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Multi-emissive Nile Red-loaded β -cyclodextrin-derived C-nanodots for specific detection of phenylalanine

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Multifunctional carbon-based nanostructures are attracting considerable attention because of their low toxicity, excellent optical and chemical properties, and intriguing applications. Currently, the development of carbon-based nanostructures for sensing remains a challenging topic in the biosensing field. Here, we report an unprecedented example of a multi-emissive supramolecular system composed of carbon-nanodots, derived from β -cyclodextrin units by a one-pot thermal process, and Nile Red (NR) hosted in the cyclodextrin cavities (Cdots- β CD/NR). The Cdots- β -CD nanostructures were characterized by spectroscopic techniques, NMR, and atomic force microscopy. The supramolecular Cdots- β CD/NR system exhibited multi-fluorescence emissions at 410 nm and 650 nm, corresponding to the Csp²-core of the carbon nanodots and the encapsulated NR, respectively. The as-prepared Cdots- β CD/NR nanosystem showed high specificity for the detection of phenylalanine (Phe) with a limit of detection (LoD) of 0.49 μ M, a limit of quantification (LoQ) of 1.25 μ M over a dynamic range of 2–100 μ M for the red fluorescence emission (650 nm), and a dynamic range of 20–400 μ M for the blue fluorescence emission (410 nm). Good specificity for Phe over tyrosine was demonstrated and supported by molecular modelling simulations. The proposed sensing strategy exhibited good correlation with the results obtained from standard mass spectrometry when applied to human blood samples collected from PKU patients.

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Introduction

The monitoring of biochemical markers is gaining increasing attention in the diagnostic field as an approach to enable personalized healthcare and telemedicine.^{1,2} Recent efforts have focused on the development of sensing strategies for biomarker detection using optical or electrochemical analyses integrated into miniaturized wearable devices,³ implantable sensors,⁴ and portable sensing platforms.⁵ Innovative sensing

approaches are being achieved by combining key enabling technologies such as nanotechnology, advanced nanomaterials, molecular biology, microfluidics, and artificial intelligence.⁶ Their application has proven effective in sensor and diagnostic research and in motivating patients with chronic metabolic diseases. Hyperphenylalaninemia and phenylketonuria (PKU) are inherited metabolic disorders affecting amino acid metabolism. In PKU patients, mutations in the PAH gene result in absent or significantly reduced activity of the enzyme phenylalanine hydroxylase (PAH), which catalyzes the conversion of phenylalanine (Phe) to tyrosine (Tyr). As a consequence, Phe cannot be converted to Tyr and accumulates in body fluids, where it exerts toxic effects. The typical clinical symptoms of PKU patients include intellectual disability, microcephaly, and other neurological corollaries such as epilepsy or movement disorders.⁷ PKU patients must adhere to a strict, lifelong low-Phe diet in order to prevent or mitigate these clinical manifestations. However, maintaining compliance with a restricted diet remains a very complex issue for PKU patients. The only reliable method for tracking their clinical progress with the diet is the regular monitoring of blood phenylalanine levels.

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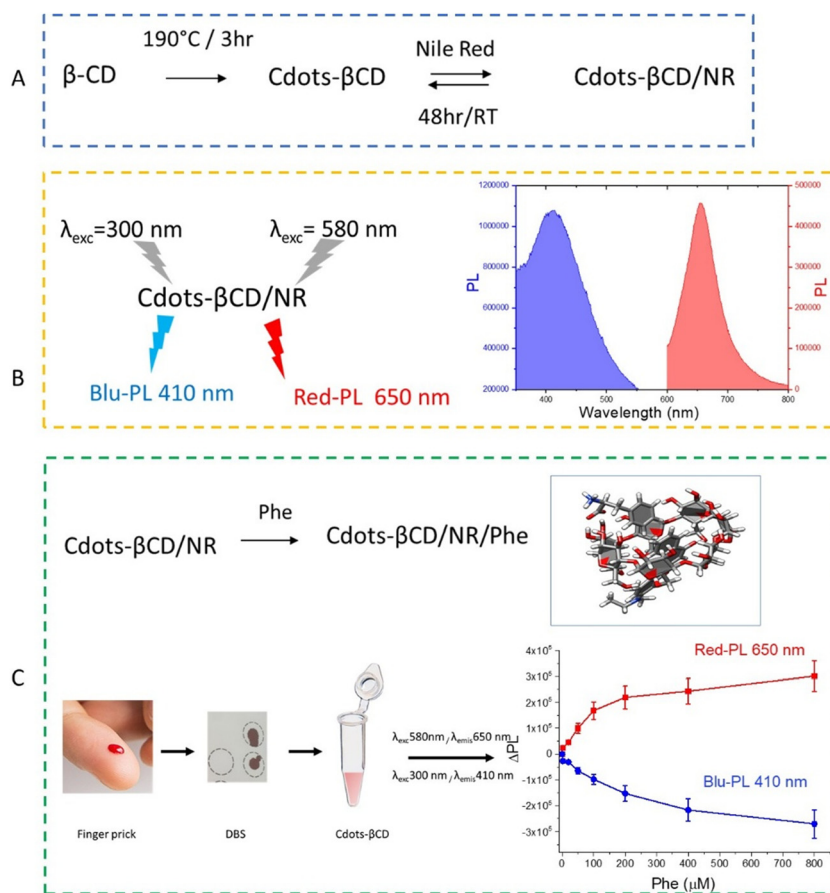
Routine monitoring enables clinicians to assess the effectiveness of dietary management to implement any necessary adjustments. Currently, the official and most widely accepted method for Phe monitoring is dried blood spot (DBS) testing. This technique involves collecting a drop of blood on a special filter paper, which, once dried, is sent to a clinical metabolic centre for analysis. Phenylalanine levels and phenylalanine-to-tyrosine ratios are measured by tandem mass spectrometry. However, this monitoring method is quite long for data return (4–5 days), and patients do not receive immediate feedback on their dietary compliance. In this regard, an integrated sensing strategy for Phe monitoring would supply quick results and feedback, assisting the diet management. During the last few years, several enzymatic and non-enzymatic approaches for Phe detection have been reported in the literature. Most of them are based on colorimetric^{8,9} or electrical detection,¹⁰ and some of these have been successfully integrated into miniaturized devices.¹¹

Thanks to their peculiar chemical, optical, and electrical properties, nanostructured materials such as metal nanoparticles, nanoclusters, and carbon-nanodots are considered promising materials for biosensing and biomedical applications including drug delivery,¹² bioimaging,¹³ nanochemosensing,¹⁴ and photothermal therapy.^{15,16} Among these materials, the carbon nanodots

composed of an emissive Csp² inner core and a specialized outer shell are appealing systems in biosensing applications.¹⁷ Several properties, including easy surface functionalization, good photoluminescence, and the ability to form host-guest complexes with fluorophores or electrochemical probes, enable the use of these materials in non-enzymatic recognition approaches.¹⁸

The use of host-guest complexes with emissive probes such as palmitin has been reported in the literature for the specific recognition of Phe.¹⁹ Similarly, Nile Red (NR: 9-diethylamino-5*H*-benzo[*x*]phenoxazine-5-one) is a fluorescent molecule well-known for its low water solubility and effective emission properties in apolar media, making it an effective emissive probe for hydrophobic environments. The host-guest β -cyclodextrin/NR complex has been extensively investigated in the literature. Recently, Ray and co-worker demonstrated the effective encapsulation of the monomeric form of NR within the hydrophobic cavity of β -CD, leading to the formation of an emissive and water-soluble β CD/NR nanosystem.²⁰

Based on the above consideration, herein, we describe an innovative multi-emissive and water-dispersible supramolecular system composed of β -cyclodextrin-derived carbon-nanodots (Cdots- β CD) loaded with Nile red (Cdots- β CD/NR) for the specific detection of Phe in human blood. The Cdots- β CD nanostructures, prepared *via* a mild thermal process using



Scheme 1 Schematic representation of (A) Cdots- β CD nanostructures and Cdots- β CD/NR supramolecular system preparation, (B) dual emissive properties of Cdots- β CD/NR, and (C) Phe detection process through Cdots- β CD/NR/Phe supramolecular complex formation.

β -cyclodextrin as the precursor, act as a blue-emissive probe, while the hosted Nile red serves as an excellent red-emissive probe.^{21–23} The Cdots- β CD/NR system exhibits multi-emission at 410 and 650 nm, corresponding to the C-dots carbon inner core and the encapsulated NR, respectively. Optical absorption and emission spectroscopy, dynamic light scattering (DLS), Z-potential analysis, and Fourier transform infrared (FTIR) spectroscopy are employed to characterize the supramolecular nanosystem prepared, as depicted in Scheme 1, and to evaluate its ability to recognize Phe in both aqueous media and human blood samples. When phenylalanine (Phe) was added to the β CD/NR system, the red-PL signal at 650 nm increased significantly, whereas the blue-PL signal at 410 nm decreased. In contrast, the addition of tyrosine to the same inclusion complex did not induced appreciable fluorescence changes. These observations demonstrate that the β CD/NR system can be used for the selective recognition and discrimination of Phe in aqueous solution.

Methods and materials

Chemicals

All chemicals were obtained from Sigma Aldrich at the highest possible purity and were used as received. Milli-Q-grade water was used in all preparations. All solvents were of spectro-photometric grade.

Instrumentation

UV-vis optical absorption spectra were recorded on a Perkin-Elmer 365 spectrophotometer (Long Island, NY, USA) using quartz cuvettes with a path length of 10 mm. Fluorescence emission spectra were acquired on a Horiba spectrofluorometer using a quartz cuvette with an optical length of 10 mm. All products were suspended in MilliQ-deionized water and the spectra were recorded at different excitation wavelengths in the range 360–520 nm to investigate the emission of Cdots- β CD (3/3 nm slit width) and at 580 nm to measure the NR fluorescence emission (5/5 nm slit width). A two Fourier transform infrared spectrophotometer with attenuated total reflectance modules (FTIR-ATR, PerkinElmer, Long Island, NY, USA) was used to confirm the formation of Cdots- β CD and Cdots- β CD/NR complex. Dynamic light scattering (DLS) and Z-potential analyses in water confirmed the formation of nanosized and slightly negatively charged Cdots- β CD structures. The measurements were performed using a Zetasizer NanoZS90 analyser (Malvern Instrument, Malvern, UK) equipped with a 633 nm laser, at a scattering angle of 90° and 25 °C temperature. The size of the particles was calculated from the diffusion coefficient using the Stokes–Einstein equation. Atomic force microscopy (AFM) analyses were performed using a Bruker-Innova microscope operating in high amplitude mode and ultra-sharpened Si tips (MSNL-10 from Bruker Instruments, Karlsruhe, Germany). Geometry set-up: radius of curvature 2 nm, tip height 2.5 nm, front angle 15°, back angle 25°, side angle 22.5°, resonance frequencies of 90–160 kHz, and nominal spring

constant of 0.07 N m^{−1}. The images were reconstructed by acquiring 512 × 512 lines and a scan rate of 0.8 Hz. The AFM images were analyzed by using the SPMLABANALYSES V7.00 software. Samples were prepared by drop casting onto a pre-cleaned silicon substrate and were allowed to dry under gentle nitrogen blow-drying to ensure proper adsorption. NMR (nuclear magnetic resonance) spectra were acquired on a Bruker 400TM spectrometer (Bruker, Germany) and calibrated on the solvent residual proton (HOD, 4.72 ppm).

Preparation of Cdots- β CD

β -Cyclodextrin (β CD, 200 mg) was thermally treated at 190 °C in air for 3 hours. The resulting dark-reddish solid was washed with 1 mL of Milli-Q water, then suspended in water and sonicated for 5 minutes. To remove excess precursor and other byproducts, the water dispersion was centrifuged at 13 000 rpm for 5 minutes, and the supernatant was filtered through a 0.2 μ m pore membrane filter. The resulting red-yellowish colloidal solution containing Cdots- β CD was purified through dialysis in Milli-Q water using a dialysis membrane with a 10 kDa molecular weight cutoff for 24 hours. The product was finally lyophilized at −50 °C.

Preparation of the Cdots- β CD/NR system

To 3 mL of aqueous dispersion of Cdots- β CD (1 mg mL^{−1}), an excess of Nile Red (NR) was added (5mg), then the dispersion was stirred for 48 hours under dark conditions. The suspension was centrifuged at 13 000 rpm for 10 minutes to eliminate the excess of free Cdots- β CD. The yellow supernatant containing the excess of Cdots- β CD was discarded. The pellet was washed with Milli-Q water. Finally, the reddish pellet was resuspended in water and centrifuged at 3000 rpm for 5 minutes to remove the free NR. The pink supernatant containing the Cdots- β CD/RN complex was separated and used.

Sensitivity experiments

To 2.5 mL of the Cdots- β CD/NR (1 mg mL^{−1}) sample, different volumes (1.25, 11.26, 18.84, 31.64, 25.63, 51.77, and 105.62 μ L) of Phe stock solutions (4 mM and 10 mM) were added to obtain final Phe concentrations of 0.0, 2.0, 20.0, 50.0, 100.0, 200.0, 400.0, and 800.0 μ M. Each sample was replicated three times. Fluorescence signals were recorded for each sample after Phe addition using excitation wavelengths of 580 nm and 300 nm (slits 5/5 nm).

Selectivity experiments

To 2.5 mL of the Cdots- β CD/NR sample, different volumes (1.25, 11.26, 18.84, 31.64, 25.63, 51.77, and 105.62 μ L) of Tyr stock solutions (4 mM and 10 mM) were added to obtain final Phe concentrations of 0.0, 2.0, 20.0, 50.0, 100.0, 200.0, 400.0, and 800.0 μ M. Each sample was replicated three times. Fluorescence signals were recorded for each sample after Tyr addition using excitation wavelengths 580 nm and 300 nm (slits 5/5).

Human subject recruitment

The whole blood sample was obtained from PKU patients. The enrolled patients were clinically followed up in our Referral Center for Inherited Metabolic Disorders, located at the Polyclinic of Catania, Italy. Informed consent was obtained from all subjects involved in the study, including minors whose informed consent was signed by their parents (resolution no. 27819-July 10, 2020, of Ethic Committee-Polyclinic of Catania and resolution no. 1327-July 20, 2020, of Polyclinic of Catania). During a follow-up visit, blood was collected from each patient. Immediately, a few microliters of each whole blood sample were spotted on a Whatman 903 filter paper (Eastern Business Forms, Greenville, SC, USA), to obtain a dried blood spot (DBS). Each DBS was dried away from direct sunlight or heat sources, and after about 4 h, it was ready for mass spectrometry measurements. All samples were collected after 3 h of fasting.

Tandem mass analysis

Measurements were carried out using the Neobase 2 nonderivatized MS/MS kit (Wallac Oy, Turku, Finland), according to the manufacturer's instructions. In brief, using an automatic puncher (PerkinElmer Panthera Puncher), filter paper disks (3.2 mm in diameter) were punched out from the DBS into the wells of a microplate, and 125 μL of a working solution was added into each well. The plate was shaken in the incubator/shaker (TriNest incubator, Wallac Oy, Turku, Finland) at 30 $^{\circ}\text{C}$ for 30 min at a speed of 750 rpm, and then 100 μL from the content of each well was transferred to another microplate to avoid the presence of the paper disk during the injection inside the mass spectrometer. The electrospray ionization-tandem mass spectrometry (ESI-MS/MS) analyses were performed on a 210 MD QSight spectrometer (PerkinElmer, Waltham, MA, USA) equipped with a triple quadrupole. The analysis was performed in the flow injection mode using a QSight HC autosampler MD (PerkinElmer, Waltham, MA, USA) and a QSight binary pump (PerkinElmer, Waltham, MA, USA); the volume of injection was 10 μL , and the flow rate was 0.02 mL min^{-1} . The MS parameters were set up as follows: capillary voltage, 5 kV; source temperature, 175 $^{\circ}\text{C}$, drying gas, 105 L h^{-1} ; and nebulizer gas, 130 L h^{-1} . The analytical measurements were performed in multiple reaction monitoring modes (MRMs), using stable isotope internal standards for quantitative analysis.

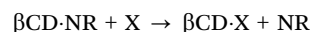
Computational methods

The formation of host-guest complexes between the β -cyclodextrin cavity (βCD) and Nile Red (NR), phenylalanine (Phe), or tyrosine (Tyr) analytes was investigated using density functional theory (DFT). Initial geometries for the inclusion complexes were generated by considering both primary- and secondary-rim orientations. The structures were optimized using the GFN2-xTB method combined with CREST conformational sampling.

The geometry optimizations were performed with the r2SCAN-3c composite functional, including the RIJCOSX approximation and implicit solvation (CPCM, water). Tight

SCF convergence criteria were applied throughout. The vibrational frequency calculations were performed at the same level of theory in order to obtain the Gibbs free energies (G). For each complex, the most stable configuration (in terms of the lowest Gibbs free energy among the sampled orientations) was selected for thermodynamic analysis. All the Gibbs free energy values were calculated in water.

To assess the competitive displacement of Nile red by Phe and Tyr, free energies of the exchange reactions were computed:



where X = Phe or Tyr. The corresponding free energy change was calculated as:

$$\Delta G_{\text{swap}}(\text{X}) = G_{(\beta\text{CD-X})} + G_{(\text{NR})} - G_{(\beta\text{CD-NR})} - G_{(\text{X})}$$

Phe recognition in human blood samples

The DBS sample was treated to extract the amino acid fraction. Briefly, the DBS was incubated with 125 μL of homemade extraction solution for 30 min at 45 $^{\circ}\text{C}$. The DBS was then removed, and the resulting extract was evaporated for 25 min at 90 $^{\circ}\text{C}$. Finally, 200 μL of the Cdots- βCD /NR dispersion was added to the extracted fraction and stirred for 10 minutes at room temperature. The resulting dispersion was then analyzed by photoluminescence (PL) spectroscopy upon excitation at 580 nm.

Results and discussion

Synthesis and characterization of Cdots- βCD

The preparation of the Cdots- βCD nanostructures was performed using a one-pot mild-thermal procedure recently developed by our research group starting from different precursors, including polymers,²⁴ chitosan,²⁵ glucose,²⁶ agarose,²⁷ βCD ,²⁸ and βCD derivatives.²⁹ The proposed approach does not require any additional chemical reagents or organic solvents. In detail, an amount of βCD precursor was heated at 190 ± 3 $^{\circ}\text{C}$ in air for 3 hours. After separation and purification processes, the Cdots- βCD nanostructures were fully characterized in terms of their optical, dimensional, and structural properties. The operative temperature was carefully selected to avoid degradation of the precursor. Indeed, it has been reported in the literature that β -cyclodextrin, as well as its derivatives, undergoes thermal decomposition at temperatures above 250 $^{\circ}\text{C}$ under both air and nitrogen atmospheres.³⁰ The successful formation of the Cdots- βCD nanostructures was verified by optical absorption spectroscopy. In particular, the absorption spectra exhibited a typical optical band at around 270 nm corresponding to the π - π^* transition related to the sp^2 -hybridized carbon domains and a broad band in the 320–450 nm region corresponding to the $\text{n}-\pi^*$ transition (Fig. 1A). To confirm the semiconductor properties of this material, the optical band gap was calculated using the Tauc plot, obtaining a value of about 3.6 eV, in agreement with those of other carbonaceous nanodots (Fig. S1).³¹

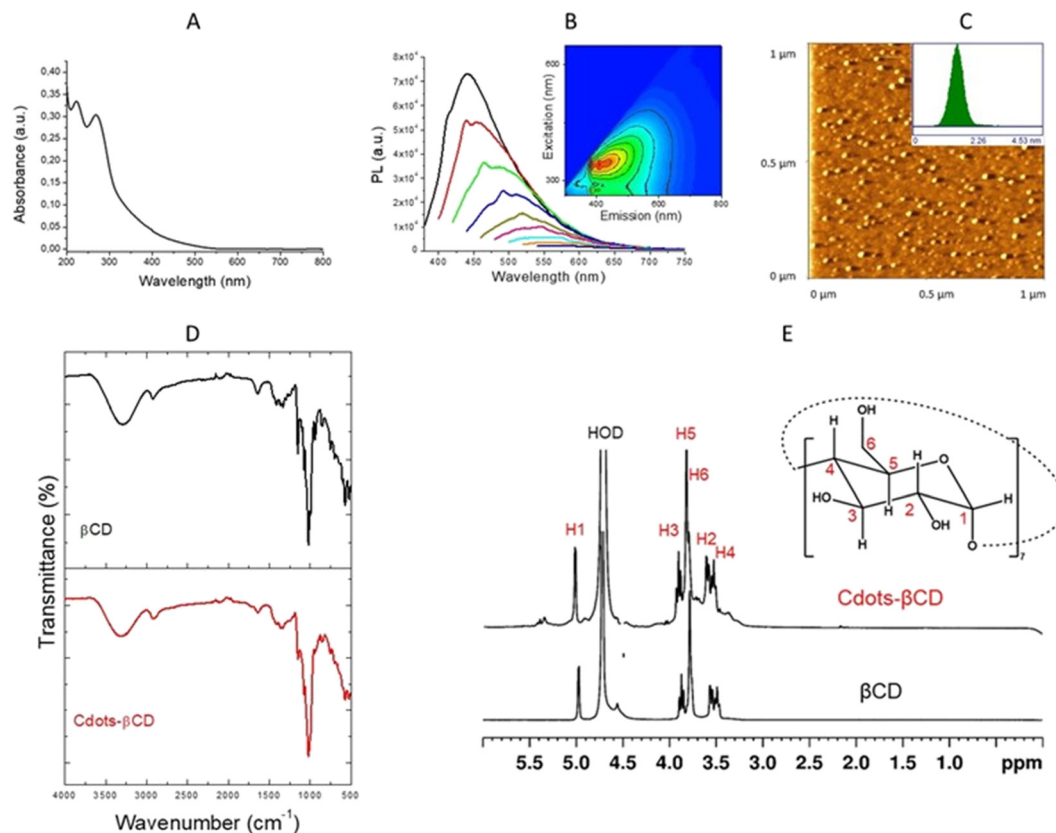


Fig. 1 Cdots- β CD nanostructures: (A) UV-vis optical absorption: 0.5 mg mL⁻¹ solution; (B) photoluminescence emission spectra: 0.5 mg mL⁻¹, slit 2/2 nm, at various excitation wavelengths; inset: 2D photoluminescence map with emission wavelength as a function of excitation wavelength, with fluorescence intensity represented by different colours along the z-axis; (C) representative AFM image (1 μ m $\times$ 1 μ m scan); inset: size distribution; (D) ATR-FTIR spectrum of Cdots- β CD (red line) and β CD precursor (black line); and (E) ¹H-NMR spectra of β CD and Cdots- β CD (400.13 MHz, D₂O, 297 K).

Fluorescence emission spectra were recorded at different excitation wavelengths (in the range of 360–520 nm), revealing an excitation-dependent emission behaviour characteristic of carbon-based nanodots (Fig. 1B). Using quinine sulphate as the reference, the photoluminescence quantum yield (Φ_{PL}) of Cdots- β CD in water was calculated to be 1.7% at an excitation wavelength of 360 nm. The inset in Fig. 1B shows the 2D photoluminescence map with emission wavelength as a function of excitation wavelength confirming again the emission in the range of 400–520 nm.

AFM and DLS experiments were conducted to confirm the nanosized and spherical-shaped morphology of the nanostructures. AFM images indicated the presence of isolated spherical carbon cores with a height of 1.6 ± 0.6 nm (Fig. 1C and Fig. S2). DLS measurements performed on the aqueous dispersion gave an average hydrodynamic diameter of 7.12 ± 3.6 nm with a Z-potential of -9.80 ± 0.984 mV (Fig. S3). For comparison, the precursor β CD displayed a smaller hydrodynamic diameter of 1.78 ± 0.44 nm with a less negative Z-potential (-1.87 ± 1.76 mV). The presence of β CD residues in the Cdots- β CD nanostructures was confirmed by ATR. The ATR-FTIR spectrum of Cdots- β CD (Fig. 1D, red line) exhibited the main diagnostic bands observed for the β CD precursor (Fig. 1C, black line): broad O–H stretching and bending vibrations at 3289–1648 cm⁻¹, the C–H stretching of

the β CD cavity at 2919 cm⁻¹, a C–O–C vibration near 1148 cm⁻¹, and a C–O stretching band at 1018 cm⁻¹. The ¹H NMR spectrum of Cdots- β CD (Fig. 1E) displayed the characteristic signals of β CD, confirming the presence of a β CD shell in the nanostructures. In particular, the signals in the 3.2–4.0 ppm region and the doublet at 5.02 ppm correspond to the H2–H6 protons and anomeric H1 proton of β CD, respectively. Minor additional signals in the same regions suggest a heterogeneous chemical environment, likely arising from variations in the local interactions between the β CD units and the carbon nanodot surface.

Preparation and characterization of the Cdots- β CD/NR nanosystem

The preparation of the Cdots- β CD/NR supramolecular system was performed according to the procedure reported in the Experimental section. In detail, an aqueous dispersion of Cdots- β CD (1 mg mL⁻¹) was mixed with an excess of NR (1.66 mg mL⁻¹), after separation and purification, the Cdots- β CD/NR product was characterized (Scheme S1). The optical absorption and emission spectra for the Cdots- β CD/NR nanostructure are illustrated in Fig. 2A and B, respectively. The optical absorption spectra of the Cdots- β CD/NR supramolecular system exhibit the diagnostic absorption bands of Cdots- β CD at around 272 nm (π - π^*) and 345 nm (n - π^*), and two prominent

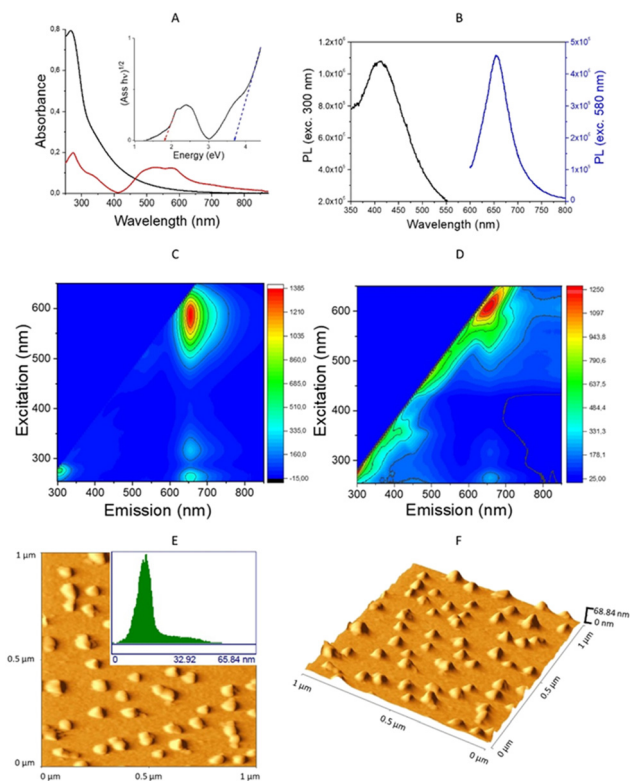


Fig. 2 Cdots- β CD/NR nanostructures: (A) optical absorption spectra of Cdots- β CD (black line) and Cdots- β CD/NR (red line) water dispersion; inset: the Tauc plot for the optical band gap evaluation; (B) emission spectrum at an excitation wavelength of 300 nm (black line) and emission spectrum at an excitation wavelength of 580 nm (blue line) of Cdots- β CD/NR in water dispersion; (C) 2D photoluminescence map for β CD/NR water solution and (D) for Cdots- β CD/NR water dispersion, with emission wavelength as a function of excitation wavelength (the fluorescence intensity represented by different colours along the z-axis); (E) representative AFM image (scan 1 μ m 1 μ m) of Cdots- β CD/NR; inset: the height distribution; and (F) representative 3D AFM image (scan 1 μ m $\times$ 1 μ m) of Cdots- β CD/NR.

peaks at around 500 nm and 600 nm indicating the successful entrapment of NR within the nanostructures (Fig. 1B, red line). For comparison the optical spectrum of the free Cdots- β CD system is also reported to show the absence of well-defined absorption bands above 500 nm (Fig. 1B, black line). The optical band gaps were calculated by the Tauc plot, which showed the variation of $(\text{Ass } h\nu)^{1/2}$ vs. $(h\nu)$ for Cdots- β CD/NR. The plot exhibits the presence of two optical energy band gaps, the first at around 3.6 eV (344.4 nm) related to the nanostructure of CD and the other at about 1.85 eV (670.2 nm) related to the NR chromophore (inset of Fig. 2A). These data well corroborate with optical investigations, showing band gaps of ~ 3.62 eV for Cdots- β CD, ~ 2.02 eV for NR in ethanol, and ~ 1.85 eV for the β CD/NR system (Fig. S1).

The interaction between NR and the Cdots- β CD nanostructures was further supported by comparing the optical absorption of NR in polar and non-polar solvents and in the inclusion complex with β -cyclodextrin (β CD). In particular, the optical absorption spectrum of NR in a polar medium (H_2O) exhibits a

band at around 462 nm (Fig. S1A, azure line), which undergoes a red shift to about 548 nm in a less polar solvent such as ethanol (Fig. S1A, blue line). Furthermore, the successful inclusion of NR within the hydrophobic cavity of β CD was corroborated by a red shift of the absorption band to approximately 600 nm. The simultaneous presence of an additional absorption band at around 462 nm suggests that a fraction of NR remains exposed to the aqueous environment, while the red-shifted band confirms the formation of the β CD/NR inclusion complex (Fig. S1A, red line). Similarly, the absorption spectrum of the Cdots- β CD/NR system displays two bands at 514 and 590 nm. The latter band, related to NR located in a hydrophobic environment, confirms the effective entrapment of NR within the hydrophobic cavities of the Cdots- β CD nanostructures (Fig. S1A, magenta line).²⁰

The photoluminescence spectra of the Cdots- β CD/NR supramolecular system show an interesting multi-emission behaviour. The first emission band, at around 410 nm, is related to the Cdots- β CD upon excitation at 300 nm (Fig. 2B, black line). The second emission band, at around 650 nm, is related to NR upon excitation at 580 nm (Fig. 2B, blue line). Interestingly, the inclusion of NR within the β -cyclodextrin cavity induces a quenching of the emission band at 410 nm, as illustrated in Fig. S4. The emission properties of the Cdots- β CD/NR nanostructures were also confirmed by 2D photoluminescence mapping investigations. In particular, the comparison of β CD/NR (Fig. 2C), empty Cdots- β CD (Fig. 1B-inset), and Cdots- β CD/NR (Fig. 2D) clearly shows the double emissions at 410 nm and 650 nm only for the NR-loaded Cdots- β CD nanostructures.

The AFM investigation of the Cdots- β CD/NR nanostructures shows the presence of nanostructures with a height of 17.6 ± 8.9 nm (Fig. 2E and F). The increase in size compared to empty Cdots- β CD (1.6 ± 0.6 nm) confirms the formation of the supramolecular aggregate system. Additional AFM images are reported in Fig. S5. For comparison, AFM investigation was also performed for the β CD/NR inclusion complex. In this case, nanostructures with a height of 14.3 ± 6 nm have been observed, consistent with the tendency of the β CD/NR complex to form aggregates (Fig. S6).

Sensing of phenylalanine

To assess the effectiveness of the Cdots- β CD/NR nanosystem in recognizing Phe, PL emission and optical absorption spectra were recorded upon the addition of increasing amounts of the amino acid (0–800 μ M concentration range), as described in the Experimental section.

The characteristic absorption of Phe in solution is consistent with the increase in absorbance in the 240–280 nm region of the UV-vis spectra (Fig. S7). A clear enhancement of the emission at 650 nm, associated with the NR species, was observed with increasing Phe concentration (Fig. 3A), while the PL signal at 410 nm, related to Cdots- β CD, was progressively quenched as the Phe concentration increased (Fig. 3B). Fig. 3C shows the variation of the emission signals upon Phe addition: the red-PL emission at 650 nm ($\lambda_{\text{exc}} = 580$ nm, black line) and the blue-PL emission at 410 nm ($\lambda_{\text{exc}} = 300$ nm, red line). A marked enhancement of the

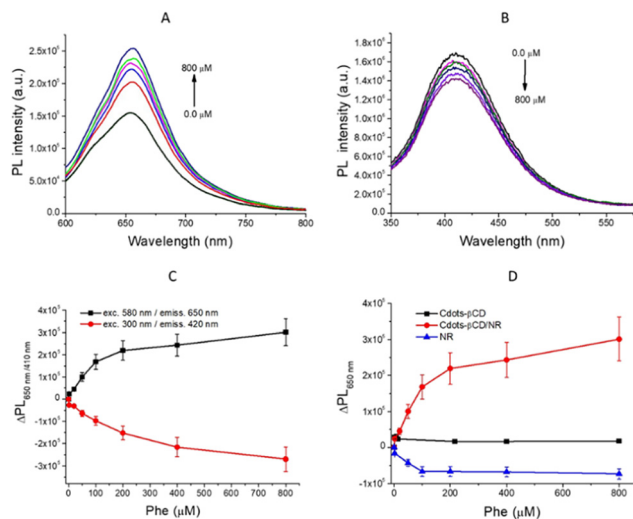


Fig. 3 Photoluminescence-based recognition of Phe by the Cdots-βCD/NR system: (A) emission spectra of Cdots-βCD/NR upon addition of different Phe amounts ($\lambda_{\text{exc}} = 580$ nm, $\lambda_{\text{em}} = 650$ nm); (B) emission spectra of Cdots-βCD/NR upon addition of different Phe amounts ($\lambda_{\text{exc}} = 300$ nm, $\lambda_{\text{em}} = 410$ nm); (C) scatter plot of PL650 nm variation (black line) and PL410 nm variation (red line) upon addition of Phe; (D) scatter plot of PL650 nm variation for Cdots-βCD/NR (red line), Cdots-βCD (black line), and NR (blue line) upon addition of Phe.

red-PL signal (650 nm) accompanied by a simultaneous quenching of the blue-PL signal (410 nm) is evident, indicating an effective interaction between Phe and the Cdots-βCD/NR system. Nile red encapsulated within the βCD cavity acts as a polarity-sensitive probe. Upon interaction with the exposed non-polar aromatic rings of Phe, the dye binds to the analyte, resulting in a pronounced enhancement of the red-PL signal at 650 nm. This behavior is consistent with the emissive sensing mechanism previously reported in the literature for Phe detection using a cucurbit[8]uril/dye complex.¹⁹ Simultaneously, the interaction of Phe with the nanostructures induces a quenching of the blue-PL signal at 410 nm through changes in the local environment and the introduction of non-radiative pathways upon interaction with Phe. As controls, the PL emission at 650 nm ($\lambda_{\text{exc}} = 580$ nm) was recorded for free Cdots-βCD, the Cdots-βCD/NR adduct, and free NR in water. The variation of the red-PL signal upon Phe addition was negligible for the empty Cdots-βCD (Fig. 3D, black line), whereas only a slight quenching was detected for free NR (Fig. 3D, blue line). In contrast, a strong PL enhancement was observed for the Cdots-βCD/NR nanosystem (Fig. 3D, red line), confirming the effective interaction between Phe and the Cdots-βCD/NR nanostructures. Similarly, quenching of the blue-signal at 410 nm was observed for the free Cdots-βCD system upon the addition of different amounts of Phe, indicating that Phe can also interact directly with the carbon-dot-based nanostructures and affect their emissive properties.

Assessment of analytical parameters

To evaluate the analytical performance of the multi-emissive Cdots-βCD/NR system, the linear response range, sensitivity, limit of detection (LoD), and selectivity performances were

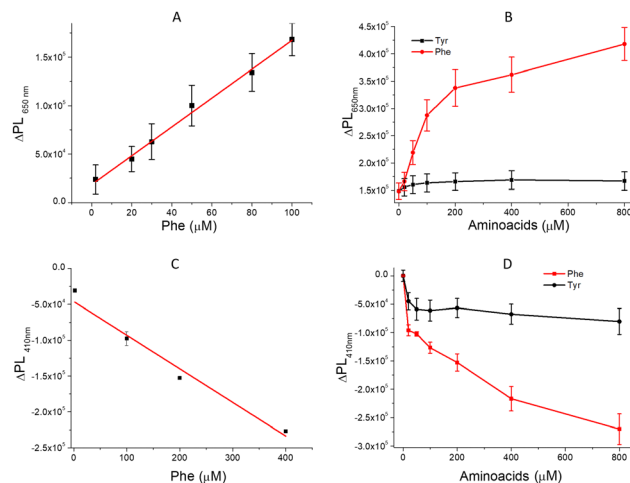


Fig. 4 Cdots-βCD/NR system analytical performance: (A) sensitivity test at $\lambda_{\text{exc}}/\lambda_{\text{em}}$ 580/650 nm ($Y = 18475.2X + 1490.0$; $R^2 = 0.99487$); (B) selectivity tests at $\lambda_{\text{exc}}/\lambda_{\text{em}}$ 580/650 nm; (C) sensitivity test at $\lambda_{\text{exc}}/\lambda_{\text{em}}$ 300/410 nm ($Y = -468.7X - 46161.1$; $R^2 = 0.92022$); and (D) selectivity tests at $\lambda_{\text{exc}}/\lambda_{\text{em}}$ 300/410 nm.

investigated. Fig. 4A illustrates the linear relationship between the red-PL response ($\lambda_{\text{exc}}/\lambda_{\text{em}} = 580/650$ nm) of the Cdots-βCD/NR system with increasing Phe amount. A dynamic linear range from 2 to 100 μM ($Y = 18475.2X + 1490.0$; $R^2 = 0.99487$) was observed with a sensitivity of about 18.47 a.u. mM^{-1} . A limit of detection (LoD) value of about 0.46 μM was obtained using the formula $\text{LoD} = 3.3\sigma/\text{slope}$ (where σ is the background standard deviation value: 2571.35). Similarly, a limit of quantification (LoQ) value of 1.25 μM was calculated using the formula $\text{LoQ} = 9\sigma/\text{slope}$.

The ability of the Cdots-βCD/NR system to selectively discriminate Phe *versus* tyrosine (Tyr) was investigated by measuring the red-PL response at different concentrations of Phe and Tyr (0, 2, 20, 50, 100, 200, 400, and 800 μM). The data obtained confirm the high specificity ($\sim 97\%$) of the proposed assay for Phe, as illustrated in Fig. 4B (red line), whereas only a very slight signal change was observed upon the addition of Tyr (Fig. 4B, black line).

Similarly, Fig. 4C illustrates the linear relationship ($Y = -468.7X - 46161.1$; $R^2 = 0.92022$) between blue-PL signal ($\lambda_{\text{exc}}/\lambda_{\text{em}} = 300/410$ nm) of the Cdots-βCD/NR system and increasing Phe concentration. A dynamic linear range of 20–400 μM was observed, with a sensitivity of about 46.1 a.u. mM^{-1} . An LoD value of about 145 μM was calculated using σ , which is the background standard deviation: 20610.6. Again, a good specificity for Phe recognition, in the concentration range of 0–800 μM , was observed as shown in Fig. 4D (red line), compared to the blue-PL signal recorded upon addition of Tyr (Fig. 4D, black line).

Additional specificity experiments were conducted by adding Phe and Tyr at concentrations of 0, 400, and 800 μM to a water dispersion of the CDs-βCD/NR nanosystem. An increase in the red-PL signal of the CDs-βCD/NR system at 650 nm was observed upon the addition of 400 and 800 μM Phe, whereas

the PL signal remained unchanged upon the addition of the same concentrations of Tyr (Fig. S8).

The Stern–Volmer plot has been used to study the quenching interaction between the emissive Cdots- β CD/NR and Cdots- β CD systems and the Phe as a quencher. In detail, the steady-state fluorescence emission of the carbon-dot core at 410 nm, recorded upon excitation at 300 nm, was monitored in the presence of increasing Phe concentrations ranging from 2 to 800 μ M. For both the free Cdots- β CD and the Cdots- β CD/NR complex, a progressive decrease in fluorescence intensity was observed without any significant shift in the emission maximum (approximately 420 nm). Fluorescence quenching follows the well-known Stern–Volmer equation. The result showed a linear relationship in the Stern–Volmer plot for the Cdots- β CD/NR system, with a K_{SV} value of about $2.2 \times 10^{-4} \mu\text{M}^{-1}$. In contrast, Cdots- β CD showed a linear relationship in the Lehrer-modified Stern–Volmer plot, yielding a higher K_{SV} value of $5.2 \times 10^{-3} \mu\text{M}^{-1}$, indicative of a higher quenching process (Fig. S9).

The stability assessment investigation evidenced that the aqueous dispersion of the Cdots- β CD/NR nanostructures retains the multi-emissive properties for at least 6 weeks (Fig. S10), confirming the good colloidal and photophysical stability of the system over time.

Molecular modelling

Molecular modelling calculations were conducted to investigate the interaction of Phe, Tyr, and NR guests with the β CD cavity. In detail, the β CD/NR/Phe(Tyr) complex formation was evaluated by comparing the Gibbs free energies of the optimized ternary adducts with those of the isolated components. Both β CD/NR/Phe and β CD/NR/Tyr were found to be thermodynamically stable, with calculated formation free energies of -26.4 and $-34.5 \text{ kJ mol}^{-1}$, respectively (Fig. 5). Geometrical analysis provides a more detailed explanation for the different fluorescence responses. In both complexes, NR remains included within the β -cyclodextrin cavity, while the amino acid approaches from the opposite rim. However, the interaction pattern differs markedly. In the β CD/NR/Phe system, Phe shows a more extended hydrophobic/aromatic contact surface with NR. Although the aromatic rings of the amino acid and NR are

not arranged in an ideal parallel π – π stacking geometry, quantitative descriptors indicate a more effective aromatic contact in the β CD/NR/Phe complex. The centroid–centroid distance between the amino acid aromatic ring and the selected aromatic region of NR is comparable in the two systems, being 6.73 Å for Phe and 6.58 Å for Tyr. The minimum atom–atom distance is also very similar, 3.36 Å for Phe and 3.33 Å for Tyr. Therefore, the key difference is not a single shorter contact, but rather the extent of the contact surface. Indeed, in the β CD/NR/Phe system, Phe shows a larger number of close contacts with NR, with 9 aromatic contacts below 4.0 Å and 24 contacts below 5.0 Å, compared to 7 and 19, respectively, for Tyr. Moreover, the angle between the aromatic planes is also smaller for Phe (30.4°) than for Tyr (40.1°), indicating a more favorable mutual orientation of the phenyl ring with respect to the NR aromatic scaffold. These results suggest that thermodynamic stability and fluorescence enhancement are governed by different structural factors. Tyr shows a slight stable ternary complex due to the interaction with β -cyclodextrin through hydrogen bonding, particularly *via* the phenolic OH group. However, this stronger anchoring to the β -cyclodextrin wall reduces the direct aromatic interaction between Tyr and NR. Conversely, Phe establishes a broader hydrophobic/aromatic interface with the dye. This arrangement may rigidify NR and further shield it from the aqueous environment, providing a plausible molecular explanation for the experimentally observed fluorescence enhancement. Overall, the calculations support a cooperative ternary-complex mechanism rather than a displacement process. NR remains included within the β -cyclodextrin cavity, while Phe interacts with the exposed aromatic region of the fluorophore from the opposite rim. The larger aromatic contact surface in the β CD/NR/Phe complex rationalizes why Phe produces a stronger fluorescence enhancement than Tyr, despite the latter being thermodynamically more stabilized through β CD-amino acid hydrogen bonding.

Phe recognition in PKU patients

The Cdots- β CD/NR nanosystem was tested on human blood samples collected from PKU patients. The human participant recruitment criteria used are detailed in the Experimental section. The blood samples were investigated by the tandem-MS technique to quantify the Phe concentration, as described in the Experimental section (Fig. S11). Herein, we report the data from a PKU sample. The mass spectrometry analysis revealed a Phe concentration of about 216 μ M. The DBS sample, investigated as described in the Experimental section using our sensing approach based on the Cdots- β CD/NR system, exhibited a red-PL enhancement ($\lambda_{\text{exc/em}} = 580/650 \text{ nm}$) of about $3.88 \times 10^4 \text{ a.u.}$ The interpolation of this value with the calibration line (Fig. S12), considering a sample volume of 3.2 μ L composed of 1.6 μ L of plasma, provided a Phe concentration of about 252 μ M. These data clearly indicate a correlation of the proposed Cdots- β CD/NR sensing strategy compared with the standard mass-spectrometry investigation.

Table 1 includes a list of comparable Phe sensing strategies including enzymatic and non-enzymatic approaches as well as

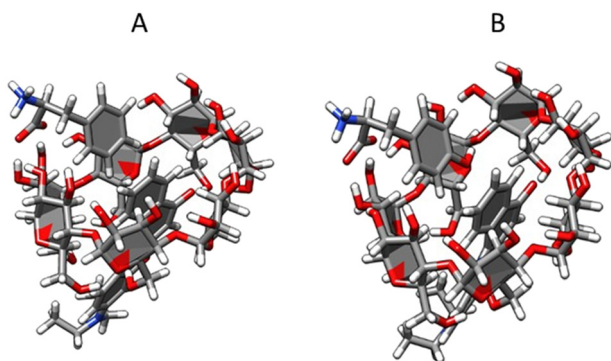


Fig. 5 Molecular modelling: optimized geometries for the supramolecular (A) β CD/NR/Phe and (B) β CD/NR/Tyr complexes.

Table 1 A comparison of different Phe sensing approaches

Sensing strategy	Transduction method	LoD	Linear range	Ref.
Enzymatic (LAAO)	Colorimetric detection of Au nanoparticles	10 μM	17–500 μM	32
Enzymatic (PDH/NAD ⁺)	Colorimetric detection of Au nanoparticles/MMT complex	34.5 μM	48–2000 μM	33
Non-enzymatic	Electrochemical; (DPV) DNA aptamer immobilized onto gold nanostructures	0.23 μM	0.72–6000 μM	34
Non-enzymatic	Electrochemical; specific sonogel-carbon electrodes	31.92 μM	—	35
Non-enzymatic	Electrochemical; mesoporous silica with Fe ₂ O ₃ nanoparticles	0.085 μM	0.14–0.27 μM	36
Non-enzymatic	Electrochemical (CV); graphene quantum dots decorated with Fe ₃ O ₄ nanoparticles	—	0.5–650 μM	37
Enzymatic (PHD/NAD ⁺)	Colorimetric detection of neo-formed tris(bipyridine) ruthenium(II) mediator	0.33 μM	0–7 μM	9
Enzymatic (PHD/NAD ⁺)	Colorimetric detection of <i>in situ</i> formed Au/CTAB nanoparticles	43 μM	50–2000 μM	11
Enzymatic (LAAO)	Colorimetric detection of TMB	0.19 μM	1–90 μM	38
Enzymatic (PHD/NAD ⁺)	Photothermal-contrast of <i>in situ</i> Au-nanoparticle formation on paper membrane	3.6 μM	10–1000 μM	39
Non-enzymatic	Fluorescence; measurement of NBD-F derived Phe through chromatographic separation on pillar-array microchip	0.119 μM	0.4–4 μM	40
Enzymatic (PHD/NAD ⁺)	Colorimetric detection of <i>in situ</i> formed Au/CTAB nanoparticles	1 μM	60–2000 μM	41
Non enzymatic	Electrochemical; on ERGO on a gold electrode	3.5 μM	120–600 μM	42
Enzymatic (PAL)	Colorimetric detection of pH indicator dye	20 μM	20–3000 μM	8
Non-enzymatic	Photoluminescence of MOF@Eu	0.04 μM	20–166 μM	43
Non-enzymatic	Photoluminescence of graphene quantum dots (420 nm)	0.05 μM	0.1–5 μM	44
Non-enzymatic	Photoluminescence (650 nm and 410 nm) of the Cdots- β CD/NR nanosystem	LoD = 0.46 μM LoQ = 1.25 μM	(Red-PL) 2–100 μM (Blu-PL) 20–400 μM	This work

Note: LAAO: flavoenzyme l-amino acid oxidase, MMT: thiazolyl blue tetrazolium bromide, NAD⁺: nicotinamide adenine dinucleotide, PHD: phenylalanine dehydrogenase, PAL: phenylalanine ammonia lyase, NBD-F: 4-fluoro-7-nitro-2,1,3-benzoxadiazole, TMB: 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate, CHA: catalytic hairpin assembly, ERGO: electrochemically reduced graphene oxide modified electrode.

different transduction strategies, such as electric and optical. Our approach is mainly novel in terms of multi-emission read-out, specific supramolecular architecture, green synthesis approach and application to real blood samples from PKU patients.

Conclusions

A water-dispersible, nanosized, multi-emissive supramolecular system based on β -cyclodextrin-carbon nanodots loaded with Nile red (Cdots- β CD/NR) was prepared *via* a two-step process starting from a β -cyclodextrin precursor. The Cdots- β CD/NR supramolecular nanosystem was characterized for its physico-chemical and optical properties, as well as biosensing capacity. Cdots- β CD/NR exhibited multi-fluorescence emission in the blue region (410 nm) with a dynamic range of 20–400 μM and in the red region (650 nm) with a dynamic range of 2–100 μM . The supramolecular system exhibits a limit of detection (LoD) of about 0.46 μM and limit of quantification (LoQ) of about 1.25 μM for the detection of Phe. Moreover, good selectivity for Phe over tyrosine was confirmed and further supported by molecular modelling simulations. The proposed sensing strategy was successfully tested on human blood samples collected from PKU patients, and a good correlation with standard mass-spectrometry measurements was obtained. The simple and green preparation method, free of organic solvents and additives, and excellent optical performance and effective sensitivity

make Cdots- β CD/NR appealing in the research of novel biosensing systems for Phe detection.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: <https://doi.org/10.1039/d6tb01046f>.

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