

One-Pot Synthesis and Design of Chiral Carbon Dots Using Response Surface Methodology

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Abstract

One-Pot Synthesis and Design of Chiral Carbon Dots Using Response Surface

Methodology

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Carbon Dots (CDots) are a vibrant class of fluorescent nanoparticles with tunable physico-chemical and optical properties that owing to their extremely small sizes ($<20\text{nm}$), ease of fabrication, low cost of preparation, unique optical properties, and low cytotoxicity, they hold great promise in a myriad of applications. While their small size enables them to traverse through natural biological barriers, their surface chemical functionalities allow them to efficiently interact with their environment. In addition, CDots have been shown to retain some of the properties of their precursors, also known as active structure preservation during synthesis that has generated interest in exploring the potential applications of CDots.

In this work, we successfully synthesize and transfer the chiral properties of an amino acid precursor (L- and D-proline) to the surface of the CDots. The retention of the active sites potentially associated with the antibacterial properties of proline can be achieved by preserving the chirality of proline based CDots, leading to an increased ability to penetrate bacterial membranes. Instead of relying on an empirical one-factor-at-a-time approach to study the influential condition for this goal, we used Response Surface Methodology (RSM) to efficiently optimize the reaction conditions (temperature, time, and molar ratio) with the goal of preserving the maximum residual chiral signal of proline on the surface of CDots. We further evaluated the

antibacterial response to the maximum preservation of the chiral properties in CDots versus its absence. Our observations evidence enhanced antibacterial activity for highly chiral samples confirming the correlation of proline activity with its stereochemistry.

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and understanding during this challenging period. Your love, encouragement, and support have been a constant source of strength, and I cannot imagine a better audience for all my presentations.

Contributions of Authors

This thesis presents an original research study conducted by Arezoo Setayesh under the supervision of Dr. Rafik Naccache who designed the project and provided financial support and guidance throughout the entire research period. Furthermore, the antibacterial analysis presented in Chapter 3, Sections 3.3.5 and 3.4.3 was carried out in collaboration with Ms. Laura Dominguez and Dr. Brandon Findlay from Concordia University.

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List of Abbreviations

CDots	Carbon Dots
CD	Circular Dichroism (Spectroscopy)
MWCO	Molecular Weight Cut-off
¹HNMR	Proton Nuclear Magnetic Resonance
PL	Photoluminescence
QY	Quantum Yield
TEM	Transmission Electron Microscopy
XPS	X-ray Photoelectron Spectroscopy
XRD	X-ray Diffraction
TGA	Thermogravimetric Analysis
RSM	Response Surface Methodology
DOE	Design of Experiment
BBD	Box-Behnken Design
CCD	Central Composite Design
OFAT	One-Factor-At-The-Time
MIC	Minimum Inhibitory Concentration

Chapter 1. Material Introduction and Background

1.1 Nanomaterials

Nanomaterials are defined as materials having at least one dimension of less than 100 nm in their structural component ¹. Nanomaterials have two major properties that have attracted significant attention, which is the increase in surface area to volume ratio and the possible appearance of new properties at the nanoscale. Therefore, many publications set the highest limit for a nanomaterial's size at 50 nm or 100 nm, with this decision being supported by the fact that when nanoparticles reach or exceed these sizes, some of their physical properties start to resemble those of the bulk ².

1.1.1 Classifications of Nanomaterials

Nanomaterials can be generally categorized into four types namely: inorganic-based, organic-based, carbon-based, and composite-based according to their origin and chemical compositions. They can take on a variety of morphologies, including spheres, sheets, and rods, among others, which can all be classified according to their dimensionality (**Fig 1.1**). Nanomaterials with zero-dimensional (0D) order have all three dimensions at the nanoscale (e.g., quantum dots and carbon dots), one-dimensional (1D) materials have two external dimensions in the nanoscale (e.g., nanorods), and following the same concept, two-dimensional (2D) materials have only one external dimension at the nanoscale (e.g., graphene nanosheets). The third category of nanomaterials include 3D nanomaterials, which have no external dimensions at the nanoscale yet exhibits internal nanoscale-like properties. Nanocomposites are an example of 3D nanomaterials described as materials with a bulk matrix and a dispersion of nanofillers that could be selected from any 0D, 1D, and 2D categories of nanomaterials ³.

Among the four categories of nanomaterials, carbon-based nanomaterials are one of the most thoroughly studied materials owing to their properties that include high carrier mobility, high electrical conductivity, flexibility, optical transparency, and relatively low toxicity. These properties have made them suitable for use in optoelectronic devices ⁴, electrochemical sensors ^{5,6}, cancer phototherapy ^{7,8}, antibacterial systems ^{9,10}, etc.

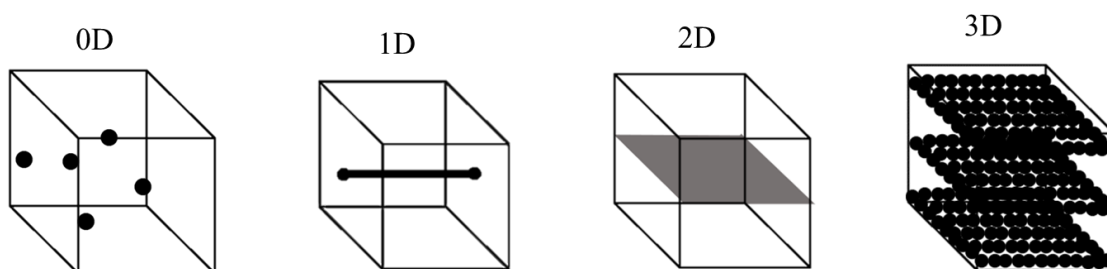


Fig 1.1 Classification of nanomaterials based on their dimensionality.

1.1.1.1 Carbon-based Nanomaterials

Carbon has six electrons that enable it to form single and multiple bonds and create different chemical structures from C-C bonds in the classical alkane molecules to the multiple C=C or C≡C bonds found in graphite and acetylene, respectively. This property also allows carbon to create nanomaterials with sp^3 and sp^2 hybridization ranging in size from 1 to 1000nm. The first member of the carbon nanomaterials family, fullerene, was not discovered until 1985 by Kroto and coworkers ¹¹ while performing an experiment related to the formation of long-chained carbon in interstellar space. The interesting 60 carbons cluster of fullerene was suggested to resemble the structure of a “soccer ball on Texas grass”. Later, other members of the carbon allotropes were discovered and are now known as carbon nanotubes (CNTs), graphene and nanodiamonds (**Fig 1.2**). These nanomaterials evidenced structures, which were either sp^2 or sp^3 with respect to the

carbon atom hybridization. More recently, a new member of the carbon nano family was discovered, namely carbon dots (CDots), and these materials possess a mixture of sp^2 and sp^3 hybridized carbon atoms along with heteroatom doping in the core and surface of the dots. Thus, these dots are not considered allotropes of carbon but simply referred to as a carbon dot, carbon nanoparticle or carbon quantum dot in the literature.

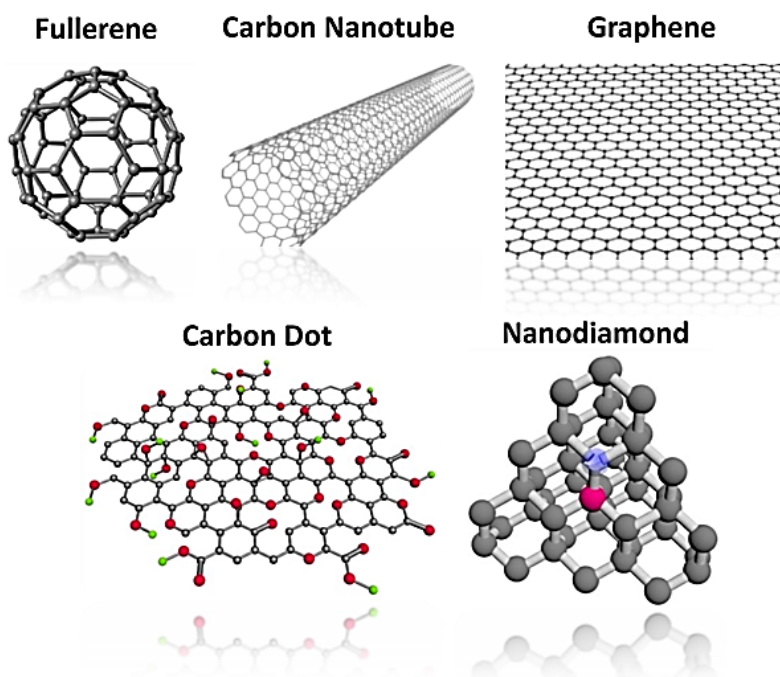


Fig 1.2 Carbon nanomaterials and their unique chemical structures. Top - from left to right: fullerene, carbon nanotubes and graphene. Bottom – from left to right: carbon dots and nanodiamonds. Reproduced with permission ¹².

1.2 The Emergence and Synthesis of Carbon Dots

In recent years, considerable attention has been directed towards CDots, which are classified as 0-D carbon-based nanomaterials possessing interesting and tunable optical properties. The accidental discovery of CDots in 2004 by Xu and colleagues ¹³ during the purification of single-walled carbon nanotubes generated from an arc-discharge technique revealed the presence of

extremely small particles in the electrophoretic profile that can fluoresce and produce different colored emissions upon irradiation with UV light at 365 nm. This discovery spurred several groups to investigate the emergence and properties of these fluorescent dots, as depicted in **Fig 1.2**. Sun and colleagues ¹⁴ synthesized these dots via laser ablation in 2006 and functionalized them with Polyethylene Glycol (PEG) to enhance their bioavailability, coining the term "Carbon Dots" for the first time. However, the long and complicated preparation procedure and the high-temperature requirement (900 °C) for synthesis, along with multiple post-synthesis steps, proved to be highly inconvenient.

Following the discovery of CDots, Li and colleagues proposed a one-step preparation method for CDots in 2010 ¹⁵, which produced size-dependent fluorescence emission similar to that of quantum dots by cutting off graphite honeycomb layers into various small fragments of graphite. The researchers reported blue, green, yellow, and red-emitting emission spectra for CDots by exciting the sample mixture at 240, 300, 360, 420, 500, and 580 nm. Meanwhile, numerous research groups attempted to generate CDots using comparable techniques that involved bulk carbon sources such as graphite. However, these techniques still posed challenges due to the poor production yield and uncontrollable size distribution of the particles produced.

In 2013 ¹⁶, a group of researchers from China suggested the use of hydrothermal reactors for the mixture of citric acid and ethylenediamine under several operating temperature from 150°C to 300°C. Their results showed a successful production of amorphous CDots, or as they called them “polymer dots” due to the carbonization of the two precursors used in the reaction. This was a discovery that led scientists to explore this new form of amorphous CDots and the potential precursors that could be used in this technique. This simpler pathway without the requirement for numerous synthesis and post-modification steps quickly became the widely accepted synthesis

approach. Today, several strategies exist for the preparation of CDots, which are classified into two categories: bottom-up and top-down. Top-down approaches mainly involve the conversion of large carbon particles into CDots, similar to the methods used in their initial and accidental discovery. In contrast, bottom-up techniques rely on small organic molecular precursors. It is noteworthy to mention that additional research is still required to gain a comprehensive understanding of the formation mechanism of CDots. However, each step taken toward this objective reveals new discoveries that provide insights into their formation mechanism and potential applications. Consequently, both the bottom-up and top-down approaches provide essential and necessary information in the field.

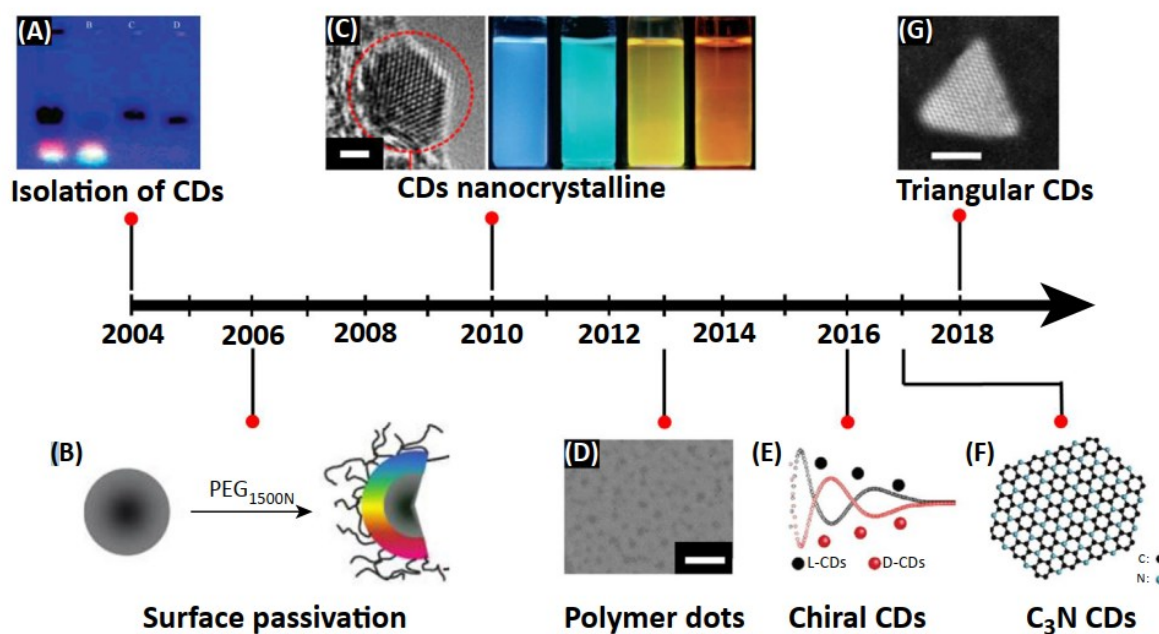


Fig 1.3 The development of techniques and strategies for the synthesis of CDots since their emergence. Reproduced with permission ¹⁷.

1.2.1 Top-Down Approaches

The top-down techniques commonly involve the breakdown of graphite and graphene oxide through laser and electrochemical oxidation (as illustrated in **Fig 1.4**, top). In fact, the accidental synthesis of CDots in 2004 resulted from the chemical oxidation of arc-discharged soot using HNO_3 . This method generated a large number of oxygen-containing groups on the surface of CDots, thereby conferring water-dispersibility upon the resulting CDots ¹³. Although this strategy has proven successful in prior studies ^{18,19}, the multiple and time-consuming post-synthesis steps required to neutralize and purify the CDots were deemed unfavorable. Furthermore, the use of strong acids and oxidizing agents poses environmental safety hazards and is thus not feasible on a larger scale ²⁰.

Another technique employed in top-down approaches is the electrochemical oxidation synthesis of CDots, which is similar to chemical oxidation. The potential difference between the cathode and anode generates oxidizing species, resulting in the exfoliation of CDots from the carbon source (i.e., the anode or the electrolyte) ²¹. Although electrochemical oxidation is considered more environmentally friendly than chemical oxidation, its practical use is limited due to time-consuming purification steps and low production yields. In contrast, laser ablation can directly generate CDots by subjecting the carbon source to a high-power laser beam. However, it requires high-temperature conditions and post-synthetic functionalization of CDots to achieve the desired physicochemical properties and acceptable yield ²².

1.2.2 Bottom-up Approaches

Bottom-up approaches are typically preferred due to their facile one-step synthesis procedures, which offer a better opportunity to control the properties of CDots. Additionally, bottom-up routes

generally result in well-distributed size populations, as opposed to their broad counterparts in top-down methods. The two techniques mainly used in bottom-up approaches are hydrothermal and microwave pyrolysis syntheses (**Fig 1.4** bottom), with other techniques including thermal/combustion and solvothermal also being utilized ²⁰.

The utilization of microwave-assisted synthesis presents a superior method of sample heating in comparison to the hydrothermal technique, offering both faster and more uniform heating. Microwaves are a type of electromagnetic wave comprising both electric and magnetic components. As microwave radiation permeates the sample, the dipole field of the material attempts to realign itself with the electric field of the microwave. This perpetual attempt to reorient, in response to the oscillating electric field, causes a loss of energy via dielectric losses and molecular friction. Thus, the direct heating of the target molecules and instantaneous regulation of the radiation provide considerable advantages over conventional heating methods employed in hydrothermal synthesis. The latter relies on heat transfer from the heat source to the walls of the stainless-steel reactor followed by a second heat transfer to the walls of the Teflon container and then finally a third heat transfer to the sample itself. In addition, hydrothermal syntheses often necessitate prolonged reaction times to ensure that the reaction medium has achieved the desired temperature, which may also lead to reactant degradation, ultimately reducing yield or halting the reaction. In contrast, microwave-assisted reactions exhibit a shortened reaction time, thereby minimizing the possibility of precursor or product degradation.

In 2009, Zhu *et al.* ²³ presented the use of microwave reactors as a straightforward approach for synthesizing CDots. A mixture of PEG200 and glucose in water was subjected to microwave heating for durations of 5 and 10 minutes. The findings demonstrated that the sizes and photoluminescence characteristics of the CDots were altered with an increase in heating time.

Subsequently, the utilization of microwaves for CDot preparation garnered greater attention among researchers owing to its ability to offer expeditious and time-saving procedures^{24–26}. In 2011 Wang *et al.*,²⁷ conducted a comparative analysis of the properties of CDots synthesized using conventional hydrothermal methods and the microwave technique. The findings demonstrated that CDots produced via microwave-assisted synthesis exhibited higher photoluminescence properties and longer lifetimes, validating the superiority of this approach over the hydrothermal technique for CDots synthesis. Nowadays, advancements in technology have enabled the precise monitoring of temperature and pressure changes during reactions employing microwave reactors, offering numerous prospects for investigating and regulating the impact of reaction parameters on material synthesis²⁸.

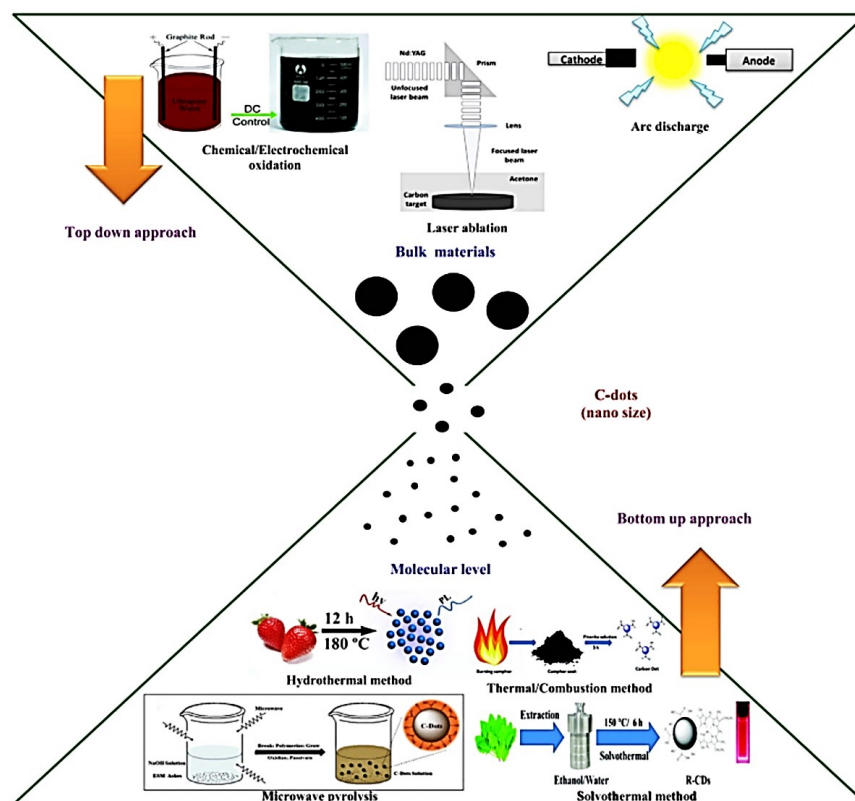


Fig 1.4 Schematic illustration of top-down (Top) and bottom-up (down) approaches to synthesis CDots. Reproduced with permission²⁹.

1.2.2 Optical properties of Carbon Dots

CDots synthesized from diverse carbon sources or using varying synthetic techniques exhibit distinct optical absorption profiles. However, they commonly demonstrate significant absorption in the ultraviolet (UV) region (200–400 nm), wherein the absorption bands correspond to the $\pi \rightarrow \pi^*$ transition of C=C bonds and the $n \rightarrow \pi^*$ transition of C=O/C=N bonds. The absorption characteristics of CDots are contingent upon variations in the heteroatom doping (e.g., oxygen, nitrogen, sulfur, etc.), content of the carbon core, the nature of surface functional groups, as well as the size of π -conjugated domains^{30,31}.

Apart from their absorption characteristics, CDots are also known for their appealing photoluminescence (PL) properties, which have been a subject of interest in numerous investigations. Several research groups have aimed to comprehend the mechanism and strategies for enhancing the PL properties of CDots. Notably, Hu *et al.*³² achieved highly luminescent CDots using three different carbon sources that varied in their number of carboxyl groups. Among the three carbon sources investigated, citric acid, with the highest number of carboxyl groups, yielded the highest fluorescence quantum yield as compared to malic acid and 2-hydroxyisobutyric acid. The fluorescence quantum yield represents the ratio of photons emitted to the number of photons absorbed, with a higher value indicating a more efficient PL process.

The remarkable fluorescence properties of CDots, coupled with their superior stability as compared to conventional fluorescence dyes, have made them a compelling choice for biomedical applications. CDots have demonstrated resistance to intensity decay phenomena, such as photobleaching and photo blinking. Photobleaching is the permanent and irreversible alteration of covalent bonds, leading to a reduction in fluorescence intensity. CDots have been found to be

resistant to this phenomenon, even after exposure for several hours or days ^{33,34}. Photoblinking is a commonly observed phenomenon in many fluorescent systems, characterized by an equal relaxation of photons in radiative and non-radiative pathways, resulting in "on" and "off" states for the emission of light. The resistance of CDots to photobleaching and photoblinking has made them particularly attractive for use in biosensing applications ^{35,36}, cancer therapy ³⁷⁻³⁹, and anti-microbial systems ^{40,41}. Despite these advancements, the origin of PL in CDots remains a controversial topic with no clearly defined mechanism. Rather, what is well-accepted is that PL depends on the graphitic conjugated core, and surface defect states that seem to dictate the fluorescent properties of the dots.

1.2.2.1 Graphitic Core driven Fluorescence Properties

CDots, which possess a graphitic π -conjugated carbon core can display size-dependent fluorescence emission properties. The fluorescence mechanism for the majority of nanoparticles with sizes smaller than the Bohr radius has traditionally been elucidated via the quantum confinement effect, analogous to inorganic quantum dots. In the case of CDots, this theory can explain the relationship between CDot size and emission profile, whereby the fluorescence properties of CDots are influenced by the band gap that separates the conduction and valence bands. Consequently, CDots with smaller sizes have larger band gaps, leading to fluorescence emission at shorter wavelengths. Conversely, as the size of the nanoparticle increases, a shift towards longer wavelengths occurs (see **Fig 1.3**). Numerous studies have documented the red-shifting mechanism of band gaps in CDots as a function of size increment ^{34,42-44}. In accordance with these findings, the photogenerated exciton in CDots is confined within the graphitic core, where the band gap decreases with increasing conjugation/size, thereby resulting in the observed red-shifted fluorescence.

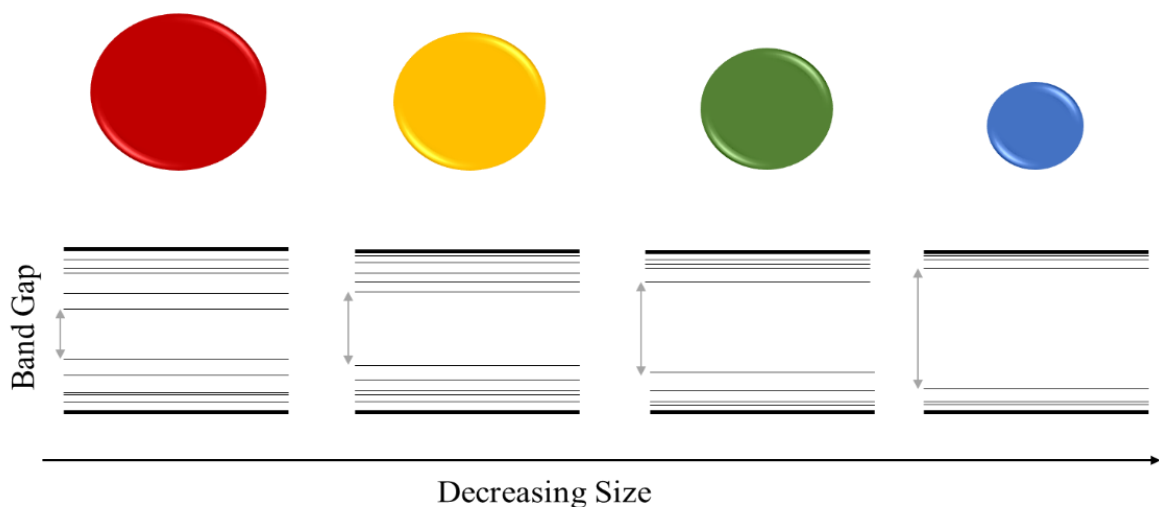


Fig 1.5 Schematic representation of quantum confinement in CDots. As the size of CDots decreases, the emission profile is blue shifted.

1.2.2.2 Surface Driven Fluorescence Properties

Surface modification plays a crucial role in nanoparticle synthesis, providing materials with stability, selectivity, and reactivity under specific environmental conditions. In the case of CDots, it is widely acknowledged that their photoluminescence properties can originate from surface states. Specifically, numerous energy traps on the surface may become excited at different wavelengths and subsequently trap the electron via non-radiative pathways. Additionally, CDots have demonstrated both excitation-dependent and excitation-independent emissions, indicating that their fluorescence emissions can either shift or remain unchanged as the excitation wavelength varies. The excitation-dependency is thought to be influenced by the energy states at the surface. In this context, chemical passivation, whether during or post synthesis, can mitigate this dependency, resulting in photoluminescence properties that are excitation-independent⁴⁵. In fact, chemical passivation, elemental doping, and other surface modifications are frequently employed

to introduce atoms and groups that can donate electrons to localized carrier traps, thus reducing the contribution of non-radiative processes.

An example of how surface groups can influence the photoluminescence (PL) of CDots is demonstrated in the study by Ding *et al.*⁴⁶ when varying the degree of surface oxidation resulted in the production of CDots with a full-color emission spectrum. The authors determined that the observed red shift in the emission peaks from 440 to 625 nm was caused by a gradual narrowing of the band gaps in CDots as additional oxygen species were incorporated into their surface structures. In contrast to traditional semiconductor quantum dots, these energy bands were found to be dependent on the surface groups and structures rather than the particle size (see **Fig 1.4**).

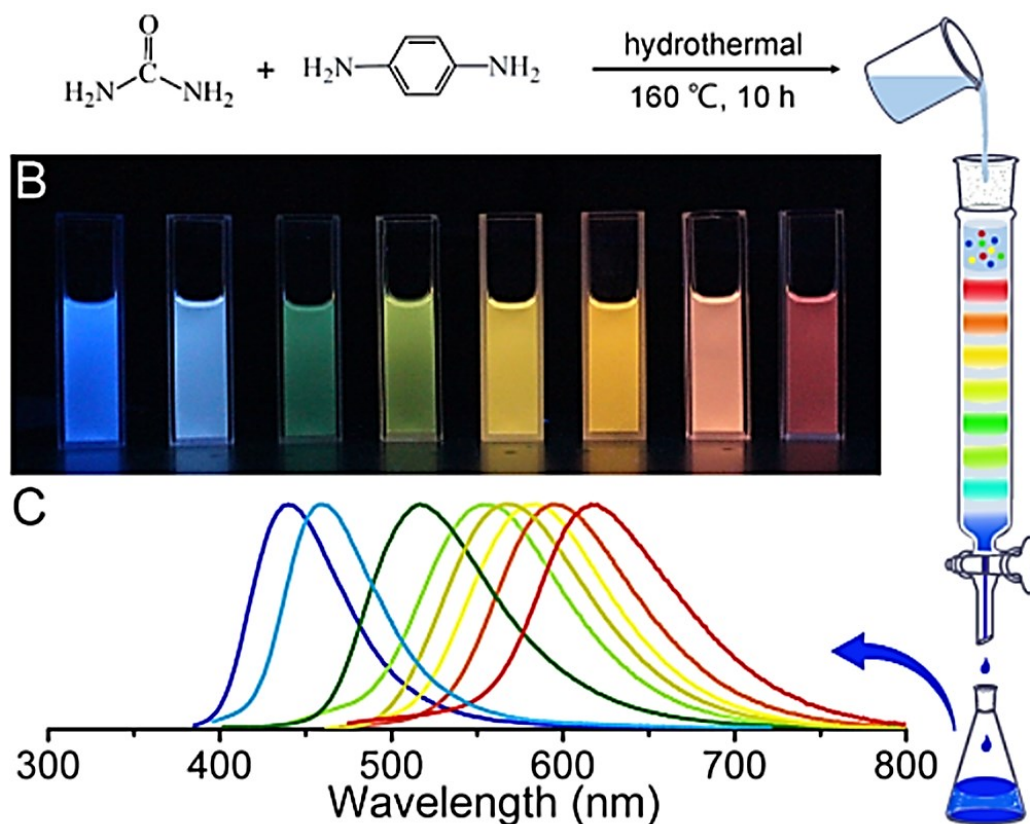


Fig 1.6 The illustration of a one-pot synthesis of CDots in water and further purification by silica column chromatography. The surface oxidation state is responsible for the shift from blue to red emission observed as the degree of oxidation increases. Reproduced with permission⁴⁶.

1.2.3 Active Structure Preservation in Carbon Dots

Recently, a highly intriguing phenomenon was discovered in CDots synthesized by bottom-up approaches, specifically, that the active structures of the precursors could be preserved in the resulting CDots. Preservation of active structures in CDots is currently defined as the transformation of some or most of the properties and affinities of the starting materials to the CDots during synthesis. To preserve the functional structures of the starting materials, the reaction temperature should generally remain below the decomposition temperature of the reactants that bear the functional groups. The primary advantage of this technique is the ability to perform a single-step synthesis without the need for further post-synthetic modification, thus saving time and resources. In contrast, the alternative approach involves indirect chemical modifications using an activating agent such as 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), which is quite labile in the presence of water, or acidic pH due to hydrolysis of the reactive *O*-acylurea structure. The lifespan of EDC could be enhanced by using N-hydroxysuccinimides (NHS), but the optimization of these two compounds is required for each application ⁴⁷. Furthermore, chemical modification of CDots can alter their surface states and their PL properties, potentially leading to a decrease in their quantum yield, or even fluorescence quenching.

In 2016, Bhunia *et al.*, ⁴⁸ reported a single-step synthesis of CDots through hydrothermal heating of folic acid at 90°C. The folic acid derived CDots effectively targeted cells that expressed the folate receptor on their surface, as was shown by fluorescence microscopy and quantitative analysis. To further confirm the true uptake of folic acid-based CDots, 6-O-(O-O'-di-lauroyl-tartaryl)-d-glucose and arginine were also used separately as precursors in the synthesis of CDots to compare their affinity to cancer cells. As shown in **Fig 1.5**, confocal microscopy images displayed significantly lower cellular uptake in comparison with the folic acid-treated CDots.

Moreover, the cytotoxicity of CDots were also evaluated in this study showing low toxicity even when incubating cells with CDot amounts well in excess of what is required for cell imaging experiments further speaking to their potential applications in this field.

Following this concept, Hou *et al.*,⁴⁹ synthesized self-functional CDots through hydrothermal synthesis of ciprofloxacin hydrochloride, an antibiotic, in accordance with the concept of CDots. The reaction temperature was maintained below the decomposition temperature of ciprofloxacin hydrochloride, by carrying out the reaction at 200°C. The resulting CDots demonstrated notable antibacterial activity against *E. coli* and *S. aureus*, comparable to that of ciprofloxacin. To determine whether the observed effect of CDots was solely due to the residual antibiotic molecules present after synthesis, silica gel column chromatography was performed for sample purification. The results indicated consistent activity of CDots both before and after purification, indicating the efficient antibacterial effect of ciprofloxacin based CDots. Furthermore, despite the pH-sensitive nature of ciprofloxacin hydrochloride, which can lead to irregular variations in PL intensity with pH, the CDots exhibited a linear and consistent decrease in PL intensity in response to pH changes within the range of 5 to 10, after six experimental cycles. This finding confirms that CDots not only retained the active properties of the starting material, but also augmented the overall efficacy of the compound.

Following the work of these groups, several studies also attempted to facilitate the use of compounds with specific activities in the form of CDots through single-step pyrolytic reactions using hydrothermal or microwave-assisted syntheses. The active properties targeted in these studies were a transfer in pH sensitivity⁵⁰, specific ion detection⁵¹, chemical interactions^{52–55}, binding affinities^{56–59}, antibacterial activities^{60,61}, catalytic activities^{62,63} and chirality^{64,65}.

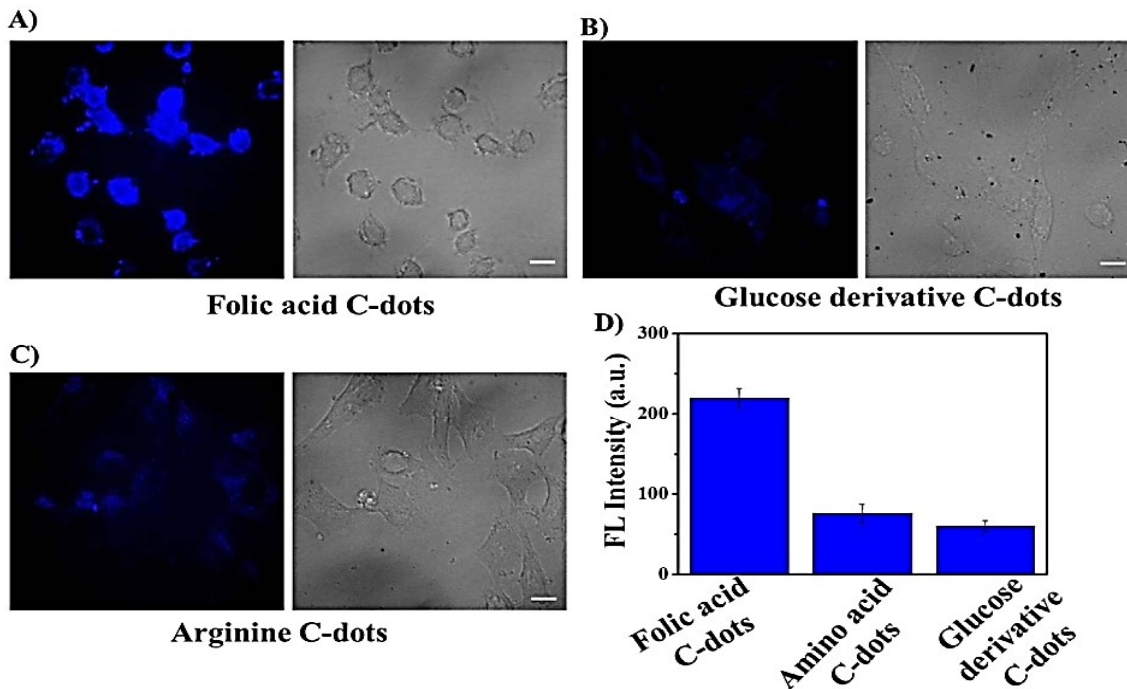


Fig 1.7 Confocal microscopy of HeLa cells treated with CDots (C-dots) made from folic acid, arginine, and 6-*O*-(*O*-*O'*-di-lauroyl-tartaryl)-d-glucose shown in A), B), and C), respectively. Emission intensities calculated from the confocal microscope images shown in E). Reproduced with permission ⁴⁸.

1.3 The importance of Chirality

Chirality is a term used to define the specific geometry of a compound when it cannot be superposed on its mirror image. The classic example of chirality is our hands that are mirror images of each other and impossible for them to coincide across all axes (**Fig 1.6 a and b**). In chemistry, chirality can be defined as a structure having an element of carbon at the center (chiral center) connecting to four different groups in a tetrahedral geometry (**Fig1.6 c**). The two chiral molecules called enantiomers are optical isomers that are similar in terms of connectivity and chemical compositions but differ in their 3D orientations in space. The right-handed and left-handed

enantiomers exhibit different optical properties when exposed to circularly polarized light, much like our hands, and are also differently recognized in biological systems.

Enantiomers are prevalent in numerous biomolecules, including sugars and amino acids. These chiral molecules exhibit drastically different biological roles and functions in nature, often being recognized by biological organisms. Carvone serves as an example of a chiral compound that displays a difference in odor between its left-handed (S) and right-handed (R) enantiomers due to the chiral olfactory receptors found in the human nose. The R-enantiomer has a spearmint scent, while the S-enantiomer smells like caraway. These distinct smells arise from the different interactions and fit with nasal receptors that subsequently communicate with the brain. This phenomenon also holds true for L- versus D-amino acids, as well as molecules that constitute our DNA.

Fig 1.7 illustrates an example of a chiral molecule that highlights the significance of spatial configuration in the binding possibilities of chiral molecules. In situation A), the configuration of the chiral substrate can fit into the receptor site due to the corresponding three-dimensional shape and color of the receptor (groups attached to the substrate are displayed in three colors). Conversely, in situation B), the two bottom groups of the chiral substrate do not align with the receptor sites due to the slight difference in the orientation of the attaching groups. Following this concept, Chirality of chemical compounds has been used in different fields namely pharmaceuticals ^{66,67}, catalysis ^{68–70}, antimicrobials ^{71,72} and bio-sensing ^{73,74} to develop enantio-selective compounds and investigate their applications.

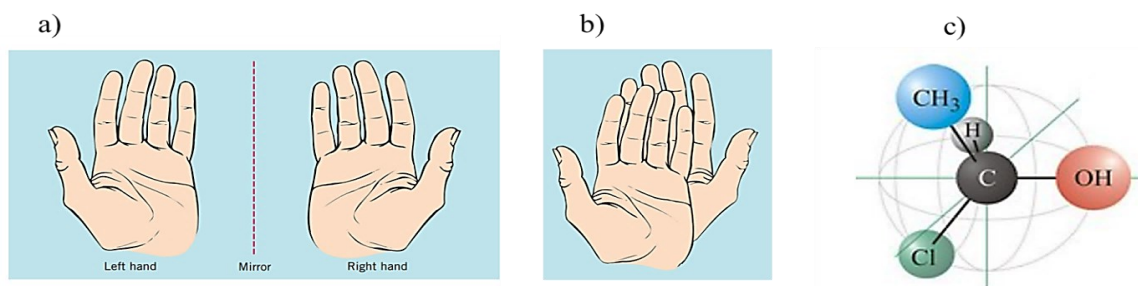


Fig 1.8 Schematic illustration for the concept of chirality. a) Right and left hands are mirror images of each other. b) Similar to enantiomers, it is not possible for the hands to superpose on each other. c) Demonstration of a chiral molecule with a tetrahedral stereocenter and four distinct groups.

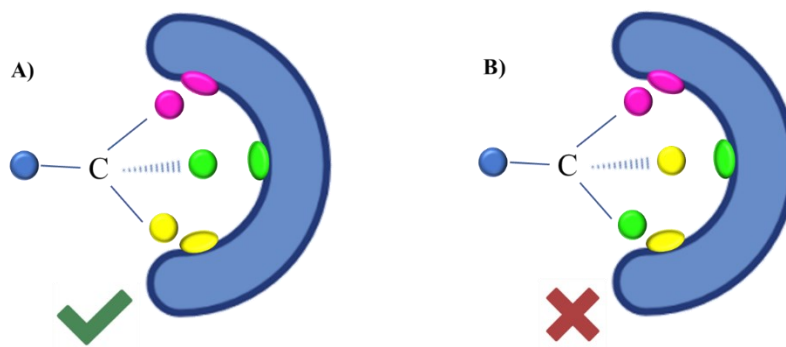


Fig 1.9 Graphical illustration of two enantiomers and their binding possibilities with the same three-dimensional receptor. A) and B) show successful and unsuccessful binding, respectively.

1.3.1 Detection of Chirality

Circular dichroism spectroscopy (CD) is an important analytical method for determining the chirality of molecules, particularly macromolecular structures such as proteins and nucleic acids. This method measures the differences in the absorption of left and right circularly polarized light in optically active substances, which are chiral substances. Circularly polarized light (CPL) is a type of light where the electric and magnetic wave planes are at a 90° phase difference from one

another and rotate as the light beam travels. When optically active molecules are present near chromophores, they preferentially absorb left and right circularly polarized light at specific wavelengths, resulting in differences in extinction coefficients for the two polarized rays. This leads to the absorption of left- and right-handed CPL by chiral molecules in varying amounts, resulting in an elliptical electric field vector, as illustrated in **Fig 1.8**⁷⁵. A CD spectrum shows the variation of this ellipticity over different wavelengths usually measured in millidegrees (mdeg).

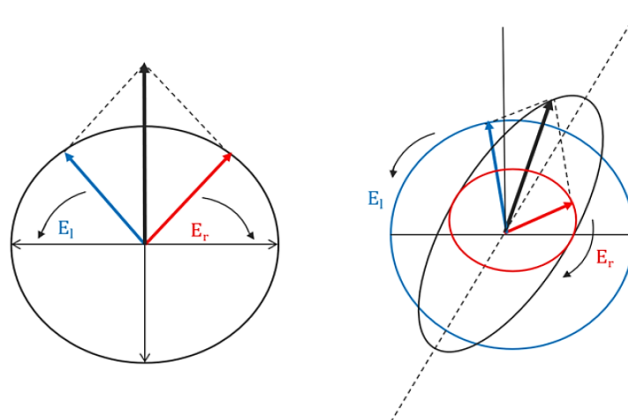


Fig 1.10 The left- and right-handed electric field (E_r and E_l) absorptions in CPL when absorbed equally resulting in a linearly polarized light and zero degree in the CD spectrum (left) versus unequally absorbed CPL resulting in elliptically polarized light (right).

1.3.2 Preserving Chirality in Carbon Dots

As previously mentioned, the preservation of active structures would enable the retention of certain features of the starting materials during the synthesis of CDots. Recently, the concept of active structure preservation has been applied to the study of chiral CDots. The first synthesis of chiral CDots following this approach was reported in 2016 by Zhang *et al.*,⁷⁶ wherein L(D)-cysteine and citric acid were subjected to hydrothermal synthesis in water at 180°C for 1 hour. The CD spectrum of the resulting CDots exhibited three distinct absorption peaks at 210nm, 245nm, and 350nm. The

$n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of CDots at 350nm and 245nm, respectively, were attributed to the passivated surface traps and sp^2 conjugated system of CDots. Notably, the peak at 210nm was thought to indicate the preservation of the cysteine chiral centers, as it was consistent with the CD absorption of pure cysteine. To compare the chiral recognition of the samples, non-chiral CDots were synthesized using only citric acid following the same procedure as a control group. The results demonstrated that chiral CDots exhibit excellent catalytic activity for electrocatalysis and enantioselective recognition of small molecules, such as L- and D-tartaric acid, whereas the non-chiral samples displayed no enantioselective recognition toward the target molecules.

Ghosh *et al.*,⁷⁷ employed guanosine 5'-monophosphate (5'-GMP) as the starting material to synthesize CDots due to its self-assembling nature as a natural nucleotide, which facilitates the formation of ordered supramolecular structures. The reaction was carried out using a microwave reactor at 160°C for 5 minutes, with the degree of irradiation being a crucial factor. Incomplete conversion was observed when exposed to lower temperatures or shorter exposure times, while precipitation occurred at higher temperatures with prolonged exposure. Structural analysis using proton NMR, conducted in both D_2O and $DMSO-d_6$, indicated that the protons in CDots were the same as those in the precursor, albeit with a slight up-fielded shift and sharper peaks in CDots. Moreover, phosphorus NMR showed a single peak attributed to orthophosphate monoester in 5'-GMP that shifted in the profile of CDots, confirming the formation of orthophosphate diester bonds. The CD spectra exhibited several small absorptions at 218nm, 230nm, 260nm, and 270nm, which suggested the presence of continuous self-assemblies of a 5'-GMP-like structure. Overall, the findings from this study indicated that the guanine and sugar components might not undergo significant rearrangement during microwave treatments. Furthermore, fluorescent hydrogels were synthesized from CDots without the introduction of externally added monpositive cations,

demonstrating the potential of these nanodots for designing intricate nanostructured materials in biomedical applications.

As research in this field progressed and more studies were dedicated to the synthesis and application of chiral CDots, the inheritance or induction of chirality from amino acids garnered significant attention. Amino acids offer diversity in terms of chemical or physical features, hydrophobicity or hydrophilicity, and aromatic functional groups while being relatively inexpensive compounds. Furthermore, they are the primary building blocks of proteins and can function as chemical messengers in cell communication. Therefore, the use of amino acids as chiral precursors emerged soon after the suggestion of the one-step synthesis of CDots through active structure preservation. Among amino acids, L- and D-cysteine remain the most frequently used precursors to induce chirality in CDots through facile one-step synthesis to date.^{78–82}

Branzi *et al.*,⁸³ conducted a study on the formation mechanism of cysteine-based chiral CDots synthesized at room temperature in a reaction catalyzed by copper(II) salts. The study suggested that several factors related to cysteine molecules influenced the formation of chiral carbon dots, including free thiol groups that participated in redox reactions producing radical species, amine and carboxylic functional groups that improved the stability of the oxidized state, and hydrogen atoms that assisted in radical hydrogen transfer reactions producing carbon-centered radicals. The authors collected CD spectra of the reaction mixture at various reaction times to examine the evolution of the chiroptical signal during the cysteine decomposition.

As depicted in **Fig 1.9**, a sharp increase in the CD signal with a maximum at 219 nm and a minimum at 250 nm was observed, which reached its highest intensity at 1.5 hours and remained nearly constant up to 2 hours. However, the intensity began to decrease drastically after two hours, which could indicate the decomposition of the grafted molecules that make up the "chiral shell"

and their conversion into the "achiral core". Additionally, based on the position of the absorption bands and PL properties of the samples between 0.4 and 84 hours, it was concluded that the formation of achiral chromophores is more favorable over longer periods. Notably, CDots did not exhibit PL properties at reaction times below 0.5 hour. Finally, the authors concluded that the cysteine molecules or cysteine derivatives (amides, disulfide, or sulfonic acid) grafted to the outer shell of the nanoparticles through amidic bonds are the possible source of the chiroptical activity of the cysteine-based chiral CDs generated by radical assisted decomposition. In other words, the core-shell structure of CDots would allow them to preserve some of the properties of their precursors on their surface and consume the rest as the reaction prolonged.

Other amino acids, aside from cysteine, have also been investigated using similar techniques, with or without the use of citric acid as a co-carbon source, for the synthesis of chiral CDots. Among the amino acids used are alanine⁸⁴, glutamic acid⁸⁵, lysine⁸⁶, aspartic acid^{87,88}, proline⁸⁹, and tryptophan^{90,91}. Although amino acids have demonstrated their potential for use as chiral inducers in CDots, the optimal conditions for preserving chirality in these systems have not been thoroughly investigated. Moreover, the initial goal of preparing chiral CDots through active structure preservation was to transfer some of the properties and affinities of the chiral precursors to a carbon-based nano system. To achieve this objective, most studies have been performed at low temperatures (<200°C) which is below the melting point of amino acids to ensure the preservation of their structure. Thus, it is necessary to conduct further research to evaluate the impact of conditions other than temperature that could directly impact the extent of chiral residues in CDots.

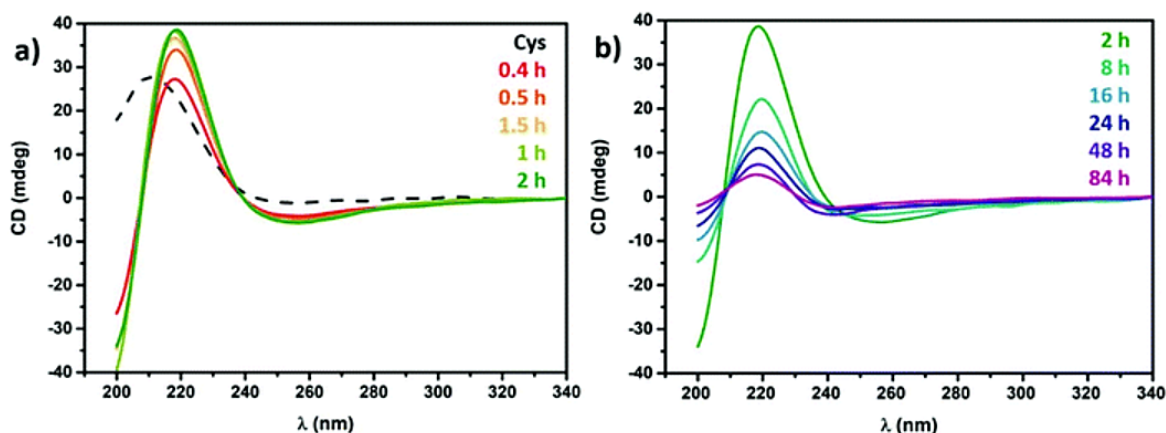


Fig 1.11 The variation of the chiroptical signal during the course of the reaction period: (a) 0–2 h and (b) 2–84 h. Reproduced with permission ⁸³.

1.4 Statement of the Problem

CDots, a newly discovered member of carbon nanoparticles, can be synthesized using microwave or hydrothermal techniques by selecting precursors with specific properties. The efficient bottom-up and single-step synthesis of carbon dots from relatively inexpensive and versatile molecules can be tailored through reaction parameters, allowing for the transfer of chiroptical properties from precursors to CDots by preserving the active structure. However, the lack of knowledge about the co-dependence of various reaction parameters during synthesis necessitates advanced approaches to address this knowledge gap. By studying the interactions between different reaction parameters, such an approach would enable us to tune the properties of CDots to meet specific requirements. Advanced statistical tools are now available to chemists, enabling them to gain knowledge about their experiments while ensuring the safety of their materials to prevent degradation. By determining the individual and synergistic effects of various parameters that could potentially affect the desired outcome of an experiment, researchers can estimate the best operating conditions

and avoid conducting redundant experiments. Furthermore, a mathematical model can provide valuable insight for predicting future responses while performing efficient experimentation protocols.

Thus, the primary objective of this project is to identify the key parameters that significantly affect the chiral signal of CDots and determine the optimal combination of these parameters. By preserving the maximum residual signal from the chiral precursors, we aim to leverage the potential enantiomer selectivity of CDots for various applications. Inspired by nature's understanding of stereochemistry, we tested the chiral CDots against different classes of bacteria to investigate their potential ability to distinguish between chiral and non-chiral CDots, as well as left-handed and right-handed CDots. Amino acids served as the primary chiral source in this system, as they are a fundamental building block for most bacteria. Therefore, we also anticipated observing potential antibacterial effects from amino acid-based CDots.

1.5 Project Objectives

Following a previous study in our group⁸², this work focuses on the development of chiral CDots through the transfer of chirality of the amino acid, proline, to CDots by carefully studying the synthesis conditions to elucidate their impact on the chiral outcomes of the dots. Proline is known to play a significant role in the innate immune system by facilitating the production of antimicrobial peptides that effectively penetrate bacterial membranes and inhibit their growth by targeting fundamental intracellular processes⁹². In addition to the potential antibacterial properties of proline that could be enhanced at the nanoscale, CDots have also exhibited slight antibacterial activity through non-specific mechanisms of generating reactive oxygen species⁹³. Given this knowledge, we synthesized chiral CDots using proline as the chiral precursor and citric acid as the

co-carbon source through a microwave reactor. We conducted comprehensive characterizations on the samples to evaluate their properties, including size distribution and morphology using TEM, surface chemical composition using FTIR and XPS, optical properties using UV-Vis and fluorescence spectroscopies, chiroptical activity using CD, crystalline structure using XRD, and thermal stability using TGA.

In addition to material synthesis and characterization, we conducted an advanced mathematical study on the effects of reaction conditions that could impact the chiral response of CDots using Design of Experiment (DOE) techniques. We developed a prediction model that could help us identify the best combination of parameters for maximizing the preservation of residual chiral signal from CDots. Furthermore, this work extended beyond material characterization and statistical analysis to biological studies of chiral CDots against several classes of gram-positive and gram-negative bacteria strains including *E. coli* ATCC 25922, *B. subtilis* ATCC 6051, *S. aureus* ATCC 29213, *P. aeruginosa* 113951, and *S. aureus* CANWARD 115852. Subsequently, MIC and time-kill assays were performed to gain a better understanding of the anti-bacterial properties of the chiral CDots and the effect of chirality in their mechanism of action, in addition to their enantioselectivity.

Chapter 2. Technical Introduction and Background

2.1 The Evolution of Design of Experiment (DOE)

Thales of Miletus, commonly known as the "father of science," is credited as the first individual to explain the workings of the universe based on empirical observations of tangible objects, rather than relying on the prevalent mythological narratives of the time. Building on Thales' approach, scientists have continued to emphasize the importance of experimental techniques and systematic data collection to support their observations and hypotheses over the past two millennia. However, the most formidable challenge beyond formulating a testable hypothesis has been the design and control of experimental conditions to achieve a desired outcome. While the primacy of different approaches in "scientific method" is highly controversial, design of experiment (DOE) introduced by the British polymath, Sir Ronald Aylmer Fisher in the early 20th century attracted significant attention ⁹⁴. Sir Fisher is widely acknowledged as the pioneer of contemporary experimental design and statistics. In his book "The Design of Experiments," published in 1935 ⁹⁵, Fisher introduced the concept of design of experiments, which deviated from the conventional trial-and-error approach to experimentation that was common at the time. Fisher asserted that a well-designed experiment should systematically test multiple variables while controlling for extraneous factors that could influence the outcome. He suggested using statistical methods to analyze the experimental data and draw meaningful conclusions. Fisher proposed the use of randomization, replication, and blocking to provide a scientific framework for designing experiments that would yield reliable and reproducible results. His contributions have been highly significant and have had a long-lasting impact on the field of experimental design and statistics. DOE is now recognized as a statistical approach to plan, implement, examine, and interpret experiments with the objective

of identifying the factors that have a significant impact on a particular response variable of interest, while at the same time reducing the number of experimental runs required ⁹⁶.

2.2 The Fundamental Principles of DOE

The fundamental principles of DOE are used to design experiments that are efficient, effective, and provide meaningful results that can be analyzed and interpreted using statistical methods, thereby enhancing the efficiency of the experimental approach. These principles often include randomization, replication, blocking, and factorial experimentation ⁹⁷.

2.2.1 Randomization

Randomization is a technique used to mitigate the potential impact of unknown biases that could distort the results of an experiment. The random allocation of conditions to experimental units, to ensure that each unit has an equal chance of receiving any particular condition. An instance of bias in an experiment is the occurrence of instrument drift when comparing a baseline procedure to a new procedure. If all the tests using the baseline procedure are performed prior to those using the new procedure, the observed difference between the procedures may be wholly attributed to the instrument drift. To avoid drawing erroneous conclusions, the sequence of testing the baseline and new procedures should be randomized, such as conducting tests in a random order of B, A, A, B, A, B, etc. This would enable the instrument drift or any unidentified bias to average out.

2.2.2 Replication

Replication is the process of repeating an experiment to take into account the variability in the outcomes. This procedure is essential for improving an experiment's precision and accuracy, especially when the sources of variability are uncontrollable nuisance variables. Nuisance factors

are extraneous variables that may impact the outcome of an experiment but are not the primary focus of the researcher. These factors can result in variability and bias, which can impede the researcher's ability to draw sound conclusions about the effect of the primary variable of interest. Examples of nuisance factors include extraneous environmental conditions, measurement error, and individual differences among subjects or objects studied. Adequate management of nuisance factors is imperative in experimental design to ensure that the results are both reliable and valid. Replication serves to improve the signal-to-noise ratio in situations where uncontrollable nuisance variables introduce noises. This technique is an exact duplication of the experimental conditions from the outset. When certain factors prove challenging to manipulate, a specialized design referred to as a Split Plot can be employed. A split-plot design is an experimental design utilized when changing some of the factors of interest in an experiment is difficult or impractical. This design involves dividing the experimental units into smaller units, where each subunit is randomly assigned to a different level of the hard-to-change factor. This enables researchers to examine the effects of both the hard-to-change factor and other factors of interest on the response variable, while minimizing the impact of the hard-to-change factor on the experimental error. Split-plot designs are frequently employed in agriculture and other fields where experimental factors may be challenging to manipulate or where spatial or temporal dependencies exist in the data^{98–100}.

2.2.3 Blocking

Blocking is a method employed to enhance the precision of experiments by eliminating the influence of known nuisance factors. These factors, such as batch-to-batch variability, can be a source of error in experiments. A blocked design is implemented by applying both the new and baseline procedures to samples of materials from one batch, followed by samples from another batch, and so forth. The impact of the batch-to-batch differences is eliminated, and the difference

between the new and baseline procedures is not influenced by these differences. Although blocking limits complete randomization, it improves precision by removing batch-to-batch variability from the experimental error.

2.2.4 Factorial Experimentation

Factorial experimentation is a statistical method used to estimate the effects of individual factors and their combinations. This type of experiment involves variations of all factors, which are geometrically constructed in a factorial design. Data is collected at the vertices of a ρ -dimensional cube, where ρ represents the number of factors studied. A full factorial design requires 2^ρ runs to collect data from all vertices. However, due to the exponential increase in combinations with the number of factors, fractional factorial designs can be constructed to reduce the number of runs required. As the number of factors in an experiment increase, the size of the fractions used in fractional factorial designs becomes smaller and smaller, such as $1/2$, $1/4$, $1/8$, $1/16$, and so on. Fractional factorial designs involve collecting data from a specific subset of all possible vertices and require $2^{\rho-q}$ runs, where 2^q represents the fractional size of the design. For instance, in an experiment with only 3 factors, a full factorial experiment requires 8 runs, whereas a one-half fractional factorial experiment (represented by an inscribed tetrahedron) requires 4 runs (**Fig 2.1**). This geometric arrangement of experimental design is a crucial consideration in the efficient use of resources in the design of experiments.

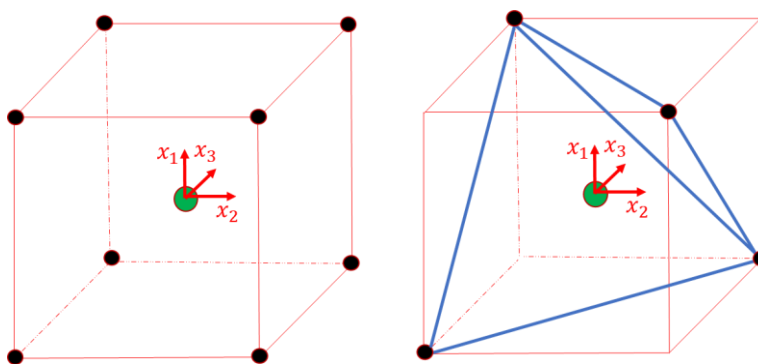


Fig 2.1 Schematic illustration of full factorial (left) and fractional factorial (right) for three variables. Black dots represent the number of experiments, and the dimension of the cube corresponds to the number of variables.

2.3 Classifications of DOE

The design of experiments typically encompasses various general and commonly adopted methodologies that are classified into five distinct categories: one-factor-at-a-time, factorial design, Plackett-Burman design, Taguchi orthogonal array, and response surface methodology (RSM). These approaches aim to describe and explain information variability under hypothesized conditions that reflect such variation.

2.3.1 One-Factor-At-A-Time (OFAT)

During the synthesis of a new compound, a chemist must make critical decisions that determine the outcome of the reaction. While laboratory skills are essential for performing efficient experiments with minimal operational errors, it is equally important to comprehend the conditions that enable the operator to control and predict the desired outcome. Chemists frequently use the one-factor-at-a-time (OFAT) approach to identify the effective parameters that could potentially be the determining factors in a reaction. This approach entails varying only one factor in the

reaction process while keeping all other factors constant and repeating the process for all factors, respectively as shown in **Fig 2.2**.

OFAT may be useful in identifying the concept of cause and effect in a reaction or presenting an introductory way to practice laboratory skills for new studies. However, it is not a good strategy for optimization. This is mainly due to its inability to account for potential interactions between factors. Accordingly, it is possible that optimizing each factor individually through OFAT may not result in the optimum combination of factors. Therefore, it may not provide the most desirable outcomes for optimization, particularly when interactions between factors are unknown or not considered. Also, it is worth noting that the choice of initial guess for parameters that have not yet been studied could affect each optimization operation using the OFAT approach. Nevertheless, such parameters are required for the sake of the procedure, and their selected values can have a considerable impact on the outcome of the optimization.

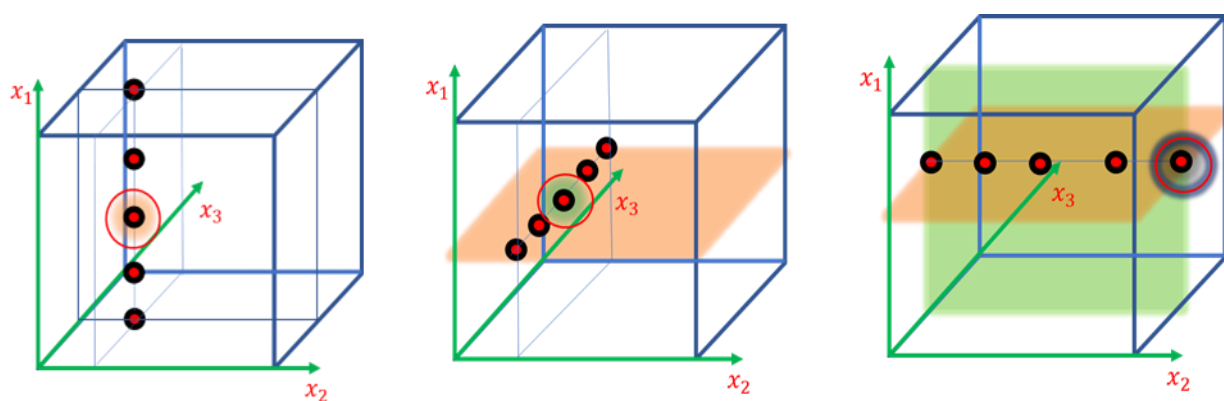


Fig 2.2 Schematic illustration of OFAT design for the optimization of a reaction with three variables (x_1 , x_2 , and x_3) showcasing the lack of consideration for the interactions of the variables at each step.

2.3.2 Factorial Design

In contrast to the OFAT approach, factorial designs seek to maximize the amount of information obtained from the experimental runs. By altering more than one factor simultaneously, factorial designs can capture potential interactions between factors, thus enabling more efficient and effective optimization. This method also assists in identifying crucial factors that have a significant impact on the reaction outcome, allowing researchers to concentrate on them and further optimize them. Factorial designs, including fractional factorials, are also known to increase the precision of other types of designs as they incorporate internal replication. The effects of each factor are calculated as the difference between the averages of all runs at two levels of a factor, such as "high" and "low".

Despite the apparent lack of replication in a factorial design, half of the data points are collected at high levels and the other half at low levels of each factor, resulting in a significant number of replicates. Furthermore, the design provides replication by incorporating factors with nonsignificant effects. Since each factor is varied with respect to all other factors, information on all factors is collected in each run. Every data point is utilized multiple times in the analysis and estimation of every effect and interaction. The two-level factorial design also has added efficiency as it spans the factor space, placing half of the design points at each end of the range, providing the most powerful way of determining the significance of a factor's effect. However, factorial designs can present a challenge due to their tendency to become intricate to analyze, especially when an abundance of factors is involved. This is because the higher the number of factors, the greater the number of experimental runs necessary to encapsulate all conceivable combinations of factor levels, resulting in potential escalations in experimental time and cost. Furthermore, interactions between factors can get difficult to comprehend and may require specialized statistical

techniques for analysis. Nonetheless, these challenges can be alleviated through other experimental designs and the application of suitable statistical tools.

2.3.3 Plackett-Burman Design

The Plackett-Burman technique is a statistical experimental design utilized to screen a large number of variables within a relatively small number of experiments. Its application is widespread in industrial research and development for determining the most critical factors that influence a process or product. Under the Plackett-Burman approach, the variables of interest are categorized into two levels, namely high and low. The construction of the design matrix ensures that each variable appears in half of the experiments at its high level and the other half at its low level. This optimization seeks to reduce the correlation between the variables and maximize the number of variables that can be screened with minimal experiments to investigate the significance of their effect on the desired outcome. Upon completion of the experiments, the data undergoes statistical analysis to recognize the most notable variables that impact the outcome of interest. This procedure is also referred to as the screening process, wherein the factors are subsequently subjected to a comprehensive analysis through techniques such as response surface methodology (RSM) to further optimize the process or product ¹⁰¹.

2.3.4 Taguchi Orthogonal Array

Taguchi orthogonal array is a type of experimental design that is highly fractional and is commonly utilized to estimate main effects with a limited number of experimental runs. This design is not restricted solely to two-level factorial experiments but can also be used to investigate main effects in experiments with factors that have more than two levels. Moreover, designs are also available to analyze main effects for certain mixed-level experiments, in which the factors being studied

have differing numbers of levels. The Taguchi method necessitates the definition of a quality characteristic in relation to an ideal or target value. Fixed targets for any quality characteristic can be categorized into three types: the closer to a nominal value, the better; the larger, the better; and the smaller, the better. A classic illustration of the effectiveness of Taguchi designs can be observed in the operations of the Ina Tile Company of Japan during the 1950s. The company faced the challenge of producing a large number of tiles that did not conform to the prescribed dimensions. Upon investigation, the quality control team discovered that the temperature fluctuations in the kiln employed for baking the tiles were the root cause of non-uniform tile dimensions. Given that building a new kiln was prohibitively expensive, temperature was deemed a noise factor. By employing Taguchi designed experiments, the team determined that elevating the lime content of the clay, a control factor, made the tiles more resilient or robust to temperature fluctuations in the kiln, enabling the production of uniform tiles ¹⁰².

Although the Taguchi orthogonal array was originally developed for use in manufacturing, its applications have expanded to various fields of science. As an example, Anıl *et al.* ¹⁰³ presents a simple, rapid, and reliable solid-phase microextraction (SPME) method coupled with gas chromatography-mass spectrometry (GC-MS) for the quantitative determination of 16 polycyclic aromatic hydrocarbons (PAHs) in water. To optimize the extraction conditions of PAHs using the SPME method, the Taguchi experimental design was utilized to obtain highly enriched analytes, followed by quantitative determination of PAHs in water using GC-MS. The study identified the critical design factors were extraction time, stirring speed, and temperature for the enrichment of all 16 PAH species from aqueous samples.

2.3.5 Response Surface Methodology (RSM)

The Response Surface Methodologies (RSM) were initially introduced by George E.P. Box and K.B. Wilson in the early 1950s ¹⁰⁴. Their aim was to provide an efficient way to maximize desired production through a sequence of experiments. The Response Surface Methodology (RSM) was originally developed to simulate experimental responses as stated by Box and Draper in 1987 ¹⁰⁵, and subsequently adapted for modeling numerical experiments. The distinction between the two lies in the nature of the error generated by the response. In physical experiments, imprecision may result from measurement errors, whereas in computer experiments, numerical noise can occur due to incomplete convergence of iterative processes, round-off errors, or the discretization of continuous physical phenomena, as indicated by Giunta *et al.* ¹⁰⁶, and Toropov *et al.* ¹⁰⁷. RSM assumes that the errors are random.

The RSM techniques are typically categorized into two main types known as Central Composite Design (CCD) and Box-Behnken Design (BBD), with a few sub-categories. The primary difference between CCD and BBD is that the former can go beyond the defined range for each factor by using five levels, while the latter uses three levels but always stays within the range. Therefore, CCD provides a more comprehensive observation of the set of data, but on the other hand, the number of experiments required is also higher compared to BBD with the risk of introducing more noises. The selection of the experimental design could impact the precision of the approximation and the expenses incurred in constructing the response surface.

The graphical representation of the response surface can be achieved by depicting it in three-dimensional space or as contour plots, which aid in visualizing the shape of the response surface. Contours refer to curves of constant response that are drawn in the x_1, x_2 plane while keeping all

other variables constant. Each contour corresponds to a specific height of the response surface, as shown in **Fig 2.3**.

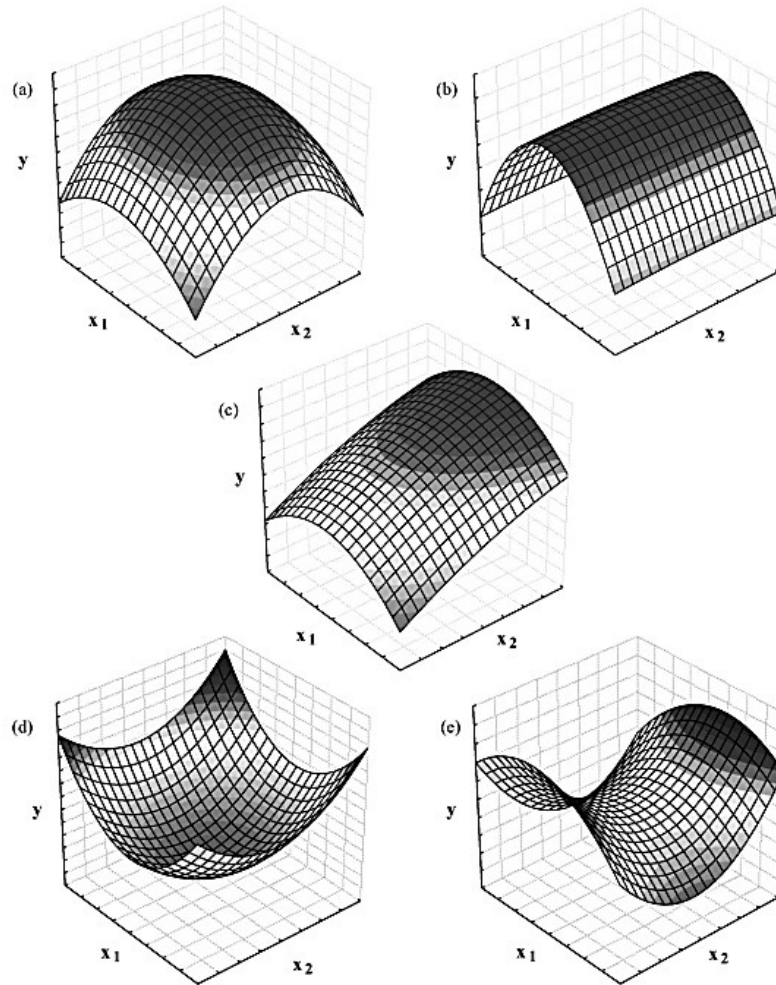


Fig 2.3 Surface response profiles resulting from a quadratic model for optimizing two variables: (a) maximum response, (b) plateau response, (c) maximum response outside of the experimental region, (d) minimum response, and (e) saddle surfaces. Reproduced with permission ¹⁰⁸.

2.4 Mathematical Concepts and Terminologies in DOE

In the context of DOE, numerous terminologies and mathematical concepts exist which facilitate the interpretation of analysis. The reaction parameters are factors that are often referred to as the

input variables or independent variables while the corresponding response is the dependent variable. Levels are the specific values or setting of a factor that is chosen for an experiment. Accordingly, high and low levels are the maximum and minimum values for the particular factor. DOE employs a polynomial equation as a mathematical model to portray the connection between input factors and output response. This equation is a polynomial function utilized to predict the response value at various combinations of factor levels due to their flexibility and capacity to approximate a wide range of response surface shapes. The general expression for a polynomial equation can be represented as:

$$Y = \alpha_0 + \sum_{i=1}^p \alpha_i X_i + \sum_{i=1}^p \sum_{j=1}^p \alpha_{ij} X_i X_j + \sum_{i=1}^p \sum_{j=1}^p \sum_{k=1}^p \alpha_{ijk} X_i X_j X_k + \dots \quad (2.1)$$

Where the α_0 represent the intercept for p input factors $(X_i, X_j, \dots, X_p)$ and Y stands for the desired response or the dependent variable. Typically, two values, referred to as levels, are designated for each factor in an experiment, one denoted as high and the other as low. These levels are coded as $+1$ and -1 , respectively. In order to detect observable differences in the response variable while remaining within the expected operational range, it is generally advisable to set the factor ranges at an appropriate distance apart, without exceeding reasonable limits. While the use of only two levels may suggest linearity in effects, the assumption of monotonicity, or a nearly monotonic relationship, is adequate for the response variable. However, at least three levels of the factors are necessary to detect curvature. A monotonic relationship is a functional relationship between input factors and response variables wherein the direction of change in the response variable is consistent with the direction of change in the input factors. In a monotonic relationship, as one factor increases, the response variable will either consistently increase or decrease. It is important to note, however, that not all relationships between input factors and response variables are monotonic. In

certain cases, the relationship may be non-monotonic, where the response variable first increases and then decreases (or vice versa) as the value of the input factor changes.

Interaction occurs when the effect of one factor on the response variable is dependent on the level of another factor. This phenomenon is visually represented as two nonparallel lines on a graph that plots the averages from four combinations of high and low levels of two factors. The α_{ij} term in **Eq 2.1** account for two-way interaction, which can be regarded as corrections to a model of simple additivity of the factor effects, i.e., the model with only the α_i terms in **Eq 2.1**. The assumption of a simple additive model presumes that the factors have separate and independent effects on the response variable, which is not always a reasonable assumption.

Analysis of Variance (ANOVA) is a statistical method utilized to evaluate and assess the statistical significance of differences in the mean response values among various factor combinations. A significance level of 0.05 is commonly used to test the significance of a term or the chosen model at a 95% confidence level. Multiple polynomial models, including linear, quadratic, cubic, and 2FI are often applied with appropriate modifications of terms to identify the best regression model for the given dataset. In addition to a low p -value (<0.05), the high value of lack-of-fit and F -value provide information about the influence of each term in the model. For the model to be accepted for optimization, it should have a significant p -value and an insignificant lack-of-fit.

To assess the adequacy of the model, both adjusted and predicted R-squared values are recommended to be at least 0.6 and as close to 1 as possible. Moreover, the coefficient of variation (CV%) is also a statistical metric used in DOE that quantifies the level of variability in a given data set relative to its average (Mean). CV% is a dimensionless quantity that is expressed as a percentage to compare the variability among different data sets. The calculation of CV% involves the ratio of the standard deviation (SD) to the Mean of the data set, multiplied by 100%. The use

of CV% in DOE is common to compare the variability among different treatments or experimental conditions. For instance, if two treatments have the same Mean but different standard deviations, the treatment with the higher standard deviation will have a higher CV%, indicating higher variability. Moreover, the coefficient of variation can also be used to evaluate the precision of measurement instruments or experimental procedures. In this context, a CV% of less than 10% is often desirable. Furthermore, the concept of adequate precision in DOE refers to the signal-to-noise ratio and is also used to compare the range of predicted values at the design points with the average prediction error. A value of greater than 4 is indicative of adequate model discrimination.

2.5 Implementation of DOE

To achieve an efficient experimental design, it is advisable to follow a systematic step-by-step procedure. The initial step involves defining the problem and establishing clear objectives. This entails identifying the problem that requires resolution and establishing the objectives of the experiment. Additionally, it involves determining the response variable to be measured, as well as the factors and their corresponding levels that may influence the response variable. Once the problem and objectives have been clearly defined, the next step in experimental design involves selecting an appropriate experimental design that is best suited to address the problem and achieve the objectives. This entails deciding on the number of runs or trials required to obtain reliable results, as well as determining the ranges and levels of the factors to be studied. After finalizing the experimental design, the next step is to conduct the experiment. This involves carrying out the experimental runs in accordance with the planned design, while meticulously recording the corresponding factor levels and response variables for each run. Following the experimental runs, the acquired data must undergo thorough analysis to estimate the effects of the factors on the response variable using statistical methods. This analysis involves assessing the significance of the

effects, and identifying any potential interactions that may exist between the factors and the response variable. After analyzing the data, the obtained results can be used to optimize the process or system under investigation. By utilizing the statistical findings, the optimal factor settings that produce the desired response can be determined, taking into account any constraints that may be present. To ensure the accuracy and reliability of the results, it is important to validate the findings of the experiment. This can be achieved by running confirmatory experiments or conducting additional data analysis to verify the initial results. After determining the optimal factor settings that produce the desired response, the final step is to implement these changes into the process or system being studied to facilitate achieving the desired outcome.

2.5.1 Implementation of DOE For CDots

In recent years, several investigations have employed Response Surface Methodology (RSM) to optimize the parameters affecting the synthesis of CDots. In 2018, Nemati *et al.*¹⁰⁹ proposed the use of CDots as a straightforward and cost-effective compound for detecting trace amounts of caffeine in food samples. CDots were synthesized through a microwave-assisted reaction between citric acid and sodium thiosulfate. To comprehensively evaluate the effects of environmental factors on the fluorescence sensitivity of CDots, the researchers conducted their study by DOE-RSM technique based on central composite design (CCD). The study examined four input variables, namely incubation time, pH, temperature, and the concentration of CDots, to evaluate their influence on the fluorescence enhancement ratio ($F-F_0/F_0$). The application of CCD in this study facilitated the determination of the detection range of CDots in the presence of various environmental factors that influence their sensing capability. While an optimum concentration of 0.7 mg/ml of CDots was shown to be suitable for the sensing application to avoid self-quenching, the incubation time of 90 seconds yielded the highest fluorescence enhancement ratio for their

CDots. Apart from this study, several other research groups have also investigated the sensing and catalytic activity of CDots using DOE techniques ^{110,111}.

To investigate the effect of reaction conditions on the photoluminescence properties of CDots, Karakoçak *et al.* ¹¹² reported the optimal reaction conditions that yield red-emissive CDots. The synthesis process considered three input variables, namely time, molar ratio of amine to acid, and concentration of citric acid, to investigate their effects on the photoluminescence properties of CDots through a microwave-assisted reaction of citric acid and ethylenediamine. The results indicated that the concentration of citric acid did not exert any significant influence on the response of the CDots. Conversely, the reaction time and the molar ratio of amine to acid were found to have a significant impact on the quantum yield (QY) of the CDots. The authors determined an optimal molar ratio of 0.75, which was found to be in close proximity to values reported in previous studies conducted using the OFAT methodology. Nonetheless, by incorporating a predictive model derived from DOE optimization, this study enables manipulation of reaction conditions by modifying one factor within a predetermined optimum range, while maintaining a consistently high photoluminescence intensity through adjustments to another factor with the aid of the proposed equation. This capability empowers the manipulation of reaction conditions to meet specific requirements or constraints that would not be achievable using traditional one-factor-at-a-time methodologies.

The implementation of DOE was not solely limited to the Central Composite Design (CCD) technique for CDots. In 2019, Cruz *et al.* ¹¹³ employed Box-Behnken design (BBD) to optimize the synthesis of CDots derived from polyurethane. Their findings revealed that temperature was the most influential factor on their QY among the three variables of time, temperature, and concentration of H₂O₄. Their observations were in consistent with the initial DOE study conducted

on CDots in 2015 ¹¹⁴ using CCD technique that further confirms the reliability of the different techniques in RSM. Subsequently, in 2022, Wang *et al.* ¹¹⁵ synthesized N, S-codoped CDots from citric acid and D-Penicillamine (DPA). Microwave power, time, and the molar ratio of DPA/CA were selected as the influential factors. The findings indicated that time and microwave power exerted the most substantial impact on the quantum yield of the CDots. Ultimately, QY of 91.55% was experimentally attained under optimal conditions, which closely approximated the value estimated via RSM.

Similar observations have been reported by other studies that investigated the impact of reaction time on the formation of CDots. According to Victoria *et al.*, ⁸² the surface of cysteine-citric acid CDots displayed a reduction in free thiol groups and an increase in the amount of C=C/C-C as the reaction time increased. The author attributed this to the consumption of precursors over extended reaction times, resulting in the formation of a well-hybridized core in the CDots. While the present study was carried out using the OFAT methodology and yielded comparable results to those obtained through RSM studies, the latter approaches provided a more comprehensive evaluation of parameter interactions and the significance of their effects. The similarity between the studies using the OFAT and RSM techniques could further prove that the majority of times, the DOE model is able to predict a particular response by using less amounts of experiments compared to OFAT with the advantage of having a reliable mathematical model for future purposes.

Although OFAT can aid in the initial observation of a system, material specialists always recommend utilizing 2-level screening techniques such as factorial and Plackett-Burman designs before proceeding with the optimization. This way, it is possible to determine the significance of each parameter and eliminate unwanted parameters that may appear effective in theory or demonstrate misleading significance by the OFAT technique. In conclusion, the use of RSM

techniques in conjunction with factorial experimentations enables scientists to focus not only on a single optimum point or specific level for their operational parameters but also on a range of variability while maintaining the optimal value for their product. In the case of CDots, numerous studies have attempted to understand the exact mechanism for their formation, particularly in bottom-up synthesis methods^{116–119}. Altering reaction parameters throughout this process not only facilitates control over the final properties of CDots but also sheds light on the underlying mechanisms for their formation. Therefore, the utilization of the best statistical tools and techniques is encouraged for the purpose of advancing this field.

Chapter 3. A Sustainable One-Pot Synthesis of Chiral Carbon Dots Using Response Surface Methodology: Elucidating the Mechanistic Pathways through Biological Responses

3.1 Abstract

Chirality is undoubtedly fundamental to nature, given that the distinct interactions of optically active molecules in a chiral environment are crucial for a multitude of applications. Chiral nanomaterials such as carbon dots (CDots) have attracted considerable attention due to their synthetic tunability. The synthesis of CDots via a single-step microwave-assisted technique using relatively inexpensive and versatile molecules can be customized by adjusting reaction parameters, enabling the transfer of chiroptical properties from precursors to CDots while maintaining their active structure. While tunability can be achieved through bottom-up synthesis approaches, it is often time consuming and requires multiple experiments. Herein, chiral CDots were synthesized from L-/D- proline as the chiral precursor and citric acid as the co-carbon source via a single-step microwave-assisted reaction. The duration of the synthesis, the molar ratio of chiral precursor, and the temperature of the reaction were optimized using Response Surface Methodology- Box-Behnken design. We demonstrated that chiral CDots were able to inhibit the growth of bacteria more efficiently than the non-chiral counterparts against both gram-positive and gram-negative strains. Further evaluation of the mechanism of action for CDots suggested their antibiotic activities to be bactericidal resembling the activity of Polymyxin B in our study. Our findings shed light on the capacity of biological systems to discriminate between chiral and non-chiral based CDots and can inspire new ideas in antibiotic and smart surface research.

3.2 Introduction

Chirality is a critical factor in the recognition phenomenon between biologically active molecules and their targets, especially in the case of antibacterial molecules that bind to cellular targets to act against bacteria. The tremendous advancements made in structural biology over the past few decades have provided increasingly precise insights into the interactions between active molecules and their biological targets, revolutionizing the field of medicinal chemistry. These developments have been particularly significant in the realm of stereochemistry, greatly aiding medicinal chemists in the design of new drugs.

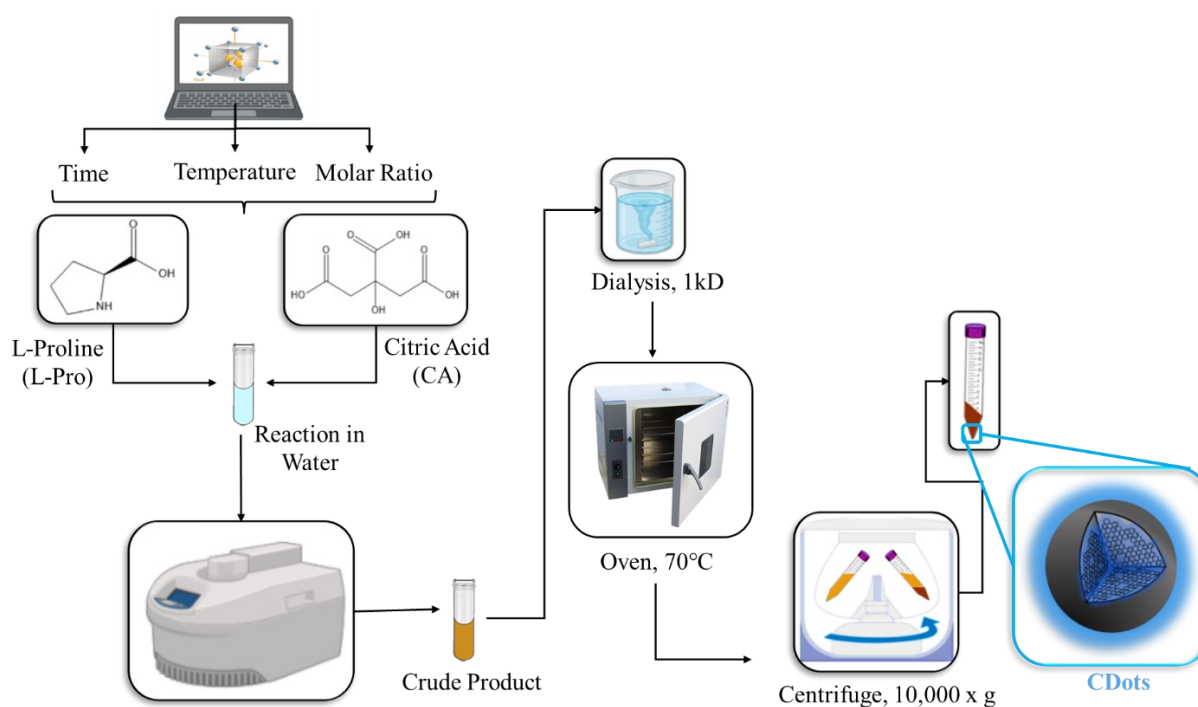
The advent of nanotechnology has brought the concept of incorporating chirality at the nanoscale to the forefront, as evidenced by its reported use in various forms, including nano-assemblies¹²⁰, nanocrystals¹²¹, and semiconductor quantum dots¹²² for a range of biological applications^{72,123}, sensing systems^{74,124}, and catalysis⁷⁰. Of particular interest for chirality at the nanoscale are carbon dots (CDots), which were introduced to the field in 2004 by Xu *et al.*¹³. CDots are photoluminescent carbon-based nanoparticles featuring tunable properties. Tunability of CDots could be defined as either chemical, optical or a combination of both when it comes to the chemical precursors and the conditions upon which the synthesis was performed. The vast range of chemical precursors that can be utilized for their synthesis has spurred numerous investigations into their potential in biosensing applications^{35,36}, cancer therapy^{37–39}, and anti-microbial systems^{40,41}. Recently, a novel approach to creating chiral CDots has emerged, whereby chiral molecular precursors are used to generate CDots with inherent chiral properties under hydrothermal conditions, without requiring additional post-synthesis modifications^{35,125–127}. This process occurs in the bottom-up synthesis of CDots, where reaction parameters are controlled to preserve or

transform some of the properties of precursors into CDots, known as active structure preservation in the literature ^{49,50,128}.

As this area of research progressed and more studies were conducted on chiral CDots, the transfer or induction of chirality from amino acids gained significant attention. Amino acids are a relatively inexpensive class of compounds that exhibit a broad range of chemical and physical characteristics, including hydrophobicity, hydrophilicity, and aromatic functional groups. Given their fundamental role as the building blocks of proteins and their capacity to act as signaling molecules in intercellular communication, amino acids have emerged as promising chiral precursors for the synthesis of CDots in biological systems ^{78,80,82,86}. Previous studies reported successful synthesis of chiral CDots from amino acids such as glutamic acid and methionine ⁸⁵, lysine ¹²⁹, alanine and aspartic acid ¹³⁰, tyrosine ¹³¹, and tryptophan ^{90,132}. However, limited studies have investigated the antibacterial effect of amino acid based CDots ⁸². Additionally, the extent to which the chiroptical properties of amino acids are retained and how it could potentially influence their antibacterial properties remains an area of limited investigation.

Proline is a non-polar amino acid that possesses a unique cyclic shape, distinguishing it from all other proteinogenic amino acids and is well-known for imparting structural rigidity to proteins. Also, proline has been found to be vital in numerous biological activities, such as protein folding, cell signaling, and the immune response ¹³³. Several studies have demonstrated that proline and its derivatives possess substantial antibacterial activity against a variety of bacterial strains ^{134–136}. In addition, the inactivity of short proline-rich peptides composed of D-amino acid residues suggests that an intracellular target biopolymer possessing chiral properties, such as a protein or nucleic acid, is likely to be present and effective on the antibacterial properties of proline ¹³⁷.

Hence, in this study, we synthesized chiral CDots from the mixture of L-/D-proline as the chiral precursor with citric acid as the co-carbon source through a single-step microwave-assisted reaction. In order to understand the effect of residual chiral centers on the surface of the CDots and investigate their potential enantiomer selectivity, we attempted to maximize the chiral signal of the synthesized CDots. Response Surface Methodology (RSM) was used for the first time to elucidate the reaction conditions (temperature, time, and molar ratio of precursors) that would result in the maximum preservation of chirality in CDots. Behnken Design (BBD) was selected as the experimental design in RSM as the main strategy to study the reaction parameters and to minimize the number of experimental runs while respecting their synergistic effect on the chiral signal. Finally, to explore the capacity of biological systems to differentiate between chiral and non-chiral CDots and to investigate the potential antibacterial properties of chiral CDots, we assessed the impact of these materials on various bacterial strains. MIC and time kill assays were performed both on chiral CDots synthesized from L- and D-proline and compared to controls of CDots bearing no chiral signal, but with the same chemical composition. Our findings have the potential to open new avenues for research and applications centered on the chiroptical properties of CDots.



Scheme 3.1 Microwave-assisted synthesis of L-Pro.CA CDots. The effect of three reaction parameters (time, temperature, and molar ratio) was evaluated on the chiral signal of L-Pro.CA CDots via RSM-BBD.

3.3 Experimental Section

3.3.1 Materials

All reagents and solvents were used as is without further purification. L-proline (>98%) and citric acid anhydrous (>99.5%) were purchased from Sigma Aldrich. D-Proline (>98%) was purchased from TCI chemicals.

3.3.2 Experimental Design and Numerical Optimization

CDots were synthesized via a microwave-assisted reaction. The impact of three selected factors, namely reaction time, temperature, and molar ratio of proline to CA on the magnitude of chiral

signal was investigated via a Box-Behnken Design (BBD). **Table 3.1** Shows the high and low values (levels) for each independent factor along with the center (medium) levels used in generating the randomized sequence of experiments in BBD. Accordingly, the independent response (Y) was set as the integration of chiral signal measured in the Circular dichroism spectra within the range of 180-220nm for all samples. The chemical similarity of L-proline and D-proline prompted the optimization of chiral CDot synthesis exclusively for the L-enantiomer, which was subsequently designated as L-Pro.CA CDots. However, the optimum condition was later expanded to the synthesis of D-Pro.CA CDots to assess and evaluate enantioselectivity and antibacterial properties of both enantiomers.

Table 3.1 The independent factors and their dependent response

Independent factors	Low level (-1)	Center Point (0)	High level (+1)
(X ₁) Time	10min	17.5min	25min
(X ₂) Temperature	160°C	175°C	190°C
(X ₃) Molar Ratio	3: 1	5: 1	7: 1
Dependent Response:			
(Y) Chiral Signal Integration	To Maximize		

3.3.3 Synthesis of CDots

For each experimental run, the mixture of 0.5M citric acid (CA) was prepared in 10ml Milli-Q water with the corresponding molar ratio of L-proline in the experimental data set and sonicated to form a homogeneous solution. Subsequently, the solution was transferred into 35ml CEM microwave vials and heated at a predetermined time and temperature in the reactor as shown in **Scheme 3.1**. The crude product from the reaction was purified from the unreacted compounds via dialysis (Spectra/Por®6 RC – Spectrum Laboratories) for 2-3 days and dried in oven to remove the solvent. The dried powder was further subjected to solid-liquid extraction with a mixture of ethanol: acetone prepared at a ratio of 2:10 and centrifuged at $10,000 \times g$ for 10 minutes and dried overnight to remove the residue of solvents. The pellets were further collected and used for characterization as the L-Pro.CA CDots.

3.3.4 Characterization of CDots

Transmission Electron Microscopy (TEM)- To obtain information about the overall morphology and the size distribution of CDots, transmission electron microscopy (TEM) was used. Dried CDots were dispersed in isopropanol/Milli-Q water (50:50, v/v) at the concentration of 1mg/ml and sonicated for 10-15 seconds. The solution was applied dropwise on the 300 Mesh Cupper (Cu-300HD) coated with holey/thin carbon films (Pacific Grid Tech) operating at 5kV.

UV-Visible Absorbance Spectroscopy- The UV-Vis absorption spectra were obtained using a Cary 5 series UV-Vis-NIR Spectrophotometer (Agilent Technologies) in the spectra range of 200-800nm. A 1cm quartz cuvette was used with wavelength changeover at 350nm and 5nm bandwidth.

Fluorescence Spectroscopy- The fluorescence emission was recorded using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). The concentration for CDots were adjusted to OD<0.1 to avoid inner filter effects. CDots were excited from λ_{250} to λ_{550} nm with 50nm intervals. The excitation and emission slits were both set to 5nm, and all data were processed using Agilent Cary Eclipse software package.

Circular Dichroism (CD)- The circular dichroism (CD) spectra were obtained using a JASCO J-815 spectropolarimeter in the spectra range of 180-400nm and in 3 accumulations. The scans were continues with the speed of 50nm min⁻¹ using a 1mm quartz cuvette. The data was analyzed using Spectra Manager II software.

Fourier Transform Infrared Spectroscopy-Attenuated Total Reflectance (FTIR-ATR)- To investigate the surface chemistry of CDots, the Fourier transform infrared spectra (FT-IR) were collected using a Thermo Scientific Nicolet iS5 equipped with an iD5 attenuated total reflectance (ATR) accessory. The spectra were obtained using 64 scans with resolution of 0.4 cm⁻¹, a gain of 1, an optical velocity of 0.4747 and an aperture setting of 100. The Omnic 9 software was used to process the data.

X-ray Photoelectron Spectroscopy (XPS)- The x-ray photoelectron spectroscopy (XPS) spectra were collected using a Thermo-Scientific K-alpha x-ray photoelectron spectrometer. Each analysis was performed on three different spots on the sample, in triplicates, with 5runs for each scan. The average for high resolution and survey was plotted.

X-ray Powder Diffraction (XRD)- To assess the crystallinity and structure of CDots, x-ray powder diffraction (PXRD) technique was used. The PXRD data were conducted on a Bruker D8 advance diffractometer equipped with a LYNXEYE linear position sensitive detector (Bruker AXS, Madison, WI). Neat samples were smeared directly onto the silicon wafer of a

proprietary low-zero background sample holder and data was collected in the 2θ -range of 10-90° in increments of 0.02°.

Thermogravimetric Analysis (TGA)- For evaluating the thermal stability of the samples, thermogravimetric analysis (TGA) was carried out using a TGA Q500 analyzer. Samples were heated from 25 to 1000 °C at a heating rate of 5 °C. min⁻¹ under an Argon atmosphere with a flow rate of 90 mL.min⁻¹.

Proton Nuclear Magnetic Resonance (1H NMR)- To observe the structural change in the chiral CDots versus the precursors, 1HNMR analysis was performed using a Bruker Fourier UltrashieldTM operating at 300 MHz. Sample analysis was carried out using the Mestrelab Mnova software. All samples were prepared in D₂O for analysis.

3.3.5 Chiral CDots in Bacterial Systems

3.3.5.1 Minimum Inhibitory Concentration (MIC)

Antimicrobial susceptibility against the CDots was measured by broth microdilution to determine the MIC of different Gram-negative and Gram-positive bacteria as per CLSI guidelines¹³⁸. In brief, 96-well polystyrene plates were used to create a concentration gradient with increasing two-fold concentrations of the antimicrobials tested. The bacterial strains tested were incubated overnight and diluted to the turbidity of a 0.5 McFarland standard, then further diluted 1:100 in MHB2 (Cation adjusted Muller-Hinton Broth). The plates were incubated overnight at 37°C, and growth or lack of growth was recorded as the presence or absence of a visible pellet after 16-20h of incubation.

3.3.5.2 Time-Kill Assays

Exponentially growing *E. coli* ATCC 29213 bacterial cells were inoculated in the tested compounds. A Magellan plate reader was used to record the absorbance. Optical Density at 595 nm was recorded every 5 minutes for approximately 22 hours. The effect of bacteriostatic, bactericidal and bacteriolytic antimicrobials was compared to the CDots to get an idea on the possible mechanism of action.

3.4 Results and Discussion

3.4.1 Synthesis and Optimization of CDots

Bottom-up approaches using small organic precursors to synthesize CDots have been employed extensively through microwave-assisted techniques to preserve some of the properties of precursors in CDots ¹³⁹. Citric acid a tricarboxylic acid, is quite prominent in bottom-up approaches of CDot preparation ^{140–142}. Enriching the surface of citric acid-based CDots with chiral properties requires precise tailoring of the conditions that favor preservation of the chiral centers during the reaction. It has also been demonstrated that the provision of heteroatoms through passivation represents a viable strategy for enhancing the reaction yield, a challenge that has been widely acknowledged in producing CDots ¹⁴³. Hence, proline, a chiral molecule, can serve not only as a chiral precursor, but also as a passivating agent to effectively enhance the reaction yield of CDots.

In this study, response surface methodology utilizing Box-Behnken design (BBD) was employed to model and optimize the reaction conditions. The parameters of time, temperature, and molar ratio of proline to CA were systematically varied to determine and model the reaction conditions. **Table 3.2** displays the results of the seventeen randomized experimental runs conducted using the BBD matrix with the corresponding integration of the chiral signal for the

synthesized CDots. Lower chiral responses were observed at higher temperatures that correspond to elevated decomposition of the reactants, especially the chiral precursor and its chiral centers. Run 15 shows the lowest value for the response (159.9-integrated area) at 190°C, 25min, and 5:1 Proline:CA ratio. Conversely, run 1 shows the highest response (547.6-integrated area) at 160°C, 17.5 min, and 3:1 Proline:CA ratio. Also, the chiral signal decreases as the ratio of chiral precursor increases from 3:1 to 7:1 that could indicate that the formation of chiral CDots is not necessarily following an enhancement by increasing the amount of enantiomer in the reaction. Instead, only limited regions, likely on the surface, can contribute to the observed chiral response and may reach saturation after a certain concentration of the enantiomer is achieved. To further investigate this observation, we also evaluated the effect of the molar ratios below 3:1 Proline:CA and no product was recovered at lower ratios, which could indicate the extremely low efficacy of the reaction at these concentrations.

Table 3.2 Experimental matrix for the synthesis of L-pro.CA CDots using BBD. Independent variables are X_1 = time, X_2 = temperature and X_3 = molar ratio of proline to CA and their dependent response is Y = Chiral integration.

Run	Factors			Response
	X_1	X_2	X_3	Y
1	17.5	160.0	3.0	547.6
2	10.0	175.0	3.0	528.6
3	17.5	175.0	5.0	411.8
4	17.5	160.0	7.0	410.5
5	10.0	175.0	7.0	305.2
6	17.5	175.0	5.0	360.9

7	25.0	175.0	7.0	310.0
8	17.5	190.0	3.0	307.6
9	17.5	175.0	5.0	423.7
10	17.5	175.0	5.0	393.7
11	25.0	160.0	5.0	444.5
12	10.0	160.0	5.0	420.7
13	10.0	190.0	5.0	349.5
14	17.5	190.0	7.0	243.3
15	25.0	190.0	5.0	159.9
16	17.5	175.0	5.0	400.4
17	25.0	175.0	3.0	321.0

Table A3.1 shows the result for the sequential model sum of squares according to the experimental runs. The quadratic model used for this experiment provides a higher order polynomial compared to a linear model and it is not aliased like a cubic model. Also, the *P*-value for the quadratic model (0.0059) is less than 0.05, which is within the significant range of this study. The adequacy of the quadratic model was evaluated with ANOVA as presented in **Table A3.2**. The significance of fitness of the model was evaluated by the very small amount for *P*-value (<0.0001) and the insignificance of the lack-of-fit (0.7373). Also, the *P*-value for each variable and its interactions should be significant to be included in the final model.

The final form of the quadratic coded model based on the estimation for coefficients of each significant term in the ANOVA table is displayed as **Eq 3.1**:

$$Y = 5.99 - 0.14X_2 - 0.15X_1 - 0.29X_3 - 0.21X_1X_3 + 0.13X_1X_2 - 0.11X_1^2 - 0.10X_2^2 \quad (\text{Eq 3.1})$$

Where X_1 , X_2 , and X_3 correspond to time, temperature, and molar ratio of L-proline: CA and the interactions between the main factors are represented by X_1X_3 and X_1X_2 . Additionally, X_1^2 and X_2^2 represent second-order terms for time and temperature, respectively. According to the equation, the coefficient for X_3 (0.14) in the model is the highest among the terms indicating that the highest influence among factors is coming from the variation of temperature. This is in accordance with previous studies showing the significance of temperature in the overall formation of CDots in addition to their chirality^{82,118,144,145}. Furthermore, the coefficients representing the interactions between time and temperature (X_1X_2), as well as the term for time and molar ratio (X_1X_3) in the model are 0.13, and 0.21, respectively. These values closely resemble the coefficients for the main factors such as time X_1 (0.15) and molar ratio X_2 (0.14). This suggests that the joint variations of these two parameters hold equal significance as the main factors on the residual chiral response.

Moreover, the values for the coefficient of determination (R-Squared= 0.98, Adj. R-Squared= 0.96, and Pred. R-Squared= 0.91) suggest a strong estimation between the quadratic model and the experimental data. The reliability of the model is also demonstrated in **Fig A3.1** as the actual data are approximately in accordance with the straight line showing the predicted amounts based on the estimation of the quadratic model. This estimation can further be validated by the confirmation test to evaluate the agreement of the model with the experimental data. Moreover, **Fig A3.2** depicts the residuals plotted against the ascending predicted response values, which demonstrate a random distribution within an acceptable range. This finding further affirms the

satisfactory nature of the residuals in the experimental data, thereby validating the ability of the model to fit the data.

The 3D plots were also generated for better demonstration of relations between the variables and the response. The effect of L-Pro:CA ratio and time on the chiral integration is shown **Fig 3.1a** which displays maximum chiral responses at lower molar ratio with a decrease in the response as the molar ratio increases. The bottom-up synthesis of CDots is based on the general assumption that prolonging the reaction will result in greater consumption of the reactants due to polymerization, which leads to the formation of well-hybridized CDots^{82,116,117}. Accordingly, the chiral response should have also decreased as the decomposition of the chiral centers takes place. Nevertheless, the variation in time appears to follow a curved pattern, whereby the chiral response initially increases with time. However, shortly after reaching the midpoint at approximately 17.5 minutes, it begins to decrease. This observation suggests that the surface of CDots may have a limited number of interaction sites available for the attachment of residual chiral centers. Moreover, increasing the concentration of CDots could lead to steric hindrance by the bulky structure, thereby potentially impeding the chiral signal. **Fig 3.1b** shows a similar trend for temperature, with an initial increase in chiral response observed up to approximately 167°C, followed by a subsequent decrease as the temperature reaches 190°C. In order to further clarify this phenomenon, experiments were conducted at lower temperatures below 160°C. However, as depicted in **Figure A3.3**, no formation of CDots was observed at temperatures below 160°C, even with 30-minute exposure time to microwave irradiation. Utilizing a shorter exposure time and lower reaction temperature to synthesize chiral CDots could confer a significant advantage to this system in terms of energy efficiency.

Therefore, to further characterize the samples, a number of conditions were selected as outlined in **Table 3.3** to perform confirmation experiments and compare the corresponding responses with the estimated responses of the model.

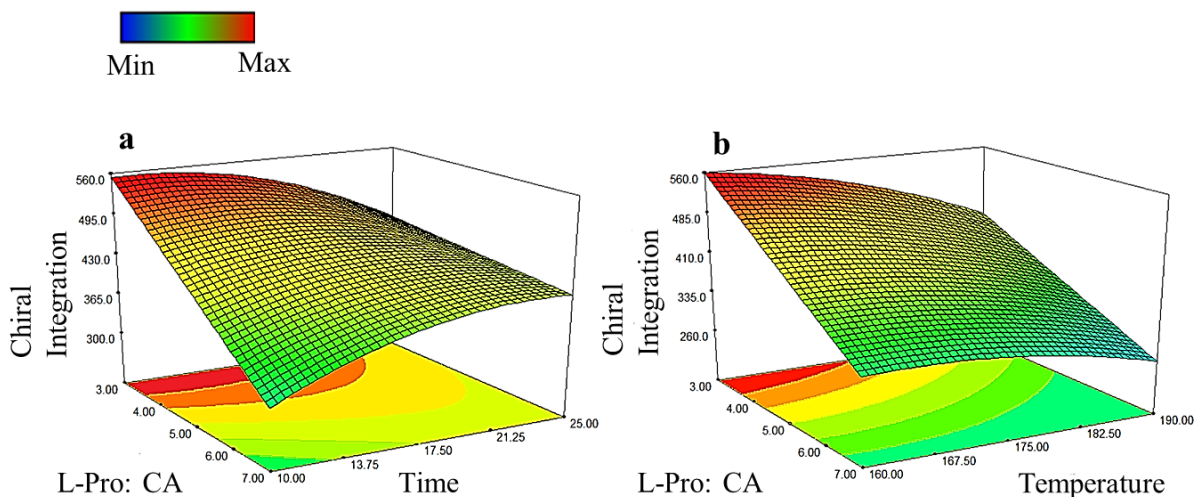


Fig 3.1 3D surface plots from optimization of chiral response using BBD showing functional relationship between the dependent response (chiral integration) and independent variables. L-Pro: CA and time (a) and L-Pro: CA and temperature (b).

Table 3.3 Results of confirmation experiments for L-Pro.CA CDots

Experiment	X ₁	X ₂	X ₃	Y _{Ex}	Y _M
	(Time/min)	(Temperature/°C)	(Molar ratio)	(Experimental)	(Model)
1	10	160	3	539.4	549.9
2	15	160	4	493.4	511.3
3	20	170	3	466.8	457.6
4	13	163	3	550.2	552.0
5	18	175	4	411.7	420.7

Based on the findings presented in **Table 3.3**, all experimental values exhibited a high degree of agreement with the estimated values derived from the model. This consistency between experimental and modeled values serves as a testament to the reliability of the model. In order to continue with the maximum chiral response observed in the study, additional characterization was performed on the sample from Experiment 4. The reaction yield for this particular sample was determined through the calculation of three independent replicates based on the mass of the precursors and the products with the value of 34 %. Based on previous research, CDot yields through microwave-assisted reactions typically falls within the range of 10-30% ¹⁴⁶.

3.4.2 Characterization of CDots

Chiral CDots (L-Pro.CA CDots) were synthesized through a microwave-assisted synthesis at the selected condition (163°C, 3:1 molar ratio, 13 min). Following each reaction, extensive purification was carried out to ensure removal of unreacted precursors. This was achieved through dialysis and solid-liquid extraction. NMR spectroscopy was utilized to determine whether the chemical groups present on the surface are chemically altered during the polymerization and carbonization processes. As illustrated in **Fig A3.4**, the most prominent dissimilarities between the NMR spectra of CDots and the reference samples are the enhanced peak broadening observed in CDots. Furthermore, the presence of numerous broad peaks in the range of 3.5 to 4.5 ppm in the CDots spectra indicates the existence of oxygen-containing functional groups, such as alcohols, esters, or carboxylic acids ^{147,148}.

We investigated the size and morphology of CDots using transmission electron microscopy (TEM). As shown in **Fig 3.2b**, CDots appeared quasi-spherical with an average size of 16.5 ± 3.5 nm and a Gaussian size distribution in the range of 8-28 nm. The optical properties of L-

Pro.CA CDots along with the precursors were characterized by UV-Visible and fluorescence spectroscopies. The appearance of the absorbance at ~ 250 nm is the characteristic of $\pi \rightarrow \pi^*$ transition attributed to the aromatic sp^2 domains, which coincides with region for the absorbance of the precursors. Additionally, the absorbance at ~ 350 nm in the UV-Vis spectra for CDots is often ascribed to $n \rightarrow \pi^*$ transition that arises due to the combination effect of the increase in O and N-doping of the structure. Furthermore, as shown in **Fig 3.2a**, the dispersion of chiral CDots in water showed a blue fluorescence with the maximum emission observed at 420 nm following excitation at 350 nm. We note that the precursors have no significant absorbance or fluorescence under these conditions.

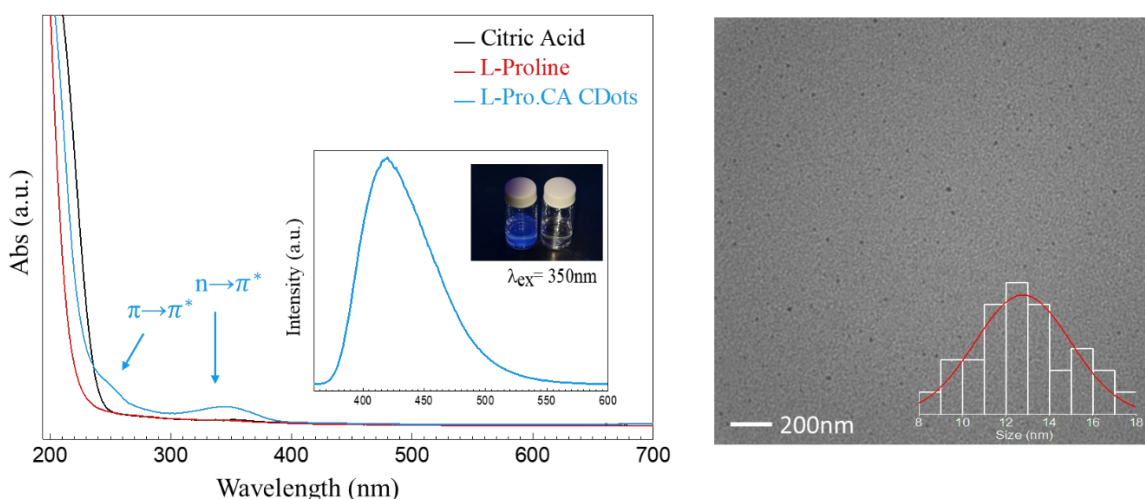


Fig 3.2 Optical characterization of L-Pro.CA CDots. UV-Vis spectra shows two peaks regarding the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions (a). The inset shows the fluorescence spectra of L-Pro.CA Cdots excited using $\lambda = 350$ nm with a blue emission centered at 420 nm. TEM images of L-Pro.CA Cdots (b).

Following absorbance and fluorescence analyses, circular dichroism was also performed on both the precursors and CDots to investigate the absorbance of circularly polarized light. Circular dichroism occurs when the left-handed and right-handed circularly polarized absorbance are

different giving a positive or negative signal in the spectra. Proline shows what is known as a coupled oscillator CD (or exciton couplet) when the two electric dipole transition moments from the two chromophores in the structure are coupled together. As shown in **Fig 3.2a**, the positive cotton effect (CE) observed for L-proline was diminished into a single negative absorbance for the L-Pro.CA CDots. This further confirms the formation of a new structure that could be the result of transforming some of the chromophores and their incorporation into the hybridized CDot system. The impact of concentration of the chiral precursor (L-proline) and L-Pro.CA CDots on the CD spectra was also evaluated. **Fig 3.2b** illustrates the CD spectra of L-proline exhibiting an intensity-dependent pattern based on the concentration of the amino acid. As the concentration changes from 10 mg/ml to 0.08 mg/ml, a decrease in intensity occurs with no significant alteration or shift in the profile. This further confirms that the exciton couplet of L-proline is not following a concentration-dependent pattern in the operating concentration but rather experiences a steady decrease in its signal. A comparable pattern is also observed in L-Pro.CA CDots with the variation of concentration from 1 mg/ml to 0.1 mg/ml showing a decline in the CD signal until it completely vanishes. The observed effect can be attributed to the Beer-Lambert law in absorption techniques, which establishes a correlation between sample absorption and its concentration. Nonetheless, the CD spectra of L-Pro.CA CDots, as illustrated in **Fig 3.2c**, exhibit a marked increase in absorbance, along with a noisy profile, at concentrations of 5mg/ml. The spectral region that exhibits this phenomenon has been attributed to the $\pi \rightarrow \pi^*$ transition of CDots, which is potentially impacted by the augmented conjugation and aromaticity within the CDots. This, in turn, can result in an increased absorption of CDots, leading to scattering and a reduced signal-to-noise ratio during measurements. To avoid this, the optimum concentration for samples was adjusted to 1mg/ml for all CD measurements.

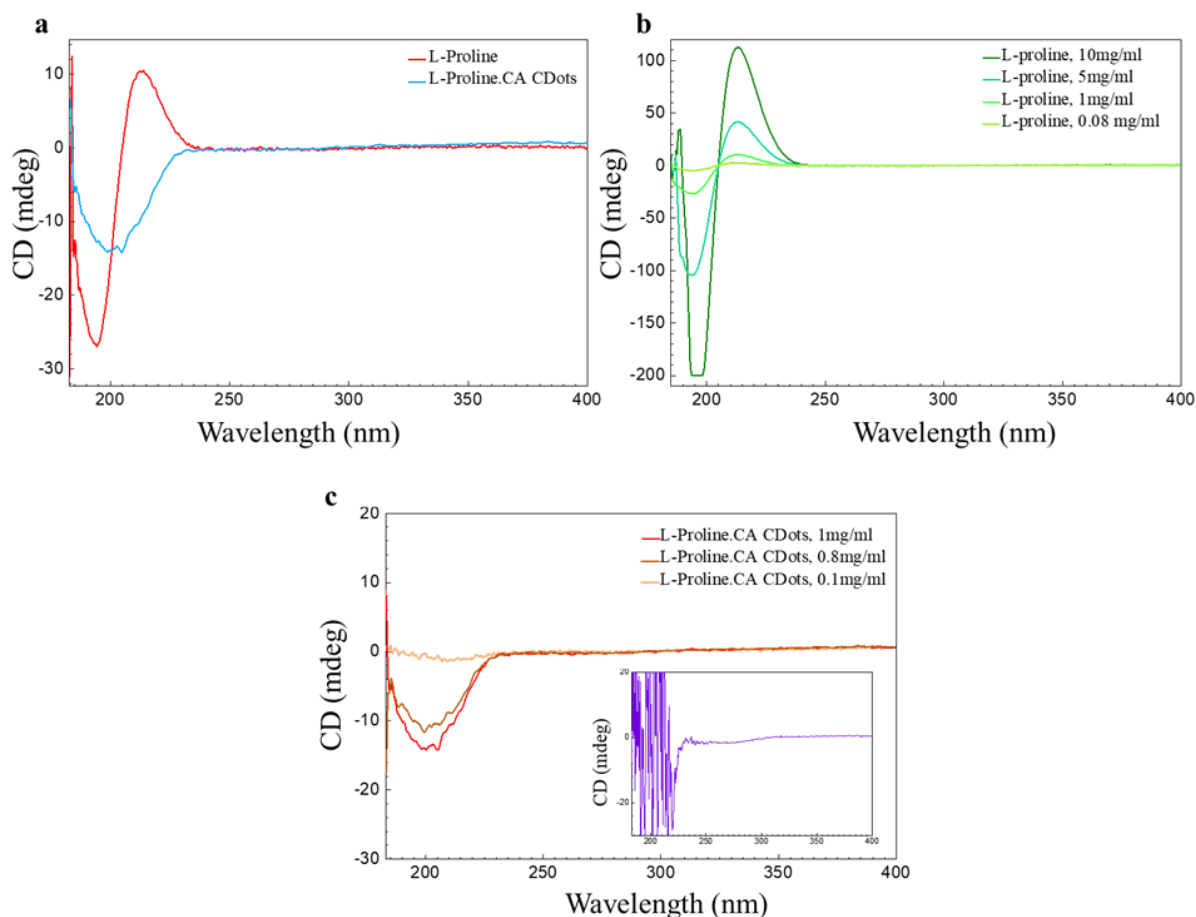


Fig 3.3 Circular dichroism (CD) spectra of L-Pro.CA CDots and L-proline (a), L-proline at varying concentrations (b), and L-Pro.CA CDots at varying concentration (c). The inset shows the CD spectra for L-Pro.CA CDots at 5mg/ml.

Subsequently, L-proline and citric acid were separately irradiated in the microwave following the same condition as the L-Pro.CA CDots. Although the circular dichroism (CD) profile of both L-proline and citric acid remained unchanged before and after irradiation, it was observed that CDots were not formed by using only L-proline as the only precursor (**Fig A3.5**). It is noteworthy to mention that the apparent reduction in the signal intensity of the amino acid upon heating may indicate the loss of certain chiral centers that could have potentially contributed to the formation of chiral CDots when combined with citric acid. Following this

observation, L-Pro.CA CDots were spiked with increasing concentration of amino acid to analyze the sensitivity of the CD profile to the presence of potential unreacted free amino acids in the solution. As shown in **Fig A3.6**, by increasing the concentration of L-proline from 5% v/v to 20% v/v, a noticeable shift occurs in the center of the absorbance from ~205 nm for the L-Pro.CA CDots without any additives to ~190 nm when containing 20% v/v free L-proline. Additionally, the CD spectra undergoes a transformation from a negative absorbance to a couplet, with a matching position as the CD profile of L-proline when analyzed independently. These observations provide further confirmation that the majority of L-proline molecules were involved in the CDots reaction, either through polymerization with the core or weak interactions and attachment to the surface. Consequently, the resulting CD signal originates mostly from CDots rather than from unattached or unreacted amino acids in the solution.

In order to study the chemical composition of CDots, Fourier transform infrared (FTIR) spectroscopy and X-ray photoelectron spectroscopies were used. **Fig 3.4** shows the FTIR spectra for the precursors (L-proline and CA) as well as the L-pro.CA CDots. The overall vibration profile of CDots exhibits major differences relative to the precursors. A notable difference is the broad band at 2500-3450 cm^{-1} observed for CDots, which is attributed to the O-H stretching of carboxylic acid and other oxygenated groups. The two distinct bands also present in the spectra of CDots at 1612 cm^{-1} and 1705 cm^{-1} along with the band at 1200 cm^{-1} are attributed to N-H bending of amides, C=O stretching of carboxylic acids, and C-O stretching of acyl groups, respectively. In addition, sp^3 C-H stretching and bending at 3000 cm^{-1} and 1400 cm^{-1} with the sp^2 C-H stretching at 2870 cm^{-1} confirms earlier studies on the sp^2 - sp^3 nature of CDs prepared from citric acid as the precursor^{82,149}.

XPS analysis was used to further expand our investigation of the surface composition of CDots. **Fig 3.4** shows the survey scan for L-Pro.CA CDots with prominent peaks at 530, 400, and 285 eV ascribed to the binding energies of O1s, N1s, and C1s, respectively. Deconvolution of the high-resolution scan of O1s state reveals two dominant peaks at 533, and 531.4 eV corresponding to C-OH/C-O-C and C=O. Also, the presence of Pyrrolic N/NH₂ and graphitic N is observed at 401.3 and 399.5 eV in the high-resolution scan of N1s. Finally, the deconvolution of C1s showed binding energies at 288.3, 285.6, and 284.5 eV that can be attributed to C-O, C-N/C=N, and C-C/C=C. These results are also in accordance with the previous studies on passivated citric acid based-CDots^{116,150,151}.

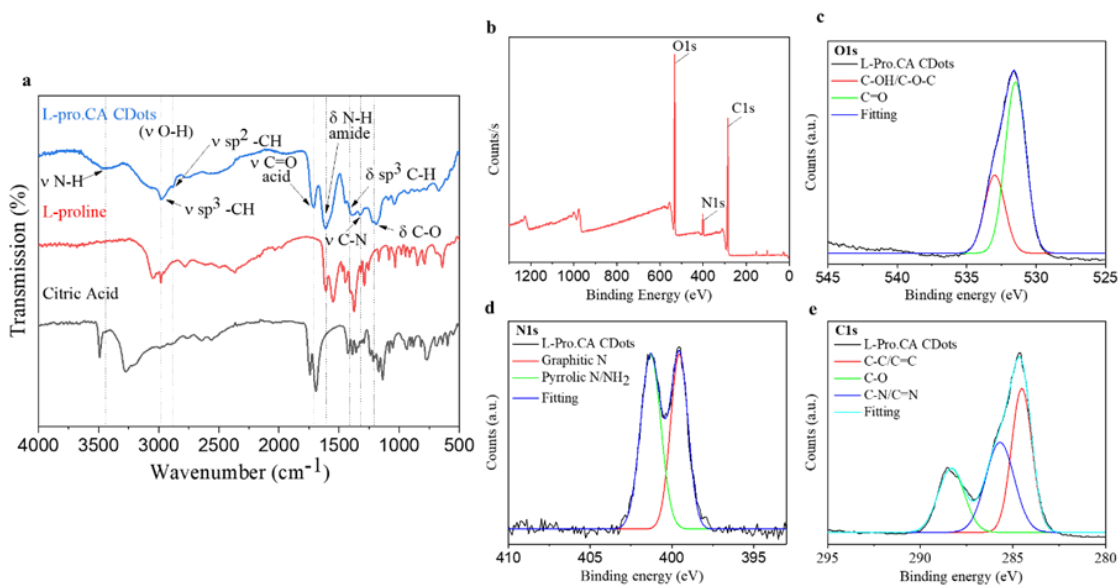


Fig 3.4 FTIR spectra of L-pro.CA CDots and the precursors showcasing the similarities and differences in the surface functional groups (a). Survey XPS scan of L-Pro.CA CDots illustrating the prominent elements in the structure (b). High resolution XPS scan for O1s (c), N1s (d), and C1s (e).

The changes to the structure of the CDots were also supported by XRD analysis (see **Fig A3.7**). The amorphous halo centered at $\sim 17^\circ 2\theta$ shown in the XRD pattern of the CDots is

attributed to the amorphous carbon as the result of thermal decomposition of precursors^{152–154}. Additionally, the L-pro.CA CDots were also evaluated for their thermal stability using TGA. As shown in **Fig A3.8**, The weight loss profiles of TGA were observed with increasing temperature, demonstrating that the chiral CDots consist of numerous functional groups and structures as evidenced by the multi-step weight loss. The primary loss of mass in the temperature range of 30-160 °C can be attributed to the elimination of residual moisture and solvent adsorbed on the surface after the process of synthesis and purification. This is followed by greater weight losses occurring between approximately 180-500 °C, which are associated with the decomposition of various functional groups on the surface. Typically, functional groups containing oxygen and nitrogen undergo decomposition at this temperature range. Specifically, oxygen-containing functional groups, such as carboxyl, lactone, and anhydride decompose at temperature of less than 500 °C, resulting in the release of CO₂¹⁵⁵. Furthermore, at temperatures exceeding 500 °C, the decomposition of phenols, ethers, and carbonyls occurs, which leads to the release of CO. The derivative curve revealed an oxidation temperature of approximately 270°C, signifying the thermal stability of the chiral CDots. The TGA profile and stability of the L-pro.CA CDots are similar to those of CDots with similar compositions reported in prior studies^{82,156}.

3.4.3 Chiral CDots in Bacterial Systems

The exact mechanism by which CDots are formed through bottom-up approaches is not yet fully understood. Nevertheless, it has been proposed that the simultaneous processes of polymerization, condensation, and hydrolysis of the precursors are responsible for the formation of CDots via bottom-up approaches, which could resemble the mechanism of peptide formation

¹⁵⁷. Proline has been demonstrated to play a crucial role in the innate immune system by means of employing antimicrobial peptides that can effectively penetrate through bacterial membranes and impede their growth via targeting of their fundamental intracellular processes^{92,158,159}. Thus, preserving the chirality of proline-based CDots could act to retain the active sites related to the antibacterial properties of proline-rich peptides, thereby enhancing their penetration into the bacterial membrane. To assess this assumption, both enantiomers of proline were synthesized as CDots to investigate the capability of bacterial strains to differentiate between them and to detect any changes in the proline structure due to decomposition or displacement of the constituent groups. To achieve this, D-Pro.CA CDots were synthesized using the same procedure employed for L-Pro.CA CDots under the selected conditions of 163°C, 3:1 molar ratio, and 13 minutes. We note that for the D-Pro.Ca, the opposite Cotton effect is observed in the CD spectra as shown in **Fig 3.5a**. In light of the demonstrated influence of temperature on the chiral signal, it is plausible that raising the temperature would promote the decomposition of the chiral centers that contribute to the signal detected by circular dichroism spectroscopy. Consequently, L-Pro.CA CDots were synthesized using the same procedure, albeit at a higher temperature to fully eliminate the chiral signal. As revealed in **Fig 3.5a**, no residual chirality on the surface of CDots were detected when temperature was elevated to 205°C indicating the decomposition of the proline structure. Following the synthesis of the dots, the MIC of the chiral precursors (L- and D-proline), as well as both D- and L-Pro.CA CDots, and DL-Pro.CA CDots (racemic mixture) were assessed for comparative analysis. **Fig 3.5b** presents the MIC values of the CDots against Gram-positive (+) and Gram-negative (-) bacterial strains, namely *E. coli* ATCC 25922, *B. subtilis* ATCC 6051, *S. aureus* ATCC 29213, *P. aeruginosa* 113951, and *S. aureus* CANWARD 115852. The results demonstrate that the chiral precursors exhibited no

antibacterial activity at the concentrations tested. However, all proline based CDots showed activity that further confirms the enhancement of the antibacterial effect in CDots versus the precursors. Also, no discernible differences were observed between the D- and L-Pro.CA CDots prepared at 163°C that could indicate an absence of enantioselectivity for CDots against the selected strains. However, it is noteworthy to mention that nearly all bacterial strains exhibited a slight preference for CDots produced at 163°C over those produced at 205°C resulting in a higher antibacterial activity. This further confirms a recognition of the lack of chiral response in the latter by the bacterial system that could potentially shed a light on the correlation of antibacterial activity of proline with its stereochemistry. Interestingly, the racemic DL-Pro.CA CDots displayed similar MIC values to those of the enantiopure CDots, underscoring the ability of a bacterial system to detect changes in the chiral centers of the dots resulting from the heating process.

This information can also offer insights into the mechanism of formation of chiral CDots and their dependence on reaction temperature. The microwave heating process may break down or displace the constituent groups and active sites responsible for the antibacterial activity of the dots, which can potentially be recognized by various strains of bacteria, both gram-positive and gram-negative.

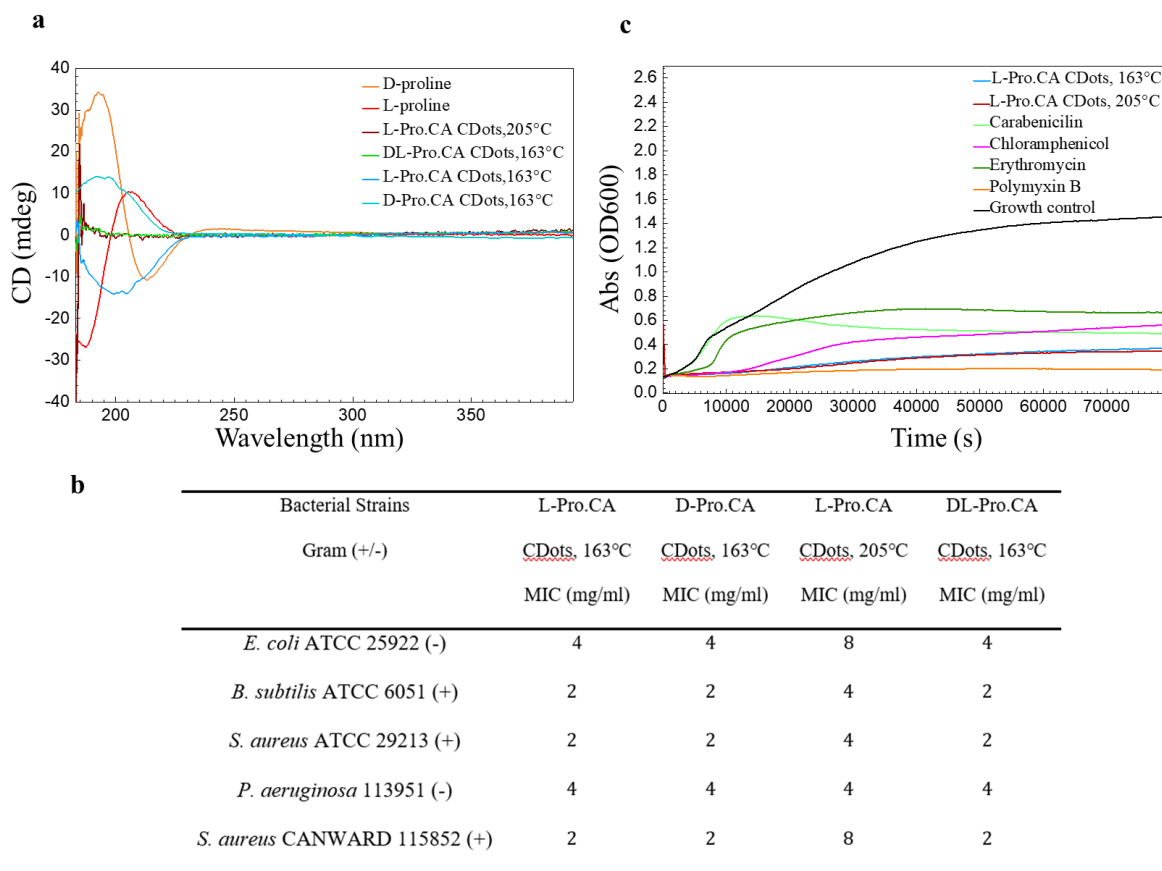


Fig 3.5 CD spectra of chiral precursors and the corresponding chiral CDots used in bacterial studies, showcasing opposite cotton effects (a). MIC values of CDots against several bacterial strains (b). Time-kill assay of chiral and non-chiral CDots prepared at 163°C and 205°C, respectively (c).

To further assess the efficacy of CDots against bacterial strains, time-kill assays were performed. This assay involves exposing bacterial cultures to different concentrations of the antimicrobial agent and measuring the bacterial growth over time. The results of the assay can be used to determine the bactericidal or bacteriostatic activity of the agent and their mechanism of action. Based on the data presented in the growth curves in **Fig 3.5b**, it appears that the mechanism of action of both chiral and non-chiral CDots are more similar to that of Polymyxin B but less aggressive, as they seem to cause a rapid decline in bacterial growth. Polymyxin B is a

polypeptide antibiotic that primarily targets the bacterial cell membrane, specifically the outer membrane of Gram-negative bacteria by interacting with the lipopolysaccharide (LPS) component of the outer membrane, and ultimately known to be a bacteriolytic. Therefore, this assay can suggest similar mechanistic behavior of CDots with Polymyxin B in terms of interacting with the cellular membrane of bacteria.

3.4.4 Conclusion

In this study, we were able to successfully transfer the chiral properties of L-/D-proline to CDots and utilized RSM-BBD to study and optimize the reaction conditions that leads to preserving the maximum residual chiral signal of proline on the surface of CDots. Based on the optimization results, the second-order polynomial model revealed that the reaction temperature had the most significant impact on the chiral response of the CDots, while the molar ratio of precursors and exposure time had a secondary influence. Moreover, the interactions between time and molar ratio, as well as time and temperature, were found to be significant in determining the magnitude of chiral response on the surface of CDots. These interactions would not have been able to be studied using conventional one-factor-at-a-time methods. The antibacterial studies confirmed that by synthesizing and transferring the chiral properties of proline to the surface of CDots, it was possible to retain some of the active sites associated with the antibacterial properties of proline. The results for MIC and time-kill assays confirmed a possible correlation between proline activity and its stereochemistry and the ability of different bacterial strains to recognize the presence and absence of chiroptical properties of the compound. Overall, this study provides valuable insights into the potential applications of chiral CDots and highlights the importance of considering the stereochemistry of active compounds when designing novel materials with enhanced properties.

Chapter 4. Conclusion and Future Works

4.1 Conclusion

In this work, the synthesis of chiral CDots was achieved successfully through a one-step microwave reaction. The chiral nature of the CDots was induced by utilizing enantiomers of proline in conjunction with citric acid during the synthesis process. By fine-tuning essential reaction parameters, such as synthesis temperature, time, and precursor ratio through Design of experiment-RSM, the chiral response of L-Pro.CA CDots was maximized.

Furthermore, the investigation of reaction conditions indicated that the chiral response of CDots was predominantly influenced by the reaction temperature, followed by the molar ratio of precursors and exposure time. Additionally, the interactions between time and molar ratio, and time and temperature were identified as significant factors determining the chiral response magnitude on the surface of CDots. Notably, conventional one-factor-at-a-time approaches would not have been able to detect the intricate interactions among various factors. Consequently, the implementation of response surface methodology (RSM) emphasized the importance of utilizing advanced statistical tools to gain insights into an experiment while ensuring the safety of starting materials to prevent degradation.

The antibacterial investigations of the CDots validated that the retention of certain active sites linked with the antibacterial characteristics of proline was feasible by transferring its chiral properties to the surface of CDots during synthesis. The outcomes from MIC and time-kill assays provided evidence supporting a potential correlation between the stereochemistry of proline and its activity, as well as the capacity of diverse bacterial strains to identify the presence or absence

of the chiroptical properties of the compound. This offers valuable insights into the potential utility of chiral CDots and emphasizes the significance of taking into account the stereochemistry of active compounds during the development of novel materials with improved properties.

4.2 Future Works

Future investigations could explore the relationship between the chiral properties of proline and its antibacterial activity by functionalizing the surface of citric acid-based CDots with proline using coupling agents. This approach could provide enhanced control over the quantity of amino acids responsible for the chiral signal on the surface. Structural studies, such as NMR, in conjunction with different separation techniques, could be employed to investigate the structural differences and potential motifs responsible for the antibacterial activity of CDots. Moreover, an evolution assay could provide insights into the possible mechanism behind the mutation of bacterial genes and the potential targets of CDots.

Further exploration of the chiral CDots synthesized in this study could encompass the evaluation of their optical sensitivity towards various analytes by monitoring changes in their CD profile and photoluminescence properties. In this context, CPL (circularly polarized luminescence) studies could provide valuable insights as well.

In order to harness the benefits of a dependable protocol for synthesizing chiral CDots with robust chiroptical properties, it is also advisable to investigate the catalytic efficacy of the dots in asymmetric reactions. Proline has demonstrated remarkable activity as a heterogeneous chiral catalyst for direct aldol condensation. This strategy may provide a streamlined alternative to the complex multistep chemical processes utilized for the incorporation of chiral sites.

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Appendix

Table A3.1 The result for sequential model sum of squares

Source	Sum of Squares	df	Mean Squares	<i>F</i> -Value	<i>P</i> -Value	
Linear	1	3	0.33	11.67	0.0005	
2FI	0.24	3	0.08	6.20	0.01	
Quadratic	0.11	3	0.03	10.23	0.0059	Suggested
Cubic	9.370E – 003	3	3.123E – 003	0.84	0.5367	Aliased

Table A3.2 ANOVA results for the modified quadratic model.

(X₁= time, X₂= temperature and X₃= molar ratio of proline to CA)

Source	Sum of Squares	df	Mean Squares	<i>F</i> -Value	<i>P</i> -Value Prob >F
Model	1.35	7	0.19	69.52	< 0.0001
X ₁	0.18	1	0.18	66.03	< 0.0001
X ₂	0.67	1	0.67	240.12	< 0.0001
X ₃	0.15	1	0.15	55.26	< 0.0001
X ₁ X ₂	0.18	1	0.18	63.19	< 0.0001
X ₁ X ₃	0.06	1	0.06	19.97	0.0016
X ₁ ²	0.05	1	0.05	16.10	0.0031
X ₂ ²	0.04	1	0.04	5.48	0.0473
Residual	0.02	9	2.77E – 003		
Lack of fit	0.01	5	2.03E – 003	0.55	0.7373
Mean	5.88		R-Squared	0.98	

C.V%	0.89	Adj. R-Squared	0.96
Adeq. precision	32.95	Pred. R-Squared	0.91

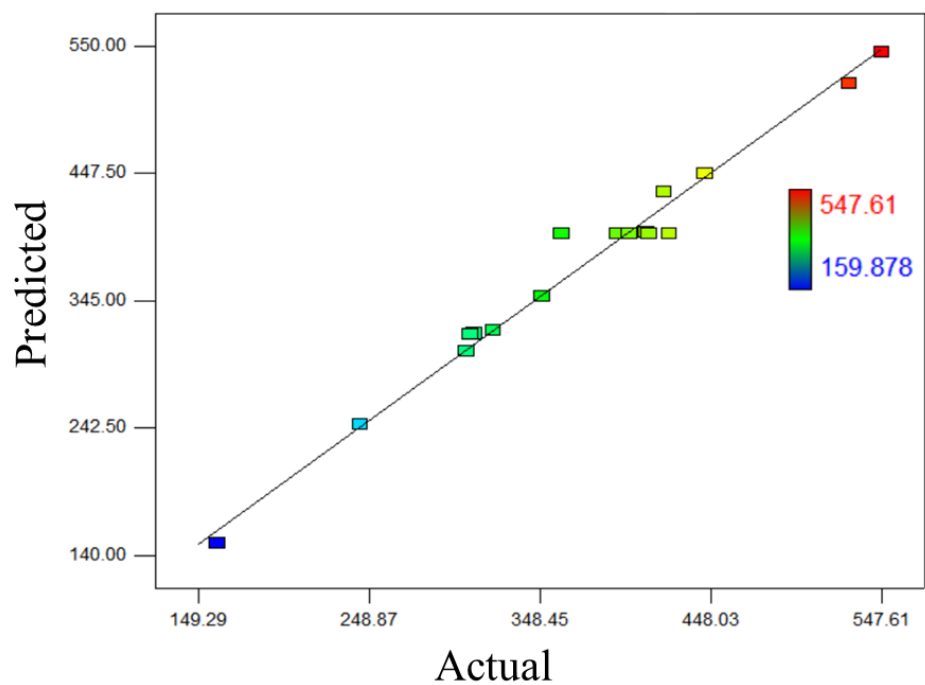


Fig A3.1 The graph of the actual response values for the chiral integration versus the predicted response values. The even distribution of the data around the predicted line shows a good estimation of the experimental result by the quadratic model.

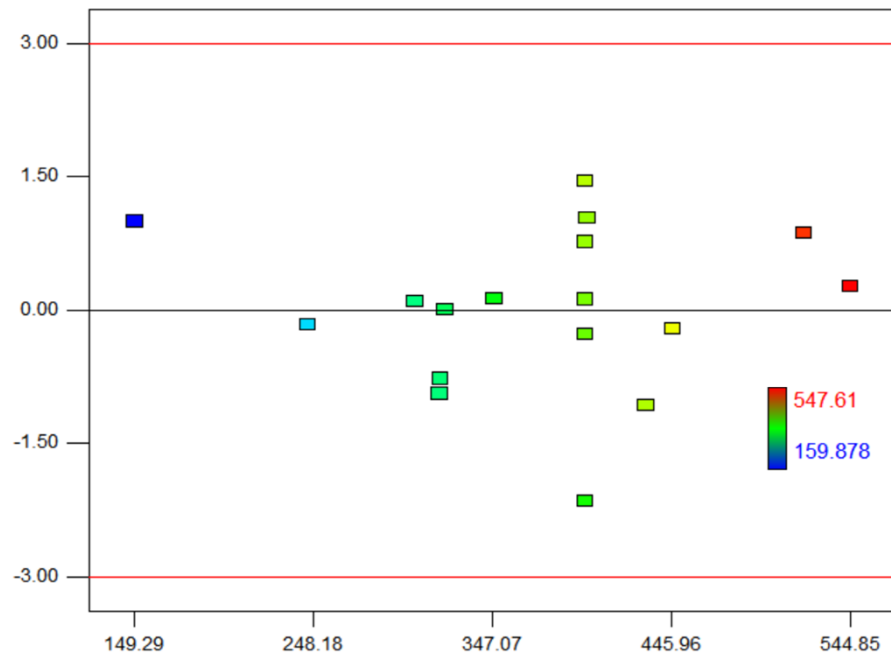


Fig A3.2 The plot of the residuals versus the ascending predicted response values. The random scatter of the plot with no specific trend indicates the independence of residuals from one another. The internally studentized residuals possess the ability to measure the magnitude of residuals in terms of standard deviation units, thereby facilitating their application in detecting outliers. Specifically, an observation that exhibits an internally studentized residual exceeding 3 (in absolute value) is typically identified as an outlier.

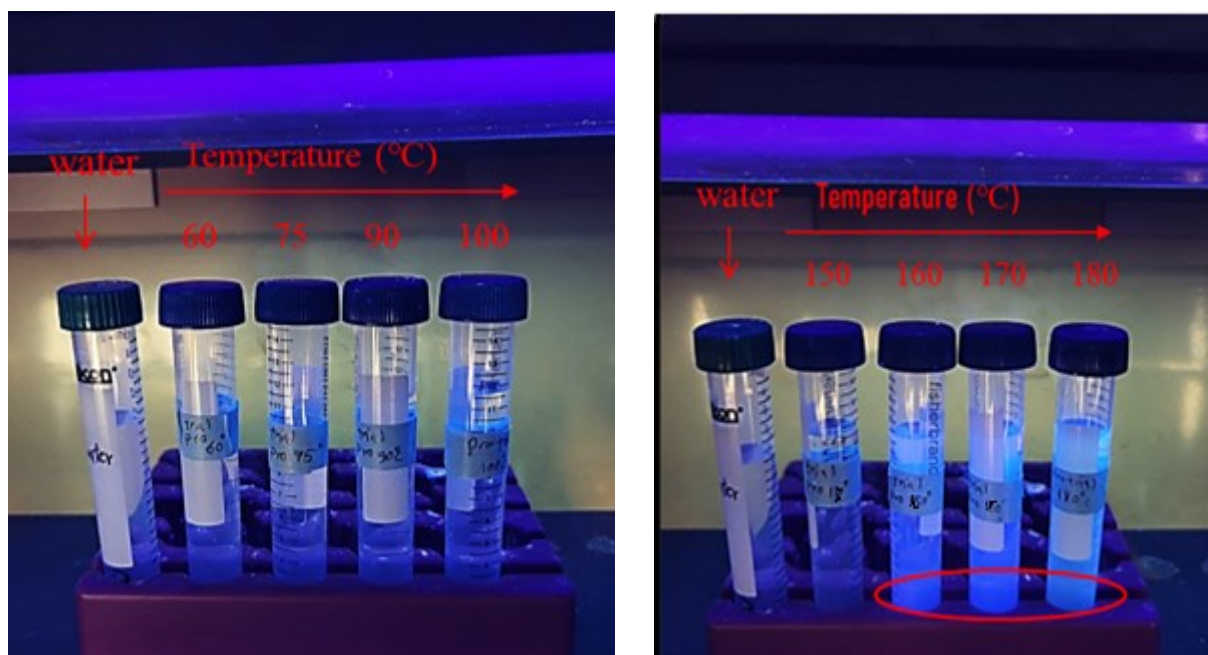


Fig A3.3 Image of various L-Pro.CA samples synthesized in a microwave reactor at temperatures ranging from 60°C to 180°C for a duration of 30 minutes which are excited under long-wavelength UV light at 365 nm.

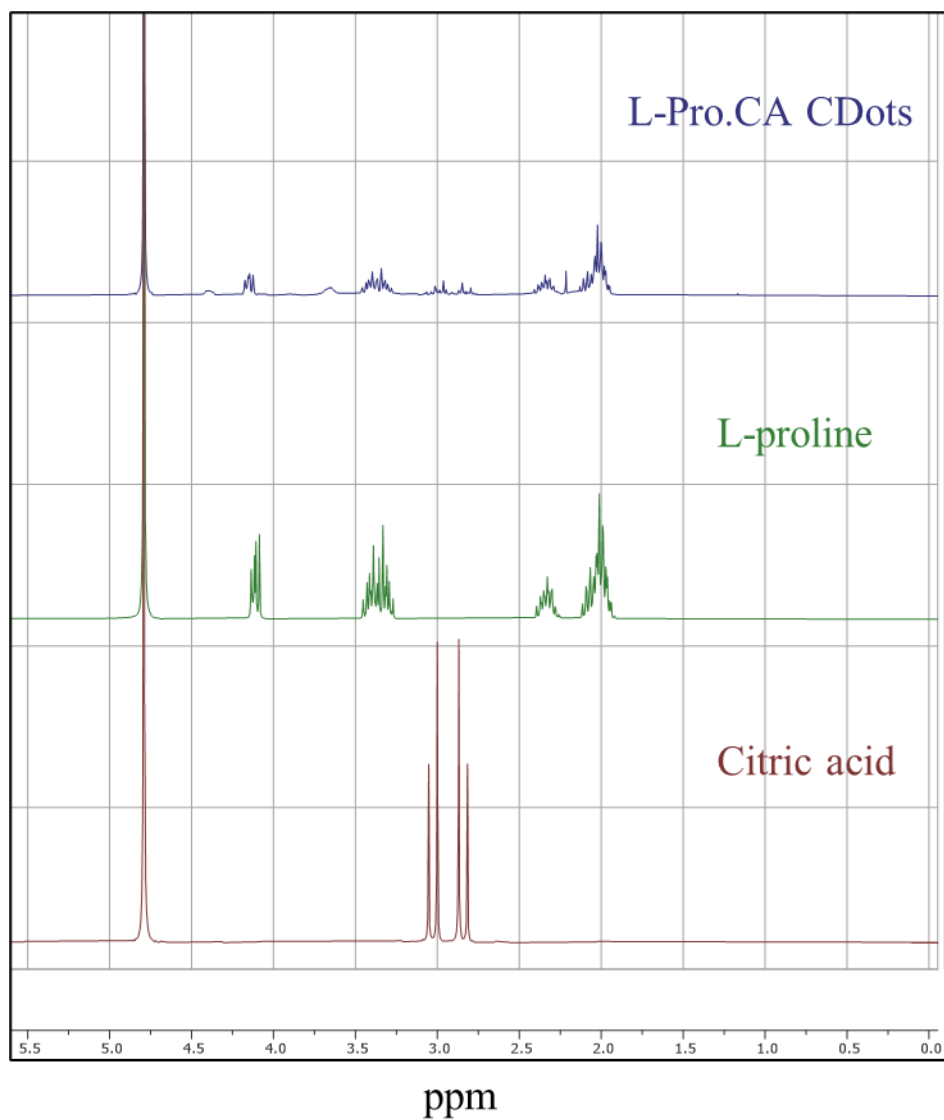


Fig A3.4 ^1H NMR spectra of the L-Pro.CA CDots, L-proline, and citric acid.

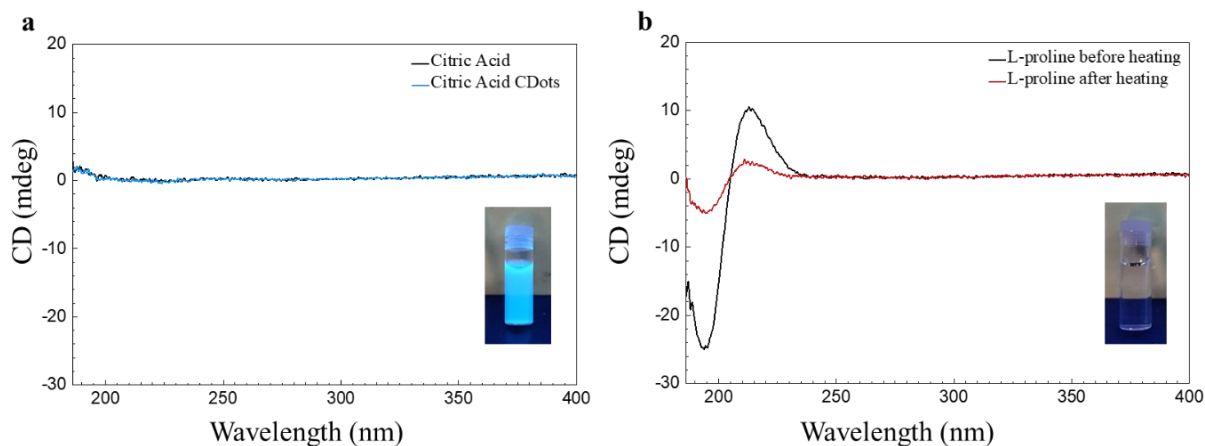


Fig A3.5 Absence of chiral signal in citric acid precursor before and after microwave in CD spectra (a). Inset: fluorescence of citric acid sample dissolved in water, indicating the formation of CDots. Heat treatment does not affect exciton couplet in L-proline, as demonstrated by CD spectra analysis (b). Inset: Fluorescence emission of L-proline after heating and dissolution in water demonstrating no sign of photoluminescence properties.

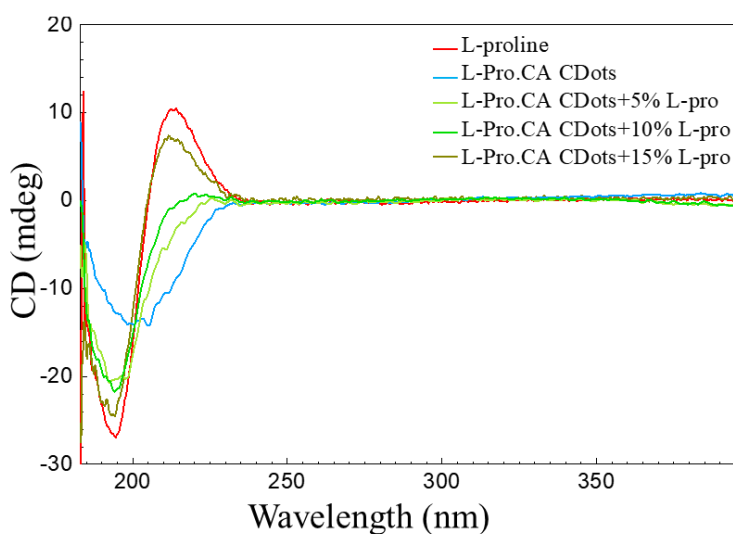


Fig A3.6 Analyzing Signal Sensitivity of L-Pro.CA CDots with Different v/v% of L-Proline. As the concentration of L-proline increases, the CD spectra transforms back to the couplet resembling the profile of the chiral precursor.

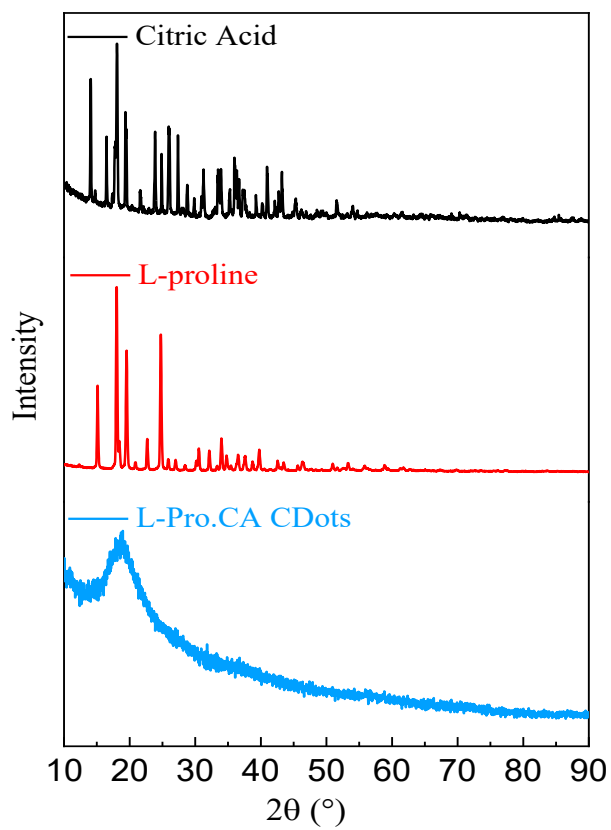


Fig A3.7 XRD pattern of L-Pro.CA CDots. The crystalline structure of precursors has transformed to an amorphous halo centered at 17° 2θ.

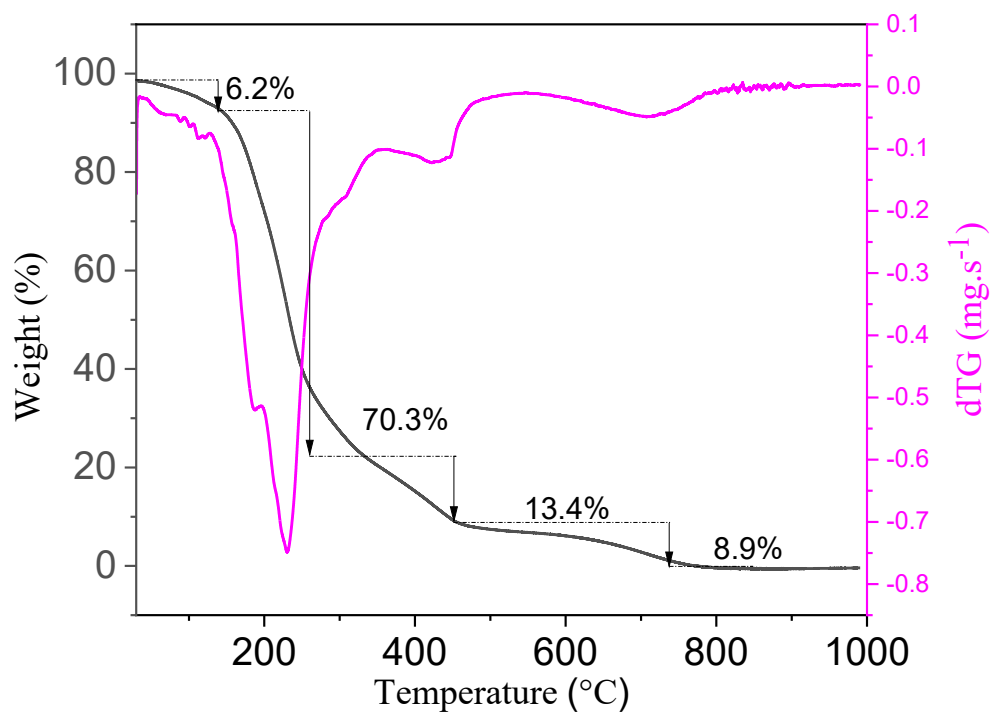


Fig A3.8 The thermogravimetric analysis (TGA) profile of L-Pro.CA CDots reveals multiple weight losses with increasing temperature. The predominant weight loss of 70.3% occurs at 260°C, indicating the thermal stability of L-Pro.CA CDots up to this temperature.