more CDs are added, the more the shape returns to be similar to the naked DNA one and the less the interaction between CDs and DNA happens. This last phenomenon is caused by the hydrogen bonds between CDs being preferred to the electrostatic interactions between CDs and DNA.

It means that the best interaction is showed when the amount of CDs is equal to the amount of genetic material.

a) b)

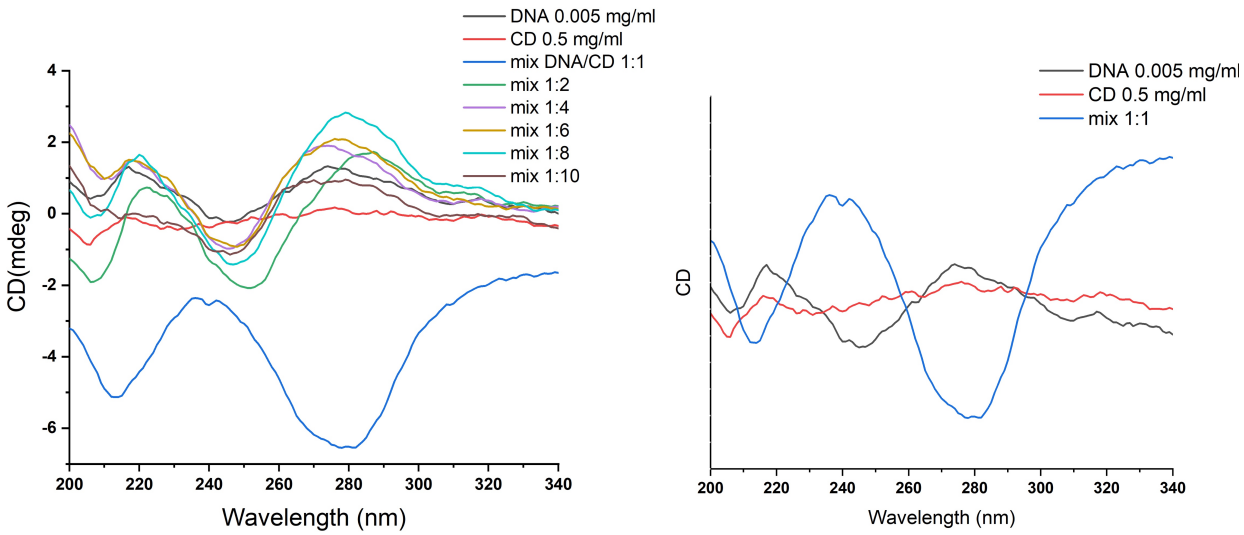


Figure 31: a) Circular dichroism spectra of DNA, CD, and mix DNA-CD in different ratio with a constant amount of DNA, b) shifted graphs of most relevant circular dichroism spectra: DNA 0.005 mg/ml, CD 0.5 mg/ml, mix 1:1 CD-DNA

3.3.3 Fluorescence spectroscopy

Another strategy to investigate the association and interaction between nucleic acids and nanoparticle is the evaluation of the changes in fluorescence spectra. Keeping constant the amount of DNA and varying the CDots amount (from 0.0005 to 0.005), fluorescence emission spectra were studied as reported in figure 32.

The fluorescence of the CDs alone was analysed in different concentrations, spectra result similar in terms of shape but different in terms of intensity. Furthermore, increasing the concentration of the particle constantly increases the fluorescence intensity as well. On the other hand, adding DNA in the solutions changes the trend of the curves and the intensity spectra do not increase smoothly.

Comparing the maximum intensity peaks of CDs alone and CD-DNA conjugates, it's possible to notice that adding DNA to a CDs solution can increase the

fluorescence emission. This gain is more visible in higher concentration CDs solutions, while, with a lower amount of CDs, this increase is less visible and almost lost. When DNA and CDs are equally concentrated (mix 1:1) there is a fluorescence quenching compared to CDs solution at the same concentration (Figure 33).

Chun-Yan Liang et al.[lxxx] affirmed that the fluorescence of Carbon Dots is quenched by the presence of DNA, this phenomenon indicates a certain interaction between DNA and CDs.

In agreement with circular dichroism results: when the weight ratio of both CD and DNA is the same, there is a strong visible interaction of the two species. On the contrary, increasing the concentration of CDs makes them interact with each other instead of binding to DNA strands.

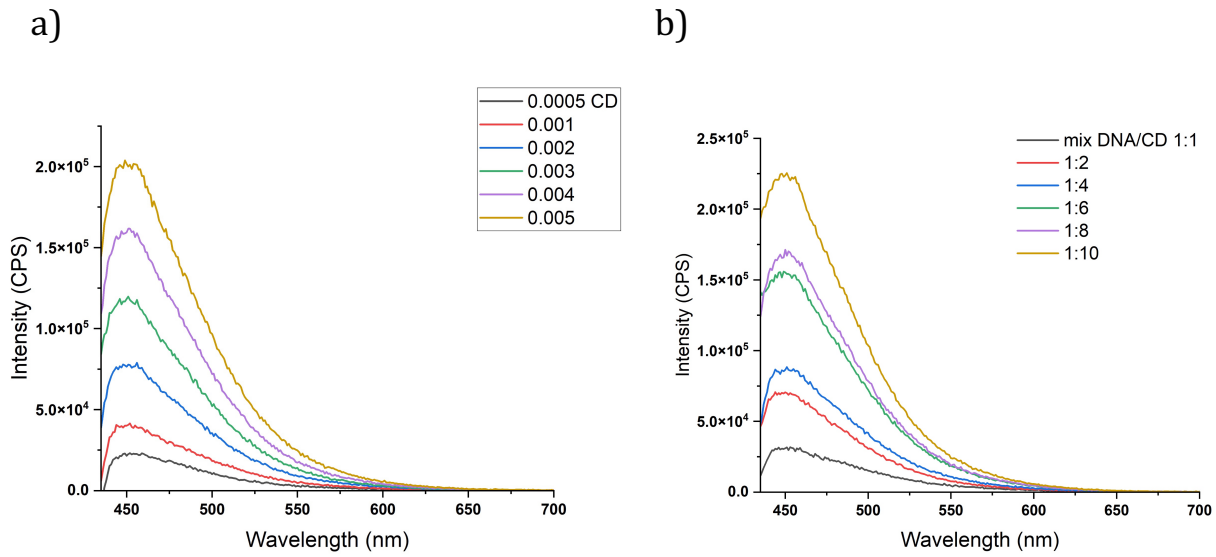


Figure 32: Fluorescence spectra of CDs in different concentrations and DNA(a), Fluorescence spectra of conjugates CD-DNA in different weight ratio with a constant amount of DNA.

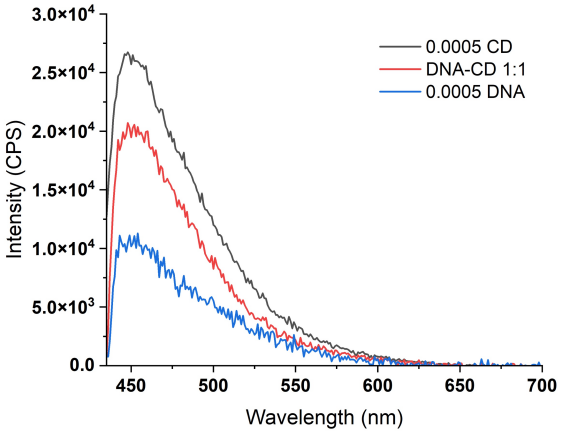


Figure 33: Comparison between mix 1:1 and the corrispective amount of CDs alone

The fluorescence decrease plotted in figure 31, is due to a static quench[lxxxi] provoked by a dipole-dipole interaction between positively charged CDs and negatively charged DNA's strands. The static quenching mechanism creates a ground-state complex which is constituted by the fluorophores and the quencher.

The mechanism of static quench act as a partial overlap of the charge density of the two species and is an effective demonstration of the bound DNA-CDs[lxxxii] as Zhang, G et al. affirmed.

3.4 DNA's affinity tests-electrophoresis

To better evaluate the effective binding between nanoparticle and DNA, an electrophoresis on agarose gel was made.

Looking at the figure 34, DNA is migrating thanks to the applied different potential to the positive pole of the board. The area that has CDs sample, doesn't show any fluorescence's signal.

However, the bands that present mixtures DNA-particles present a fluorescent line at the top of the running lane. In this line is effectively present the interaction complex. Increasing the amount of DNA, the unreacted genetic strands are running along the line to the positive pole.

For an efficient loading without waste of DNA, it's convenient to have a 1:1 weight ratio, an optimal ratio should be between 1:1 and 1:5 (CDs-DNA). Finding this ratio could be a further step of this research.

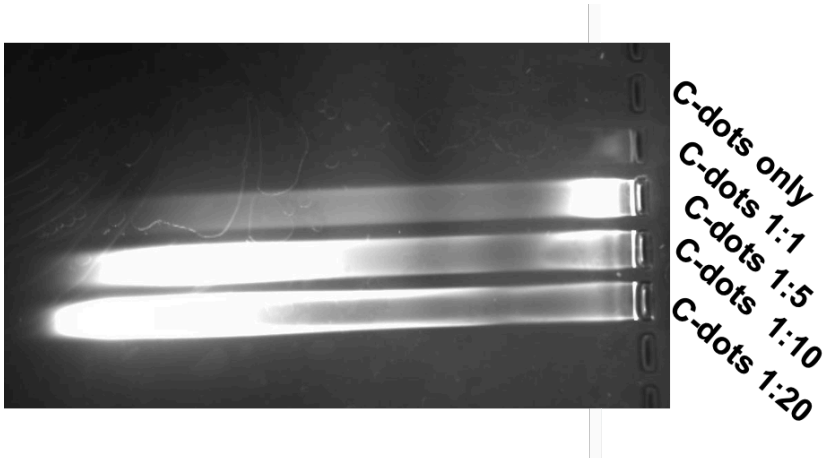


Figure 34: Electrophoresis tests on agarose gel of compounds having a fixed amount of CDs and a variable amount of DNA

3.5 Cytotoxicity

3.5.1 Cytotoxicity tests of Carbon dots

Cytotoxicity on four tumoral cell lines was analysed applying different concentrations of Carbon Dots, from 10 mg/ml to 5 µg/ml.

HEK293 (immortalized human embryonic kidney cells), MCF7 (breast cancer), 4T1 (breast cancer), ASPC1(pancreatic cancer) were used as cancer cell lines.

From the percentage viability reported in figure 32 is possible to affirm that CDs are extremely toxic for cancer cells, applied in concentrations that range from 10 mg/ml to 12.5 mg/ml. Decreasing particles' concentration to 10 and 5 µg/ml. MFC7 and 4T1 cell lines show cell viability that start to increase from 60% to almost 100%. For the ASPC-1 and HEK293, the viability remains low, in the best case almost reaching 50% (ASPC-1 treated with 5µg/ml of CDs).

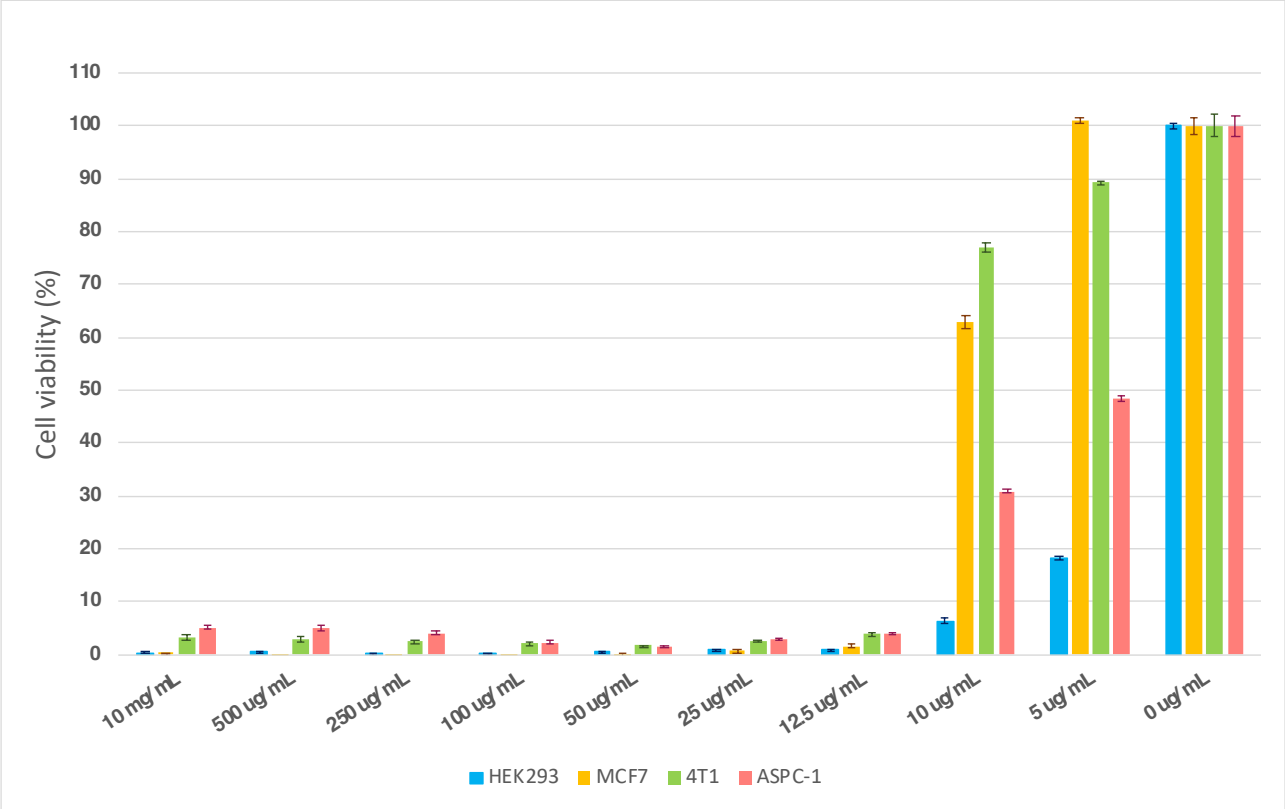


Figure 35: Cellular viability test of four different cancer cell lines studied with different concentrations of CDs

Data show a high variability according to two-way ANOVA's hypothesis test. Having F factor much greater than F critic, the variability between cell lines and between different concentrations is statistically significant. This means that changing CDs concentration or changing cell line the viability percentage will change as well.

From 10µg/ml to lower concentrations is possible to notice a discontinuity in the trend, this is due to the change of the used concentration. In this case using a concentration less than 12.5 µg/ml Cells are beginning to better tolerate particles.

It is conceivable that data have a significant positive trend easily deducible by the histogram in figure 33.

3.5.1 Cytotoxicity tests of the complex CD-DNA

Another cell viability experiment was carried out using healthy cells such as MSCs (human mesenchymal stem cells) and VSMC (brain derived human vascular smooth muscle cells). CDs alone at different concentrations and CDs-DNA in different ratio were tested on cell lines. Treating cells with CDs alone makes cytotoxicity quite high, even with extremely low concentrations. Cells are not affected by CDs' toxicity by the addition of DNA. At ratio 1:1 and 1:5, which are the best in terms of interaction, it's possible to affirm that conjugates preserve a cellular viability of 100%.

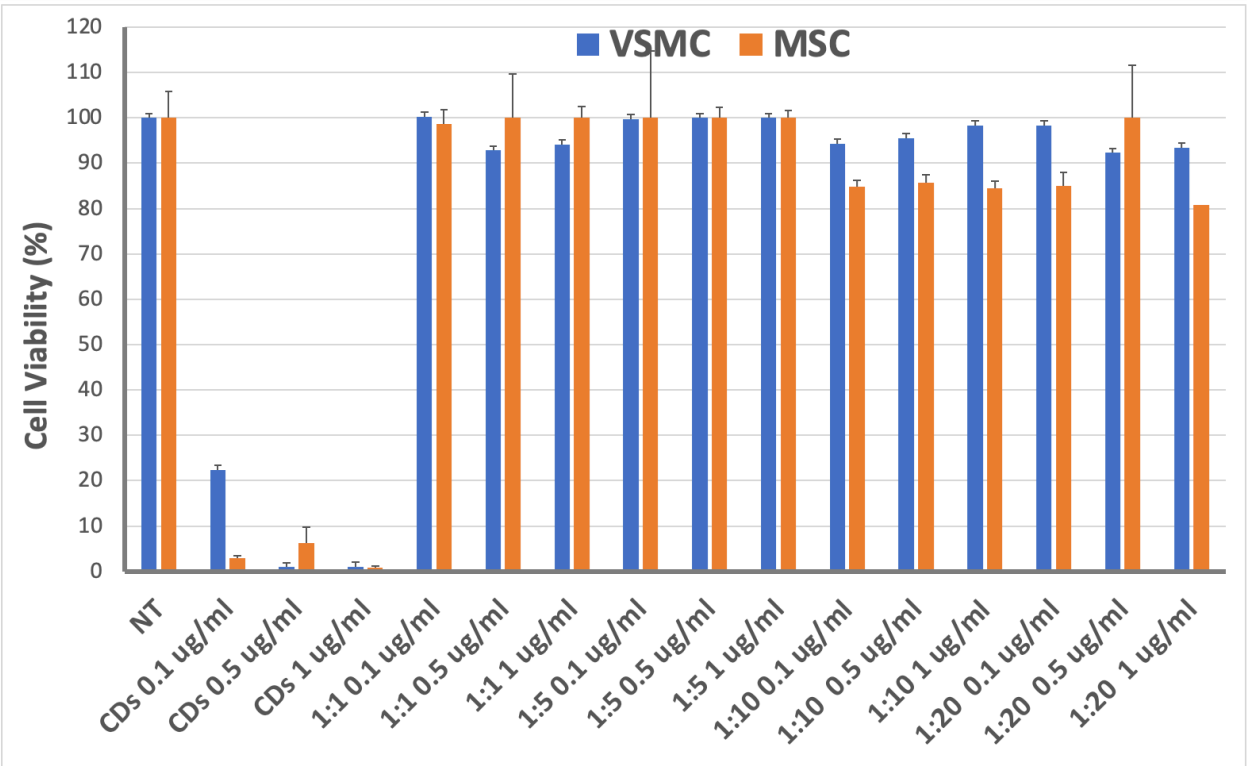


Figure 36: Cellular viability test of four different cancer cell lines studied with different concentrations of CDs and different ratio of mix CDs-DNA

Testing the two distributions with ANOVA Hypothesis test, both the cell lines have the same trend. This behaviour is confirmed by F (sample/cell) which is less than F critic. Both of the healthy cell lines are very sensitive to CDs alone, and they survive in presence of the conjugates of CDs-DNA.

On the other hand, there is no correlation concerning different solutions, all the data have two completely different behaviours. There are two different trends: using CDs alone and CDs in combination with DNA.

Conclusions

This research was focused on the production of nanometric positively charged particles for nucleic acids linkage, to promote gene delivery and therapy for cancer treatment.

Concerning CDs optical properties, UV-Vis spectra confirm the presence of π-π* transition of the C-C bonds of sp2 networks, and C=O bonds. This demonstrates the formation of benzene-like domains with functional groups. Furthermore, particles show an excitation independent blue fluorescence emission.

These 1.2 nm nanoparticles have a random polymeric structure with aromatic rich domains. This hypothesis is supported by TGA analysis.

These domains are the result of the condensation between Citric Acid and ortho-phenildiamine. Citric Acid's Carboxylic groups and ortho-phenildiamine's amino groups condense during the carbonization process to create these pseudo aromatic domains. The aromatic domains are mostly substituted with Nitrogen atoms derived from ortho-phenyldiamine's amino groups.

Seeing the strong similarities between CD's IR spectrum and PEI's one, it's deducible the major presence of the polymer into the particle. However, considering MALDI-TOF Mass Spectroscopy results, the presence of PEI is traceable to limited sections of the long polymer's chain. These sections can interconnect the pseudo aromatic domains.

The electrostatic binding between CDs and DNA is supported by UV-Vis, fluorescence and circular dichroism spectra. The highest interaction is detected having a 1:1 (DNA-CDs) weight ratio. Increasing Cdots' amount means less interaction between nanoparticles and DNA because CDs tend to aggregate and form hydrogen bonds among them.

Electrophoresis and cellular tests were evaluated with an excess of DNA, to see if it was possible to have a higher loading of DNA. From the 1:5 (CDs-DNA) weight ratio there is an excess of DNA. This confirms that the solution having an equal amount of ctDNA-particle is the most promising one in order to avoid DNA waste and promote a good loading for further delivery.

Regarding cytotoxicity tests, CDs showed toxicity for both healthy and cancer cell lines, even at low concentrations. For this reason, viability tests were carried out also with the conjugates CD-DNA. The presence of DNA makes the solution harmless and innocuous for both the healthy cell lines studied, increasing the viability up to almost 100%. This toxicity can be associated with the high presence of PEI into the particle's random coil[lxxxiii]. This delivery system can be tolerated by biological environment with the presence of the nucleic acids that are also the cargo of this gene delivery system.

Further studies has to be carried to evaluate the effective gene delivery and transmission.

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