1. Introduction

Chromophore-appended cyclodextrins (CDs) are spectroscopically active colourful systems engineered to exploit synthetically the supramolecular hosting capabilities of these glucose-based macrocycles with the optical properties of the affixed chromophores.1,2 These dual conjugates are valuable photochemical molecular devices of paramount importance that have found increasing applications in many different areas with a heavy emphasis on imaging, drug delivery, and sensing. Among chromophores, fluorophores provide high sensitivity in analytical applications and a low detection limit in optical imaging methods. Therefore, fluorophore-appended CDs (FCDs) have been profusely investigated as fluorescent chemo- and biosensors for detecting a variety of organic and biological compounds.3-6 The principle behind FCDs is the effect of the microenvironment surrounding the chromophore on their fluorescence properties. Based on their fluorescence behaviour, two types of molecular-based FCD sensors, namely “turn-off” and “turn-on” FCDs, have been described.1,6 For the most abundant “turn-off” FCDs, upon complexion of the analyte, a decrease in the fluorescence intensity is observed while for the “turn-on” FCDs an increase in the fluorescence is detected.

Fluorophores of diverse nature have been incorporated into FCD-based sensors. Classically, various types of molecular fluorophores (aryl derivatives, dansyls, nitrobenzofurans, xanthenes, cyanines, porphyrins, and phthalocyanines, among others) have been used for CND covalent tagging.6 Molecular-based FCDs are generally constructed using the tools of synthetic chemistry and chemical strategies that ensure isomeric purity and a unitary degree of substitution of native polyhydroxylated CDs since well-characterized systems are required for better performance in most of the sensing applications. Usually, monosubstituted FCDs are synthesized using multistep synthetic procedures that enable the realization of a sole FCD regioisomer.
The outstanding progress made in recent years on nanotechnology has enabled the elaboration of novel CD-modified nanoparticles (NPs). Diverse inorganic NPs (gold, silver, quantum dots and magnetic NPs) and carbonaceous nano-materials (fullerenes, nanotubes, graphene and carbon dots) provide suitable platforms for the assembly of CNDs on their surfaces. CD-modified NPs combine the supramolecular loading capabilities of CNDs with the optical, electronic or magnetic properties of NPs. When these systems are used as fluorescent sensors, NPs behave as chromophores governed by the same principles outlined for their molecular counterparts.

Among fluorescent nanomaterials, metal-free carbon dots (CNDs) are leading-edge compounds that have attracted rapidly growing interest because of their outstanding features (cost-efficient and easy preparation, water solubility, low toxicity, water compatibility, and easy functionalization). CNDs are promising candidates for numerous (bio)applications such as bioimaging, theragnosis, drug delivery and fluorescent (bio)sensing. Nonetheless, despite the extensive use of native CNDs as (bio)sensors, the reported cases of hybrid CND–CD composites are scarce and limited to their implementation in (bio)analytical applications. The “turn-off” detection of selected (bio)analytes (fullerenes, phenolic compounds, and enzymes) and, alternatively, more elaborated “turn-off-on” systems for the biosensing of steroid compounds and cholesterol have been described. In the latter case, the competitive hosting between a sacrificial quencher and the desired analyte is used for the successive depletion (“turn-off” state) and restoration (“turn-on” state) of the fluorescence, with beneficial gains in sensitivity. From the synthetic point of view, CND–CD composites are usually obtained by the post-synthetic surface modification of already synthesized CNDs. Typically, fluorescent CNDs are treated with a coupling reagent to activate the carboxyl groups on the surface of the CNDs and then reacted with amino-CDs. However, this strategy is not exempt from drawbacks.

A central issue in CNDs is the origin of the photoluminescence. Although the topic is still a subject of debate, some consensus has been established. For bottom-up CNDs obtained by the co-pyrolysis of citric acid (CA) and an amine, it is accepted that luminescence primarily results from the molecular state rather than from size differences. When α,β-bifunctional ethylenamines (α,β-diamines, β-amino alcohols or α,β-aminothiols) are used, the formation of highly luminescent molecular fluorophores containing a five-membered fused 2-pyridone skeleton has been reported. The citraconic derivative 5-oxo-1,2,3,5-tetrahydroimidazo[1,2-α]pyridine-7-carboxylic acid (IPCA) is the most paradigmatic molecular fluorophore detected (ESI Scheme S1). The plausible mechanism for the formation of IPCA involves, first, the formation of two amide bonds between CA and the amine group of the dopant agent, followed by an intramolecular nucleophilic attack by the β-heteroatom – N, O or S – of the dopant amine to form the five-membered ring fused 2-pyridone. The resulting molecular fluorophores are hypothesized to be located on the surface and/or inside the CNDs. However, as the pyrolisis proceeds, carbon cores are formed with a concomitant consumption of molecular fluorophores, a decrease in the quantum yield and an increase in photostability.

Regardless of the top-down or bottom-up strategy used, CNDs are intrinsically heterogeneous materials. Accordingly, the ulterior functionalization of CNDs with diverse compounds, including CDs, yields composites that are not well defined in terms of structure and composition. In order to avoid the drawbacks associated with the heterogeneity of CNDs and inspired by the fluorescence properties of citrazinic acid-based molecular fluorophores, we report herein the preparation of IPCA-appended CDs (IPCA-CDs). Two highly efficient synthetic chemical strategies starting from suitable pre-modified monosubstituted CDs (β- and γ-derivatives) are described. We also demonstrate that these engineered FCDs behave as CND-based CD “turn-off-on” biosensors using p-nitrophenol as a quencher. As a proof-of-concept, we validate their capabilities as a non-enzymatic cholesterol biosensor and as a sensor for β-galactosidase activity.

2. Results and discussion

2.1. Chemical synthesis and characterization of IPCA-appended FCDs

Despite the outstanding optical properties of IPCA derivatives as fluorophores, limited efforts have been devoted to develop synthetic methods that enable their efficient preparation. In the majority of the reported cases, IPCA-based fluorophores are only identified by spectroscopic (NMR and mass) and analytical techniques, and/or isolated in low yields from the prepared CNDs. In order to access the engineered FCDs that avoid the drawback of the heterogeneity of the CNDs as a fluorescent material, we envisaged two different strategies for the synthesis of IPCA-appended CDs that are based on the reaction of CA with a suitable α,β-ethylenediamine molecular motif (Scheme 1).

The first approach involves the use of diethylenetriamine (DETA) for the preliminary formation of the IPCA skeleton by a reaction with CA, followed by the covalent grafting of the molecular fluorophore obtained to conveniently functionalized α- and γ-CDs. Thus, in a first step 1-(2-aminoethyl)-5-oxo-1,2,3,5-tetrahydroimidazo[1,2-α]pyridine-7-carboxylic acid (AEIPCA) was prepared using a stand-alone procedure by the microwave-assisted condensation reaction of an equimolecular aqueous mixture of DETA and CA (300 W, 130 °C, 1.5 h). The procedure is novel, it allows the isolation of AEIPCA in a good yield (49%), and spectroscopic characterization correlates well with the already published data. AEIPCA was subsequently grafted to mono 6-vinylsulfone-modified CDs (VS-β-CD and VS-γ-CD, 4 and 5) via aza-Michael coupling, exploiting the complementary reactivity of the clickable amine and vinyl sulfone functions. The efficiency and versatility shown by the Michael-type additions of aminated and thiolated nucleophiles to VS in diverse click metal-free (bio)conjugation scenarios have been previously demonstrated by us. The synthetic approach is novel, it allows the isolation of AEIPCA in a good yield (49%), and spectroscopic characterization correlates well with the already published data.
thesis of VS-CDs was carried out with a good yield following the protocol already published by our group.\textsuperscript{35} It consists of a two-step reaction that involves (a) the microwave-assisted nucleophilic substitution of easily accessible mono 6-O-sulfonyl-CDs (Ts-β-CD and Trisyl-γ-CD, 2 and 3\textsuperscript{36}) with ethanolamine to yield the corresponding intermediates, mono-6-(2-hydroxyethyl)amino-CDs, and then (b) vinyl sulfone functionalization of these compounds by the aza-Michael reaction with divinyl sulfone (DVS). The clickable assembly of AEIPCA and VS-CDs (4 and 5) is straightforward, and the corresponding AEIPCA-appended FCDs (6 and 7) were thus isolated.

In an alternative second approach, IPCA was directly synthesized on the CD skeleton using suitable pre-modified monosubstituted α- and γ-CDs containing the α,β-bifunctional ethylenamine motif. Starting from the same mono 6-O-sulfonyl-CDs (Ts-β-CD and Trisyl-γ-CD, 2 and 3\textsuperscript{36}), DETA was first incorporated into the CD scaffold via the substitution reaction of the sulfonyl leaving groups following an already reported procedure for the synthesis of DETA-β-CD (8)\textsuperscript{37} with minor modifications, and then reacted with CA by a thermal condensation reaction (160 °C, 2 h, under pressure). The desired IPCA-CDs (10 and 11) were thus isolated.

The NMR and mass spectra of IPCA-appended CDs (6, 7, 10 and 11) as well as of all the intermediates (4, 5, 8, and 9) are in accordance with the expected chemical and formula structures (see the ESI†).\textsuperscript{25,38} UV-visible spectra in water (Fig. S1, ESI†) show two absorption peaks at 235 nm and 355 nm reported for CNDs that are related to the highly fluorescent IPCA.\textsuperscript{25,30} An additional peak at 285 nm is found in the spectra of compounds 6 and 7 having the sulfone group. As expected, IPCA-appended CDs and compound 1 share the photoluminescence profile of CNDs with a maximum emission peak at 435 nm, typical of CNDs (Fig. S2, ESI†). Regardless of the synthetic approach, the quantum yield (QY) of IPCA-appended CDs (6, 7 and 10, 11) is larger than 0.4, very close to that of compound 1 (QY 0.47) and in the range of that for quinine sulfate (QY 0.53) (Table S1, ESI†). These results demonstrate that the synthesized FCDs have spectroscopic features that resemble those previously reported for CND–CD composites.\textsuperscript{18} At this point, it is important to recall that a major difference between IPCA-appended CDs and CND–CDs is that the latter are heterogeneous materials, whereas the former are well-defined molecules (Fig. S3, ESI†).

2.2. IPCA-appended FCDs as “off-on” sensors for the quantification of cholesterol (Chol)

As discussed, CND–CDs have been used in (bio)analytical applications, in particular for the biosensing of Chol and steroid compounds, using a “turn off–on” strategy that relies on host–guest recognition,\textsuperscript{18,19} where the fluorescence is turned off by a sacrificial quencher and recovered (i.e. turned on) upon displacement by an analyte. For these purposes, p-nitrophenol (pNP) has been reported as a valuable quencher, better than o-nitrophenol or m-nitrophenol, and the mechanism has been identified as static quenching.\textsuperscript{16,41} Additionally, since the absorption spectrum of pNP partially overlaps with the excitation spectrum of the CNDs, the quenching has also been attributed to an inner filter effect.\textsuperscript{17,39–41}

Regardless of the mechanism of quenching, the feasibility of using the novel IPCA-appended FCDs as “off-on” sensors depends on the quenching and recovery of the fluorescence by the selective displacement of the quencher by the analyte. Inspired by the works on CND–CDs, we analysed the effect of pNP as a quencher and Chol as an analyte\textsuperscript{19} on the fluo-
The ability of pNP to form an inclusion complex with β-CD and quench the fluorescence of β-CD-based CND–CDs has been previously exploited to quantify the activity of β-galactosidase,17 α-glucosidase,42 and alkaline phosphatase.15,17 The assays rely on the quenching of the fluorescence by pNP released upon the action of the enzyme on an analog of the substrate that bears the pNP motif. To further explore the biosensing capabilities of the IPCA-appended FCDs, we decided to investigate these systems as sensors for the determination of β-galactosidase activity. For this purpose, we selected FCD 10. As expected, this compound proved to be a good system to quantify the activity of β-galactosidase from E. coli using p-nitrophenyl β-D-galactopyranoside (pNPG) as a substrate. The results obtained from the kinetics of 8 min show a linear response in the range of 9–280 μM mL−1 and R² = 0.98 (Fig. S7, ESIf). These values are basically the same as those reported for β-CD-based CND–CDs,15,17 indicating that the role of CNDs in the assay is limited to acting as a support for the molecules responsible for the fluorescence.

The facts that the fluorescence of AEIPCA (1), either alone or in the presence of free β- or γ-CD, is quenched by pNP (Fig. 1), and that the activity of α-glucosidase is determined by uncoated CNDs19 suggest that, providing a suitable substrate, AEIPCA (1) may be a simpler system to detect enzymes, in general, and β-galactosidase,17 in particular. To determine the role of β-CD in the quenching of the fluorescence by pNP, the activity of E. coli β-galactosidase was evaluated using AEIPCA (1), either alone or in the presence of free β- or γ-CD, and FCDs 10 and 11, where the fluorophore is covalently bonded to β- and γ-CDs, respectively, as controls. Unexpectedly, at the beginning of the experiment (i.e. t = 0), the fluorescence of FCDs 10 and 11 was 25% and 60% of that for AEIPCA (1), which is not affected by the presence of free CDs (Fig. 2),

![Fig. 1 Quenching (Q) with pNP (40 μM) and recovery (R) with Chol (100 μM) of the photoluminescence (PL) of compound 1, alone or in combination with free β- or γ-CD (compounds b and g, respectively), and IPCA-CDs (6, 7, 10 and 11). Values and transparency of the colour are normalized to those before quenching.](image)
Fig. 2 Detection of the activity of E. coli β-galactosidase with compound 1 (blue), alone or in combination with an equimolecular amount of β-CD (dark green) or γ-CD (red), and compounds 10 (light green) and 11 (yellow). Left: Evolution of the fluorescence intensity as a function of time. Right: Determination of the enzymatic activity.

despite they sharing similar QY (Table S1, ESI†). This result suggests the formation of an inclusion complex between CD and pNPG that is plausible according to theoretical calculations.43 However, quenching is evident only when pNPG and AEIPCA (1) are in close proximity or at a high local concentration, as it is the case for FCDs 10 and 11 due to the covalent bond between the fluorophore and CD:pNPG inclusion complex. From the point of view of sensing the enzymatic activity, AEIPCA (1) is as suitable as FCDs 10 and 11 because the values of the enzymatic activity are very close, with the coefficient of variation being 0.15.

3. Conclusions

The feasibility of CD-appended CNDs as sensors has been demonstrated by different authors. These sensors rely on the quenching of the fluorescence of the CNDs by a sacrificial quencher, although the heterogeneity of CNDs hinders their characterization at the molecular level. We have deconstructed the CND–CD systems to demonstrate that (i) the role of CNDs is limited to acting as a support for the molecular fluorophores produced during their synthesis and (ii) the molecular fluorophores suffice for the determination of the enzymatic activity based on the quenching by pNPG as a sacrificial quencher. Additionally, we have described two novel routes for the functionalization of CDs with the IPCA molecular fluorophore described as being responsible for the fluorescence of the CNDs. The resulting IPCA-appended FCDs (IPCA-CDs) have been demonstrated to share many of the features of CND–CDs responsible for their use as “off-on” sensors.

4. Experimental

4.1. Chemical synthesis

Anhydrous citric acid (CA), cholesterol (Chol), diethylametriamine (DETA), divinyl sulfone (DVS), ethanolamine, triethylamine (TEA), 4-nitrophenol (pNP), and 4-nitrophenyl β-galacto-pyranoside (pNPG) were obtained from Sigma-Aldrich (St Louis, MO, USA). Commercial reagents were used as received without further purification. Mono-6-O-toluensulfonyl-β-cyclodextrin (2) and mono-6-(O-2,4,6-trispropylbenzenesulfonyl)-γ-cyclodextrin (3)56 were prepared following the already reported procedures. TLC was performed on Merck silica gel 60 F254 aluminium sheets. Flash column chromatography was performed on silica gel (Merck, 230–400 mesh, ASTM). 1H and 13C NMR spectra were recorded at room temperature using a Varian Direct Drive (400, 500, and 600 MHz) spectrometer. Chemical shifts (δ) are given in ppm; J values are given in Hz. MALDI-ToF mass spectra were recorded on an Autoflex Bruker spectrometer using HCCA and NaI as matrices. Optical rotations were recorded using a PerkinElmer 341 polarimeter at room temperature. Fluorescence spectra and UV-Vis spectra were recorded using a Varian Cary Eclipse luminescence spectrometer and a Spectord 200 Plus Instrument (Analytik Jena, respectively).

4.1.1. Synthesis of 1-(2-aminoethyl)-5-oxo-1,2,3,5-tetrahydroimidazo[1,2-a]pyridine-7-carboxylic acid (AEIPCA) (1). DETA (154 mg, 1.50 mmol) was added to a solution of CA (313 mg, 1.63 mmol) in purified water (40 mL). The reaction mixture was irradiated at 300 W and 130 °C in a Milestone Star Microwave Labstation for 1.5 h. Evaporation of the solvent gave a crude product that was purified by column chromatography (acetone/water/ammonia 8:2:0.25 to 6:2:0.5), yielding pyrone derivative 1 (164 mg, 49%): Mp 204 °C (dec); IR (neat): ν

= 1636, 1535, 1509, 1490, 1446, 1411, 1368, 1293, 1255, 1104, 761, 707, 609, 590, 549 cm⁻¹; 1H NMR (D2O, 400 MHz): δ = 6.10 (d, J = 1.2 Hz, 1H), 5.91 (d, J = 1.3 Hz, 1H), 4.14 (t, J = 8.9 Hz, 2H), 3.77 (t, J = 8.9 Hz, 2H), 3.69 (t, J = 6.0 Hz, 4H), 3.32 (t, J = 6.0 Hz, 2H). 13C NMR (D2O, 126 MHz): δ = 172.71, 162.63, 153.38, 152.38, 103.14, 85.03, 47.49, 44.11, 43.66, 36.72; HR-MS (ESI⁺): m/z = found 224.1026, calcd for C10H14N3O3 [M + H⁺]: 224.1035.

4.1.2. General procedure for the synthesis of mono-6-VS-modified CDs (4 and 5). VS-functionalized CDs were obtained

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following the one-pot two-step procedure reported for the synthesis of compound 4. Briefly, ethanolamine (470 \mu L, 7.75 mmol) was added to a solution of the corresponding mono-6-O-sulfonyl CDs (2 or 3, 0.38 mmol) in DMF (10 mL). The reaction mixture was irradiated at 500 W and 80 °C in a Milestone Star Microwave Labstation for 1 h. Acetone was added (100 mL), and the resulting precipitate was filtered in vacuo and washed with acetone (20 mL) and EtO (20 mL) to yield the corresponding intermediate mono-6-deoxy-6‐hydroxylethylamino-CD as a solid that was dried in vacuo at 192 °C (dec); \[\text{acetone (40 mL) and ether (40 mL). The obtained solid was added (100 mL), and the resulting precipitate was filtered in vacuo, washed with acetone (10 mL) and EtO (10 mL), and then dried in vacuo at 50 °C for one day.}\]

(a) 6-Deoxy-6-(2-hydroxyethyl) (vinylsulfonfyl)methylamino-\(\beta\)-cyclodextrin (4) was obtained as a solid (140 mg, 85%).

(b) 6-Deoxy-6-(2-hydroxyethyl) (vinylsulfonfyl)methylamino-\(\beta\)-cyclodextrin (5) was obtained as a solid (130 mg, 70%); Mp: 235 °C (dec); IR (neat): \(\nu = 3350, 2927, 1738, 1651, 1566, 1154, 1079, 924, 849, 757, 706, 582, 529 \text{ cm}^{-1}\); \[\text{[\alpha]}_D +153.6. (c 0.25, \text{H}_2\text{O}); \text{IR (neat): } \nu = 3300, 2927, 1738, 1566, 1154, 1079, 924, 849, 757, 706, 582, 529 \text{ cm}^{-1}\]; \[\text{[\alpha]}_D +153.6. (c 0.25, \text{H}_2\text{O}); \text{IR (neat): } \nu = 3300, 2927, 1738, 1566, 1154, 1079, 924, 849, 757, 706, 582, 529 \text{ cm}^{-1}\].

4.1.3. General procedure for the VS-based click synthesis of AEIPCA-appended FCDS (6 and 7). To a solution of the corresponding mono-6-O-modified CDs (4 or 5) (0.1 mmol) and the AEIPCA molecular fluorophore 2 (1.1 mmol) in DMSO-water (1:2, 9 mL) was added triethylamine (50 µL, 0.3 mmol). The reaction mixture was heated at 50 °C for 6 h and then acetone (50 mL) was added. The resulting precipitate was collected by centrifugation and successively washed with acetone (40 mL) and ether (40 mL). The obtained solid was purified by column chromatography to yield the corresponding IPCA-appended FCDS (6 or 7):

(a) IPCA-\(\gamma\)-FCDS (6): Column chromatography (acetonitrile/water/ammonia 5:2:0.1) gave 6 (140 mg, 92%) as a solid: Mp: 92 °C (dec); \[\alpha]_D +44.8 (c 0.25, \text{H}_2\text{O})); \text{IR (neat): } \nu = 3214, 1649, 1551, 1412, 1077, 1028, 612 \text{ cm}^{-1}\); \[\text{[\alpha]}_D +153.6. (c 0.25, \text{H}_2\text{O}); \text{IR (neat): } \nu = 3300, 2927, 1738, 1566, 1154, 1079, 924, 849, 757, 706, 582, 529 \text{ cm}^{-1}\]; \[\text{[\alpha]}_D +153.6. (c 0.25, \text{H}_2\text{O}); \text{IR (neat): } \nu = 3300, 2927, 1738, 1566, 1154, 1079, 924, 849, 757, 706, 582, 529 \text{ cm}^{-1}\].

(b) IPCA-\(\gamma\)-FCDS (7): Column chromatography (acetonitrile/water/ammonia 6:2:0.1) gave 7 (59 mg, 35%) as a solid: Mp: 198 °C (dec); \[\alpha]_D +76.4 (c 0.25, \text{H}_2\text{O}); \text{IR (neat): } \nu = 3231, 1736, 1651, 1555, 1417, 1079, 1024, 996, 759, 705, 613, 527 \text{ cm}^{-1}\); \[\text{[\alpha]}_D +153.6. (c 0.25, \text{H}_2\text{O}); \text{IR (neat): } \nu = 3300, 2927, 1738, 1566, 1154, 1079, 924, 849, 757, 706, 582, 529 \text{ cm}^{-1}\]; \[\text{[\alpha]}_D +153.6. (c 0.25, \text{H}_2\text{O}); \text{IR (neat): } \nu = 3300, 2927, 1738, 1566, 1154, 1079, 924, 849, 757, 706, 582, 529 \text{ cm}^{-1}\].

4.1.5. General procedure for the in situ CA-based synthesis of IPCA-appended FCDS (10 and 11). A solution of the corresponding IPCA-modified CDs (8 or 9) (0.50 mmol) and CA (0.40 mmol) in water (10 mL) was heated at 160 °C in a sealed Teflon reactor under pressure for 2 h. After cooling to room temperature, the solvent was evaporated in vacuo and the resulting solid was purified by column chromatography to yield IPCA-appended FCDS (10 and 11):

(a) IPCA-\(\beta\)-FCDS (10): Column chromatography (acetonitrile/water/ammonia 5:2:0.1) gave 10 (280 mg, 42%) as a solid: Mp: 228 °C (dec); \[\alpha]_D +91.6 (c 0.25, \text{H}_2\text{O}); \text{IR (neat): } \nu = 3214, 1717, 1651, 1553, 1366, 1230, 1151, 1076, 1022, 946, 753, 704, 610, 576, 528 \text{ cm}^{-1}\); \[\text{[\alpha]}_D +153.6. (c 0.25, \text{H}_2\text{O}); \text{IR (neat): } \nu = 3300, 2927, 1738, 1566, 1154, 1079, 924, 849, 757, 706, 582, 529 \text{ cm}^{-1}\]; \[\text{[\alpha]}_D +153.6. (c 0.25, \text{H}_2\text{O}); \text{IR (neat): } \nu = 3300, 2927, 1738, 1566, 1154, 1079, 924, 849, 757, 706, 582, 529 \text{ cm}^{-1}\].
4.2. Fluorescence studies

4.2.1 Quantum yield. Fluorescence quantum yield (QY) was estimated using quinine sulfate dissolved in 0.1 M H₂SO₄ as a standard (QYₚₑ = 0.54) and applying the following equation:

\[ QY = QY_{st} \times \left( \frac{m_{sm}}{m_{st}} \right) \times \frac{n_{sm}^2}{n_{st}^2} \]

where QYₚₑ is the QY of the standard, m is the slope of the plot fluorescence intensity vs. absorbance at the excitation maxima for the sample (sm) and the standard (st), and n is the refractive index. Samples were dissolved in Milli-Ro water (conductivity: 18.2 MΩ cm) to values of absorbance <0.1 and the integrated emission intensities were recorded.

4.2.2. Determination of the lag time. The fluorescence of a solution of 2.5 mL of IPCA-CD (2 µg mL⁻¹) in phosphate buffered solution was monitored as a function of time upon the addition of pNP (50 µL, 10 mM). Once no change in the fluorescence was detected, Chol in EtOH (125 µL, 2 mM) was added and the recovery of the fluorescence was monitored for 15 min. The excitation and emission wavelengths were 365 nm and 435 nm, respectively.

4.2.3. Quenching of the fluorescence by p-nitrophenol. The fluorescence of a phosphate buffered solution of IPCA-CDs (2.5 mL, 2 µg mL⁻¹) was monitored as a function of the concentration of pNP. Successive amounts of pNP (10 mM) were added to reach a final concentration of up to 40 µM. After incubation (5 min), the sample was excited at 365 nm and the emitted intensity in the range of 375–625 nm was recorded.

4.3. Fluorimetric assays

4.3.1. Detection of Chol in human serum as a matrix. A phosphate buffered solution of 6 (2400 µL, 2 µg mL⁻¹) supplemented with pNP (10 µL, 2 mM) was combined with human serum (100 µL) from healthy volunteers. The samples were spiked with different amounts of Chol (20, 40, 60, and 80 µM). After incubation (10 min), the emission at 435 nm (excitation at 365 nm) was monitored.

4.3.2. β-Galactosidase activity. β-Galactosidase activity was assayed in 25 mM HEPES, pH 7.3, 20 µM MgCl₂, 200 µM pNP and E. coli β-galactosidase (14 U L⁻¹). The sensing system consisted of either 210 µM IPCA-CD or compound 1 plus IPCA-CD (equimolecular amount). The solution was excited at 365 nm and the fluorescence emitted at 435 nm was recorded every 30 seconds for 15 min. The activity was estimated as the slope of the plot I/I₀ vs. time for the first 8 min. At the optimal time of assay (8 min), the enzymatic activity of E. coli β-galactosidase was assayed in a range of 9.3–280 U L⁻¹.

Conflicts of interest

There are no conflicts to declare.

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