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Evaluation of acetylcysteine promoting effect on CdTe nanocrystals photoluminescence by using a multipumping flow system

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ABSTRACT

A simple and straightforward quantification method integrated in a fully automated multi-pumping flow system (MPFS) using water-soluble mercaptopropionic acid (MPA)-capped CdTe quantum dots (QDs) was implemented for the fluorescence quantification of *N*-acetyl-L-cysteine (NAC) in pharmaceutical formulations. The developed approach was based on NAC ability to establish surface interactions that result in enhanced nanocrystals fluorescence intensity, proportional to analyte concentration. Size and concentration of QDs, ageing, composition, concentration and pH of the buffer solution revealed to have a noticeable effect on the enhancing efficiency affecting sensitivity and linear working range of the methodology.

Under the optimal conditions, a linear working range was obtained for NAC concentrations ranging from 50 to 750 μmol L^{-1} ($r = 0.9978$), with good precision (r.s.d. < 1.6%; $n = 5$) and a sampling rate of about 75 hr^{-1}. The detection limit (LOD) was approximately 1.6 μmol L^{-1}. The method was applied to pharmaceutical preparations and the results revealed good agreement with those obtained by the reference procedure with relative deviations between −2.1 and +4.2%. Advantages of the new procedure include speed, low consumption of reagents, minor waste generation, requiring also much less work than the recommended HPLC method. The mechanism for luminescence enhancement of CdTe QDs is discussed. FT-IR spectra revealed that sulphydryl groups of NAC have a high affinity with the nanocrystals.

1. Introduction

Colloidal nanocrystals, often referred to as quantum dots (QDs), have gained increasing interest over the past decade to their unique properties such as size-tunable optical properties, wide absorbance range, narrow emission, strong fluorescence intensity and excellent photostability [1]. Although QDs have been widely used in biolabeling and bioimaging [2], a new area has recently emerged in which QDs are used in the development of fluorescence sensors for analytical applications [3]. In this regard, in literature there is already a reasonable amount of published methodologies concerning analytical quantification procedures for detection macromolecules such as proteins and nucleic acids, as well vitamins, heavy metals, etc., involving surface interactions with these nanocrystals [4–7]. So far, development of fluorescence sensing schemes for analytical applications using quantum dots has been almost exclusively based on discrete approaches (manual sample handling and measuring). These approaches present critical drawbacks namely high consumption of solutions, complex and laborious sample preparation procedures, poor reproducibility and repeatability and are time-demanding; manual handling is also more prone to errors. Continuous flow-based techniques, as expeditious solutions to overcome these drawbacks, are finding their way into quantum dots analytical exploitation. Sequential injection analysis (SIA) [8] and flow injection analysis (FIA) [9–11] approaches, using fluorescence and chemiluminescence detection, respectively, have been proposed. Fortes et al. [12] developed a multipumping flow system (MPFS) for the determinations of glicazide and glipizide in pharmaceutical formulations using CdTe quantum dots as chemiluminescence sensitizers. A single interface flow analysis (SIFA) system was implemented for the assessment of chemical oxygen demand by using CdTe nanocrystals as radical generators for organic matter photodegradation [13]. These works showed that quantum dots photoluminescence could be significantly influenced by changes on QDs surface charge or ligands that affect electron–hole recombination. Direct binding of the analyte on the QDs surface could, for instance, induce a change on the fluorescence response resulting in either a enhancing or a quenching effect.

N-Acetyl-L-cysteine (*N*-acetyl-2-amino-3-mercaptopropionic acid, NAC), is an acetyl derivative and one of the homologs of the L-cysteine, being a biologically active compound owing to the crucial role in biological systems [14]. NAC is also used as a pharmaceutical drug due to its importance as a mucolytic

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agent used to reduce the viscosity of pulmonary secretions in respiratory diseases among other potential applications mainly due to its antioxidant action [15]. For the determination of NAC in pharmaceutical formulations, the United States Pharmacopeia [16] recommends high-performance liquid chromatography (HPLC) whereas the Brazilian Pharmacopeia [17] describes a titrimetric procedure. Nevertheless, based on the-on-going research of the quality control analyses of NAC-containing pharmaceuticals, several alternatives of flow-injection procedures have been developed with different detection systems via spectrophotometric [18,19], chemiluminescence [20], potentiometric [21], amperometric [22], voltammetric [23], etc. Moreover, SIA has also been reported in the quantification of this analyte as another alternative of flow analysis with different detection systems such as fluorimetric [24] and spectrophotometric [25]. Although the development of those conventional flow systems permitted to overcome most of the shortcomings associated to the commonly used chromatographic methods, recently developed MPFS [26,27] has provided additional features and brought important advantages in terms of analytical application such as ease and simplicity of assemblage, operation and control, compact and straightforward configuration increasing versatility and minimizing solutions consumption, high mixing capacity, etc. Based on the exclusive utilization as active components of multiple solenoid actuated micropumps of very small size it is possible to assure a very simplified configuration of the flow system due to the different task they could be accountable for including sample and reagents insertion and propelling, the implementation of different strategies for solutions mixing and reaction zone establishment. Moreover, the characteristic pulsed flow ensures an effective sample/reagent mixing leading to a better and a faster reaction zone homogenization and thus improved analytical signal.

The aim of this work was to study the surface interactions between *N*-acetyl-L-cysteine and CdTe nanocrystals and, as a consequence of the pronounced fluorescence enhancement observed, to develop a simple and fast automated analytical methodology for its determination in pharmaceutical formulations providing a high analytical sensitivity and a wide dynamic working range. Due to the versatility, efficiency and feasibility guaranteed by the MPFS, important analytical parameters such as solution volumes and flow rate, reactor length, size and concentration of the QDs, buffer and sample pH, as well as QDs ageing time were evaluated for their repercussion on analytical sensitivity and reaction rate.

2. Experimental

2.1. Apparatus

For fluorescence measurement an optical fiber (QP1000-2-UV-VIS, Ocean Optics, Dunedin, USA) spectrofluorometer (USB 4000-FL, Ocean Optics, Dunedin, USA) equipped with a LS-450 light source and a CUV-UV Holder, was used. The excitation maximum at 395 nm was guarantee using the LED-395 (Ocean Optics, Dunedin, USA) as source. The designed flow manifold comprised three 120SP (Bio-Chem Valve Inc. Boonton, NJ, USA) solenoid actuated micropumps, which were of the fixed displacement diaphragm type, two of them (P_1 and P_3) were dispensing 10 μL per stroke while P_2 was dispensing 20 μL per stroke. All connections, depicted in Fig. 1, were made of PTFE tubing (0.8 mm i.d.) with lab-made end-fittings and confluence connectors. The holding coil (*L*) was serpentine-shaped in configuration and was 60 cm long. A 8 μL inner volume flow cell (176.753-QS, Hellma) completed the flow system.

A PC was used to control the flow system by means of dedicated software built in Visual Basic, which enabled to operate the solenoid pumps by RS-232 C serial binary data signals. The system was implemented in a computer equipped with a PC-LAB card model PCL-711 B (Advantech, Taipei, Taiwan) interface card. A homemade power drive based on the ULN2003 chip was used to operate the solenoid micro-pumps. The data acquisition was accomplished by using the Spectrasuite software version 2007 (OceanOptics, Dunedin, USA).

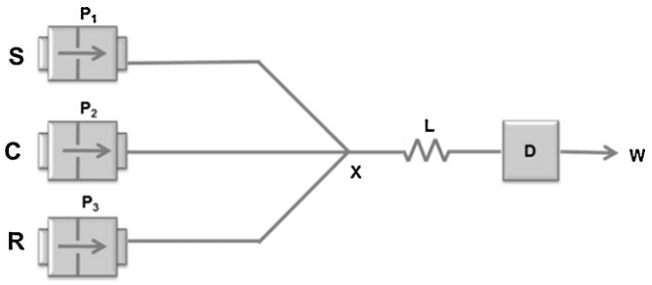

Fig. 1. Multipumping flow manifold for the determination of *N*-acetyl-L-cysteine. P_1 and P_3: solenoid micro-pumps (10 μL per stroke); P_2: solenoid micro-pump (20 μL per stroke); *X*: confluence point; *L*: reactor (60 cm); D: Fluorimeter detector (λ_{exc} = 395 nm, λ_{em} = 522–524 nm), *W*: waste; *S*: sample in 6 mmol L^{-1} acetate buffer at pH 5.2; *C*: Carrier (water); *R*: 5 μmol L^{-1} CdTe QDs.

Absorbance spectra for the characterization of the synthesized QDs were measured on a Jasco V-660 spectrophotometer. Fluorescence measurements were carried out with a PerkinElmer LS-50B luminescence spectrometer. QDs centrifugation was performed with a ThermoElectron Jouan BR4I refrigerated centrifuge.

FT-IR spectra were obtained on an ATI Mattson Genesis series (software: WinFirst v. 2.10) spectrophotometer in KBr pellets (cm^{-1}).

All pH measurements were made with a Model pH-meter GLP 22 (Crison, Allela, Spain).

The absolute emission quantum yields were measured at room temperature using a quantum yield measurement system C9920-02 from Hamamatsu with a 150 W xenon lamp coupled to a monochromator for wavelength discrimination, an integrating sphere as sample chamber, and a multichannel analyzer for signal detection. Three measurements were made for each sample so that the average value is reported. The method is accurate to within 10%.

2.2. Samples, standards and reagents

All solutions were prepared with water from Millipore Q system (conductivity ≤ 0.1 μs cm^{-1}) and analytical grade chemicals. A 4 mmol L^{-1} *N*-acetyl-L-cysteine (Sigma–Aldrich. Chemie GmbH, Steinheim, Germany) standard solution was prepared by dissolving 13 mg in 20 mL of deionized water and kept in the refrigerator.

For the preliminary studies, several NAC solutions were tested by using various buffers with different pH. Based on this, 500 μmol L^{-1} NAC was diluted with different concentrations following buffers: Na_2HPO_4/NaH_2PO_4, citric acid/Na_2HPO_4, boric acid/NaOH and sodium acetate/acetic acid [28], adjusted to different pH values. Within the range of 3.6–8, depending on the pH range of each buffer, the luminescence intensity variation ΔF (%), calculated according to Eq. (3), was evaluated. For further studies reported in this paper, the acetate buffer with pH from 3.4 to 5.9 was the selected one as described in the optimization section.

After optimization, the working NAC standards ranging from 50 to 750 μmol L^{-1} were prepared, on a daily basis, by appropriate dilution of the above stock solution. An appropriate volume of 6 mmol L^{-1} acetate/acetic acid buffer solution at pH 5.2 was transferred to those flasks containing the aforementioned working NAC standards.

Six commercially available pharmaceutical samples, one in granulated form and five in liquid form containing NAC were analyzed according to the proposed procedure. No pre-treatment was required for any of the liquid samples prior to analysis. For the granulated form, this was accurately weighed, dissolved in water and filtered. For the remaining five samples, a known amount of each sample already diluted was transferred to a volumetric flask of 10 mL. Afterwards, to those flasks were transferred 300 μL of 0.2 mol L^{-1} acetate/acetic acid buffer solution at pH 5.2, filling the volume up to the mark with deionized water. The amounts of sample were defined in accordance to the labeled NAC contents of the assayed samples.

For the synthesis of the CdTe quantum dots, tellurium powder (200 mesh, 99.8%), sodium borohydride ($NaBH_4$, 99%), cadmium chloride hemi(pentahydrate) ($CdCl_2{\cdot}2.5H_2O$, 99%) were purchased from Sigma–Aldrich (St. Louis, MO, USA); 3-mercaptopropionic acid (MPA, 99%) and absolute ethanol (99.5%) were obtained from Fluka (St. Louis MO, USA) and Panreac (Barcelona, Spain) respectively.

QDs solutions were prepared by dissolving a certain amount of the dried nanocrystals in ultrapure water and used directly.

2.3. Synthesis of CdTe QDs

Three different diameters of MPA-capped CdTe QDs were synthesized as described by Zou et al. [29] with some modifications. Briefly, the first stage consists on the reduction process of tellurium with $NaBH_4$ in N_2 saturated water to produce NaHTe. After all tellurium has been consumed, the resulting solution was transferred into a second flask containing 4.0×10^{-3} mol of $CdCl_2$ and 6.8×10^{-3} mol of MPA in 100 mL N_2 saturated solution. The pH of the solution was adjusted to 11.5 with a 1.0 mol L^{-1} NaOH solution. The molar ratio of Cd^{2+}:Te^{2-}:MPA was fixed at 1:0.1:1.7. The size of CdTe QDs was controlled by changing the refluxing time.

To purify CdTe QDs, these were precipitated in absolute ethanol to remove the contaminants and the precipitate was subsequently separated by centrifugation, vacuum dried, kept in amber flasks and protected from light, for posterior use. The particle size of the synthesized QDs was calculated by the following expression Eq. (1) [30]:

$$D = (9.8127 \times 10^{-7})\lambda^3 - (1.7147 \times 10^{-3})\lambda^2 + (1.0064)\lambda - (194.84) \quad (1)$$

where D is the diameter (nm) and λ (nm) the wavelength of maximum absorbance corresponding to the first excitonic absorption peak of the crystal.

To facilitate the preparation of the QDs solutions was also necessary to calculate the molar weight of the different sized nanocrystals. This was carried out by establishing firstly the extinction coefficient (ε) by using the expression Eq. (2) [30]:

$$\varepsilon = 3450\Delta E(D)^{2.4} \quad (2)$$

where ΔE is the transition energy corresponding to the first absorption peak and the unit is in eV. Knowing ε it was simple to reach the molar mass by measuring the absorbance of a known concentration solution and by applying the Lambert–Beer law.

In order to reach the desired molar concentration of QDs, a certain amount of the dried nanocrystals was dissolved in ultrapure water.

Table 1 shows the maximum emission wavelength (nm) and the corresponding concentrations of CdTe nanocrystals for the three sizes that have been employed in this paper.

Table 1
Data corresponding to the diameter, λ emission max., quantum yield (%), and concentrations of the nanocrystals used in this work.

Diameter (nm)	λ Emission max. (nm)	Quantum yield (QY%)	Concentration (μmol L^{-1})
1.87	~523	8	2.5; 5; 10
3	~574	29	1.25; 2.5; 5
3.8	~647	35	1.25; 2.5; 5

Table 2
Range of values used in dimensioning the MPFS system, and selected operating conditions for the NAC determination using 1.87 nm CdTe QDs.

Parameters	Evaluated range	Selected value
Sample volume (μL)	[30–80]	60
QDs volume (μL)	[30–80]	60
Carrier stream volume (μL)	[1000–1600]	1200
Reactor length (cm)	[25–85]	60
QDs/sample flow rate (mL min^{-1})	[1.2–3]	2
Carrier flow rate (mL min^{-1})	[2,3]	2.4
Acetate buffer concentration (mol L^{-1})	[0.001–0.008]	0.006
pH range	[5,6]	5.20
QDs ageing time (days)	[1–6]	2
QDs concentration (μmol L^{-1})	[2.5; 5; 10]	5

2.4. Manifold and MPFS procedure

The analytical flow manifold pictured in Fig. 1, comprised three solenoid micro-pumps (P_1, P_2, P_3) which were responsible for the individual insertion and propulsion of sample and reagent solutions. P_1 and P_3 were responsible of the insertion of the sample and QDs solution, respectively, whereas P_2 pumped the carrier stream and simultaneously was responsible for the establishment of the baseline. The versatility provided by the actuation of the micropumps allowed controlling at once the sample and reagent volumes, flow rate and the sequence and timing of sample insertion and reaction zone establishment. The developed analytical cycle began after all flow tubing had been filled with the corresponding solutions. After this first step, the baseline was established by repeated actuation of P_2 used to propel the water (as carrier) toward waste while P_1 and P_3 remained switched off. Subsequently, sample was inserted by simultaneous actuation of P_1 and P_3 for a pre-set number of pulses, generating a flowing stream (at confluence point X) resulting from the merging of QDs and sample aliquots, which were directed to fill the reactor L. These solutions when flowing throughout L establishing a reaction zone that by actuating P_2 and keeping P_1 and P_3 deactivated permitted the reaction zone to be carried out toward the detector generating an analytical signal whose magnitude was proportional to the NAC concentration. The fluorescence emission was monitored at 395 nm (λ_{em} = 522–524 nm).

3. Results and discussion

3.1. Sampling strategy, sample volume and flow rate

Aiming at the maximization of the analytical signal, some optimization studies involving physical and chemical parameters were carried out using the univariate method (Table 2). This was attained by using the QDs with a size of 3 nm at a concentration of 5 μmol L^{-1} in water and a 500 μmol L^{-1} NAC solution also in water.

Even though initial studies showed that the use of a reactor did not show a relevant influence on the analytical signal, the length of the serpentine reaction coil was assessed between 0 and 85 cm, by using an insertion of 6 pulses for both the sample and the reagent. The results demonstrated that there was a slight increase on the analytical signal up to 25 cm, although for longer coils the signal slightly dropped probably because of an increase in sample

dispersion. As a compromise between repeatability and sample dispersion for the subsequent assays a reactor of 60 cm length was selected.

Different sampling strategies, such as merging zones, binary sampling and the insertion of a unique sample volume were exploited and it was verified that merging of sample and reagent zones was the most suitable strategy as it provided enhanced repeatability and sensitivity, and a more stable baseline. Moreover, it assured lower reagent consumption and higher sampling rates. Taking into account the sample volume, the obtained results demonstrated an improvement in sensitivity up to 6 pulses of sample solution (60 μL of sample, 120 μL for reaction zone) and a subsequent stabilization for higher values, at a flow rate of 1.2 mL min^{-1}. The influence on the peak height of stopping the flow was also examined for halting periods of 30–120 s and the results showed that the signal was not affected by any of the stop periods.

Another relevant parameter in system design was the flow rate which is determined by the pulse frequency and can affect not only the reaction development but also the sample throughput. The flow rate of sample and QDs solutions was assessed in the range of 1.2–3 mL min^{-1}. The magnitude of the analytical signal seemed not to be influenced by the flow rate because the analytical signal remained practically unchanged over all tested values although the repeatability decreased for the higher flow rates. Aiming at a compromise between sampling rate and repeatability, a flow rate of 1.2 mL min^{-1} was selected for the further experiments. Considering the flow rate of the carrier, a value of 2.4 mL min^{-1} was selected since it decreased the time required for the analysis without compromising precision.

3.2. CdTe QDs and acetate buffer

Preliminary studies revealed that NAC interacted with MPA-capped CdTe quantum dots in slightly acidic medium yielding a fast fluorescence enhancement. The emitted light intensity was directly related to the amount of NAC in the sample and depended on the pH and concentration of buffer solution and on its composition. Nevertheless, the CdTe QDs solution had a noteworthy impact on the analytical signal, regarding its concentration, size and even the ageing time. Given the extensive range of chemical and physical variables affecting analytical signal magnitude that have emerged during this work, a fully automated MPFS was implemented for automating the optimization procedure and the subsequent determinations due to its high analytical efficiency and speed, ease of parameters adjustment as well as its high reproducibility and repeatability which facilitates the study of a set of reaction conditions that otherwise would be very tedious, laborious and time-consuming to accomplish.

A factor that was found to promote CdTe fluorescence, even in the absence of NAC, was the buffer solution. Knowing this, in all experiments the fluorescence intensity arising from the interaction between the acetate buffer solution and the QDs was used as the blank reading. The evaluation of the fluorescence enhancing effect, $\Delta F(\%)$, was made through the ratio between the fluorescence emission of the sample solution (F_s) and of the blank (F_0) according to the Eq. (3):

$$\Delta F(\%) = \frac{F_0 - F_s}{F_s} \times 100 \quad (3)$$

All experimental conditions were optimized in order to obtain the highest $\Delta F(\%)$.

Regarding the chemical optimization, the magnitude of the analytical signal was observed to be strongly affected by the QDs size, age, pH, composition and concentration of the buffer. A study of the effect of such variables on the analytical sensitivity allowed achieving the optimal conditions and also to understand some phenomena occurring on the surface of the QDs. By using two different sizes of the nanodots, and two concentrations for each size, some work was done to study the impact on the $\Delta F(\%)$ of diluting NAC in buffer solutions of distinct composition and concentration, over a wide range of pH, already described in the experimental section. After an extensive evaluation of the obtained results, the acetate buffer was selected for the succeeding optimization assays due to two main reasons. The first reason is that when the NAC solutions were slightly acidified, independently of the type of buffer, an increase of luminescence intensity was observed. Quite the opposite, when NAC solutions were made more alkaline, no changes on signal magnitude were noticed. This result is particularly interesting because the generality of the analytical procedures available in literature using QDs capped with carboxylic acids, such as MPA, tended to avoid acidic pH. The main reason is that at acidic pH the surface negative charge of QDs decreases and these tend to aggregate and precipitate. Since the referred analytical procedures are discrete ones reaching a stable reading under these conditions is very difficult. On the contrary, when using a flow-based methodology (like MPFS) all measurements are carried out at the same reproducible reaction time (determined by flow rate and reaction coil length), which did not require the attainment of a stable reading. Therefore the exploitation of stability detrimental reaction conditions, which would be forbidden in a batch method, is possible in flow analysis. The second reason is that under the selected range of low pH, it was also possible to see a greater increase of the signal $\Delta F(\%)$ when acetate buffer was used in comparison with other buffers. Furthermore, this buffer seemed not to interfere with the QDs/NAC interaction. Another variable that revealed to affect $\Delta F(\%)$ was the concentration of acetate buffer solution. At pH in the range 3.4–5.9, concentration values between 1 and 7 mmol L^{-1} were assayed being the highest analytical signal difference observed for 6 mmol L^{-1} which was then selected for the posterior experiments.

By using the previously selected variables, three sizes of QDs (1.87, 3 and 3.8 nm), each one at three different concentrations, were evaluated in terms of fluorescence intensity, reactivity, repeatability and reproducibility. Two distinct ranges of concentrations were established according to the QDs size: for the bigger sizes (3 and 3.8 nm), the influence of QDs concentration was evaluated from 1.25 to 5 μmol L^{-1} while for the smaller ones (1.87 nm) the QDs concentration was studied between 2.5 and 10 μmol L^{-1}. It was observed that for former the analytical signal increased up to 2.5 μmol L^{-1} and then decreased while for the later it increased until 5 μmol L^{-1} followed by a drop. This could be explained by the fact that when the concentration reached higher values it led to a decrease of fluorescence mainly due to the inner filter effect as a result of re-absorption of emitted radiation. The concentration and diameter of QDs were therefore of utmost importance affecting QDs reactivity and the sensitivity of NAC determination. After a detailed and extensive study on the evaluation of the fluorescence response of the various QDs sizes with the corresponding concentrations, the highest sensitivity (higher $\Delta F(\%)$) was verified with the 1.87 nm nanodots. Although these nanoparticles were observed to be less fluorescent than the two other sizes, they exhibited a higher reactivity. A possible explanation is that they present a greater number of surface defects that can coordinate with the different groups of the NAC, which also justifies the lower quantum yield QY%, when compared to the bigger QDs (Table 1), thus the evidently lower fluorescence.

After establishing the concentration of acetate buffer and dot size (1.87 nm) the pH was readjusted between 5 and 5.9 (the fluorescence signal remained almost constant for a pH down to 4.9 but the solution stability markedly decreased). Another variable that was considered in the assessment of the optimization

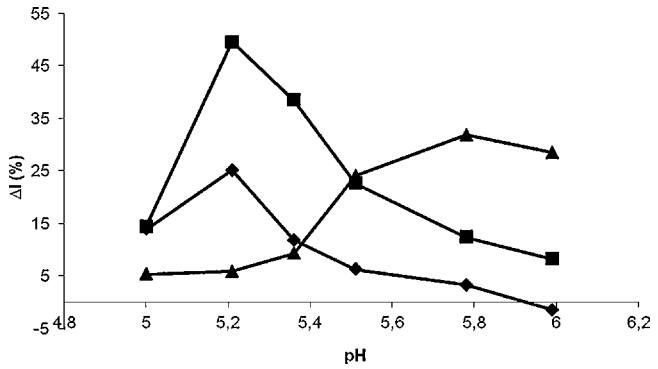

Fig. 2. Influence of the pH on the analytical signal over three days of QDs ageing time for a fixed concentration of *N*-acetyl-L-cysteine: 500 μmol L^{-1} and for a fixed concentration of acetate buffer: 6 mmol L^{-1}. (♦) 1st day; (■) 2nd day; (▲) 3rd day.

variables was the quantum dots solutions ageing. We have noticed differences on the variation of the fluorescence signal in terms of photostability/reactivity whether fresh solutions of the QDs or old ones (with some days of age) were used. For this reason a study was carried out to evaluate the fluorescence response according to the age of the QDs solutions. QDs concentrations of 2.5 and 5 μmol L^{-1} were studied over six days within the pH range already selected. Subsequently, for each day of ageing, the fluorescence intensity variation for each concentration of the QDs was observed to vary randomly over the pH range. After some repetitions, the concentration of 5 μmol L^{-1} was estimated to be the optimum value according to those parameters of validation given by an increase of approximately 78% on the fluorescence signal at pH of 5.21 in the second day of ageing (Fig. 2). Nevertheless, for the concentration of 2.5 μmol L^{-1}, an increase of approximately 47% on the analytical signal at pH 5.78 in the second day of ageing was observed as the highest value. As perceived by the results, the fluorescence emission of CdTe QDs demonstrated to be pH-sensitive with the ageing time and with the concentration of the quantum dot. Since the final decision was influenced by compromise between analytical sensitivity and wide range of linearity, a calibration curve was plotted for both concentrations. After an evaluation of the calibration curves, the concentration of 5 μmol L^{-1} was the applied one, since lower detection limit (LOD) was efficiently reached as well wider linear working range. In the graph of Fig. 2 is only plotted the influence of the pH on the analytical signal over three days of QDs age for the selected concentration of 5 μmol L^{-1}. Under the optimized experimental conditions a linear range for NAC concentrations within 50 and 750 μmol L^{-1} was defined.

3.3. Interferences

The interfering effect of several substances usually present in the pharmaceutical formulations such as excipients, like benzalkonium chloride, sodium benzaoate and sodium chloride was assessed. Samples containing NAC at a fixed concentration of 13 mg L^{-1} (corresponding to 80 μmol L^{-1}) and increasing concentrations of the excipient (up a 100-fold molar ratio) were analyzed by the developed methodology. A compound was considering as non-interfering if the analytical signal variation was ±3% when compared to the one obtained in its absence. The results (Table 3) showed that under the system operating conditions, no interfering effect was observed.

3.4. Analysis of pharmaceutical formulations

After system optimization, the proposed procedure was applied to the determinations of NAC in pharmaceutical formulations. By using a 5 μmol L^{-1} QDs solution a linear working range for NAC concentrations between 50 and 750 μmol L^{-1} was obtained. The calibration curve was expressed by the equation (Eq. (4)):

$$\Delta F = 66.315C + 100.87 \quad (4)$$

in which ΔF was the *delta* of fluorescence intensity and C was the logarithm of NAC concentration, in μmol L^{-1}, with a correlation coefficient of 0.998 ($n = 5$). The precision of the MPFS system was also satisfactory as corroborated by the r.s.d. < 1.6%, $n = 5$. The detection limit and the quantification limit (LOQ) were calculated according to Skoog et al. [31] using the following expression: LOD = $3\sigma/b$ and LOQ = $10\sigma/b$, where σ is the standard deviation of 20 measurements of the blank and b is the slope of the analytical curve. The LOD and the LOQ were 1.6 and 5.3 μmol L^{-1}, respectively; these results demonstrate the sensitivity of the proposed MPFS spectrofluorimetric procedure. The sampling frequency was about 75 hr^{-1}, with the consumption of 60 μL of both sample and QDs solution per determination. This represents a noteworthy improvement regarding the official method that requires 20 min for the determination of just one sample.

Table 3
Interfering effect of excipients on the developed methodology.

Interference	Tolerance limit[a]
Benzalkonium chloride	25
Sodium benzoate	50
Sodium chloride	100[b]

[a] 80 μmol L^{-1} *N*-acetyl-L-cysteine added.
[b] The highest value tested.

For assessing the accuracy of the results furnished by the developed procedure, the same 6 commercial pharmaceutical formulations containing NAC were analyzed according to the reference procedure recommended by the United States Pharmacopeia which relies on a HPLC methodology [16]. The results, summarized in Table 4, revealed a good agreement between both methods, with relative deviations between −2.1 and 4.2%. In addition, a paired Student's *t*-test [32] confirmed that there were no statistical differences ($t_{estimated} = 1.083$, $t_{tabulated} = 2.571$) between the results obtained by both procedures, for a confidence level of 95% ($n = 3$).

3.5. Proposed mechanism of interaction between QDs and NAC

In order to better understand the response characteristics of CdTe QDs to NAC molecules some batch assays involving steady-state fluorescence spectroscopy measurements and FT-IR were carried out by applying the reaction conditions already used in flow. For the photoluminescence emission studies, two different solutions of QDs were prepared (2.5 μmol L^{-1} CdTe QDs in 3 mmol L^{-1} acetate buffer at pH 5.2 and 2.5 μmol L^{-1} CdTe QDs in 3 mmol L^{-1} acetate buffer at pH 5.2 with 250 μmol L^{-1} NAC), and the results were compared (Fig. 3). For both solutions no significant shift of the emission spectra was observed. However, the fluorescence intensity of QDs changed reasonably. With the addition to a solution of 2.5 μmol L^{-1} CdTe QDs in 3 mmol L^{-1} acetate buffer at pH 5.2 of 250 μmol L^{-1} NAC, an increase of approximately 35% was verified in comparison to the blank (in the absence of NAC). This fluorescence enhancement is clearly lower than the 78% increase in luminescence intensity obtained with MPFS (for identical reaction conditions) confirming that the applied flow-based methodology is undoubtedly an advantageous expeditious way of implementing reactions of this type.

By comparing the FT-IR spectrum of two solutions obtained after lyophilization, one containing 250 μmol L^{-1} NAC and another one with 2.5 μmol L^{-1} CdTe QDs in 3 mmol L^{-1} acetate buffer at pH 5.2 with 250 μmol L^{-1} NAC it was possible to gather additional

Table 4
Results obtained in the determination of *N*-acetyl-L-cysteine in pharmaceutical preparations.

Samples	Concentration declared (mg)	Amount found[a]		R.D.(%)[b]
		Developed methodology	Reference method	
Fluimucil 2%[c]	20	21 ± 4	20.1 ± 0.2	2.1
Fluimucil 4%[c]	40	42 ± 2	41 ± 4	2.0
Tirocular[c]	40	39 ± 3	40 ± 2	−1.3
Fluimucil injectable[c]	100	103 ± 4	102 ± 1	1.5
Fluimucil 20%(injectable)[c]	200	196 ± 3	200 ± 1	−2.1
Fluimucil (granule)[d]	200	203 ± 3	212 ± 3	4.2

[a] Mean ± $t0.05$ (Student's t-test) × $(S/\sqrt{n})$.
[b] Relative deviation of the developed methodology with respect to the reference procedure.
[c] mg *N*-acetyl-L-cysteine per mL.
[d] mg *N*-acetyl-L-cysteine per sachet.

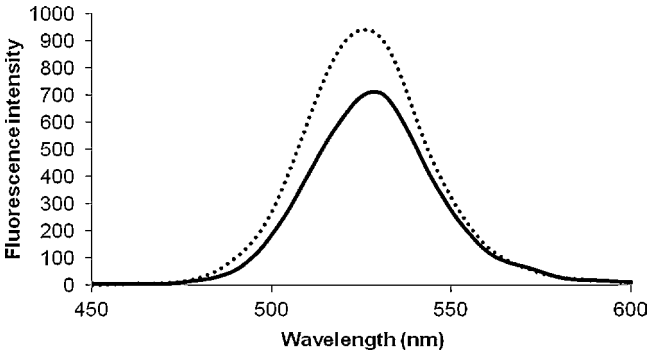

Fig. 3. Fluorescence enhancement of CdTe QDs upon addition of 250 μmol L^{-1} NAC in pH 5.2;
(solid line) 2.5 μmol L^{-1} CdTe QDs in 3 mmol L^{-1} acetate buffer at pH 5.2; (dashed line) 2.5 μmol L^{-1} CdTe QDs in 3 mmol L^{-1} acetate buffer at pH 5.2 with 250 μmol L^{-1} NAC.

information that helped to understand the interactions that may happen on the surface of the dots resulting in enhanced fluorescence emission. *N*-Acetyl-L-cysteine is a weak acid containing three important functional groups: thiol (sulfhydryl), amine and carboxylic acid. In order to identify whether some of these groups could be bonded to the surface of QDs, FT-IR measurements were performed and the spectra are displayed in Fig. 4. The thiol group has a high affinity for binding heavy metals [33] and the results obtained with the FT-IR spectra seemed to reinforce this finding. In fact, the disappearance of the entire NAC S—H band (2550–2678 cm^{-1}) upon interaction with the dots surface can be explained by complexation of incompletely bounded cadmium ions (Cd^{2+} surface traps) which contributed to improve dots passivation inducing a quantitative modification of the optical properties. These results showed to be a good evidence for the remarkable enhancement emissions of CdTe QDs promoted by NAC. Even though it has been demonstrated that the interaction cadmium–thiol strongly depends on the pH, the diameter of the QDs was clearly an aspect affecting the fluorescence increase. Effectively, luminescence enhancement was much more prominent in the case of smaller sized QDs, compared to larger ones, despite their initial low fluorescence emission. As it was already mentioned, QDs with a diameter of about 1.87 nm showed a lower QY% when compared with the other nanoparticles assayed. This happens because smaller sized QDs have a greater surface to core atoms ratio and therefore most of the electronic defects or dangling bonds are localized on the surface [34]. As it was referred, those traps are Cd^{2+} ions located on the QDs surface that did not complete all their allowed bonds. Molecules like NAC bind to those sites effectively removing the traps states [35] and increasing the intensity of emission. Albeit the experimental results suggested that the enhancement effect is mainly due to the binding of the thiol group to surface defects sites, the participation of other functional groups of NAC was not entirely rejected since they are all prone to coordinate cadmium ions. Furthermore, a slight acidic medium seems to facilitate the reaction between NAC and Cd^{2+} sites. This can be probably attributed to the only "partial" protonation of MPA carboxylic groups at the QDs surface. These groups when dissociate conferred a negative charge to the nanoparticles surface that ensures the stability in solution. On the other hand, this charged layer can difficult the diffusion of negatively charged analytes into the QDs surface and subsequently prevents the establishment of interaction with the reactive sites existing on it. If we compare the carboxylic acidic group pK_a of the two involved molecules (pK_a MPA = 4.34; pK_a NAC = 4) at pH 5.2, MPA will be more protonated than NAC. Thus, NAC has

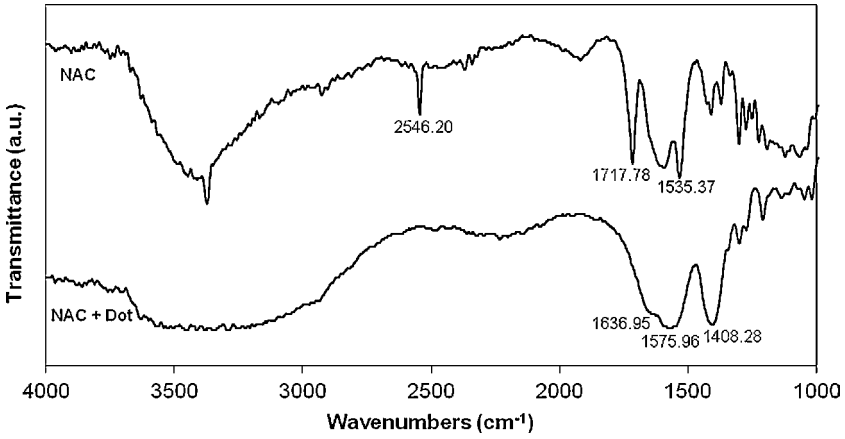

Fig. 4. Infrared spectra of *N*-acetyl-L-cysteine (NAC) and the interaction of NAC with MPA–CdTe QDs under the optimized conditions.

a easier surface access and a more strong tendency to react with Cd^{2+}[36]. This assumption was validated by the results obtained when NAC solution was acidified with HCl at pH 5.2, which provided a 35% enhancement, similar to the one obtained with acetate buffer. Despite of this, a lack of efficiency and reproducibility would hinder the reliability and efficacy of the HCl method. Moreover, this procedure would be more complex and time consuming since we would have to guarantee the uniformity of pH for all standards and samples.

4. Conclusions

The feasibility and robustness of implementing CdTe QDs in a multipumping flow system for the fluorimetric determination of NAC in pharmaceutical formulations was demonstrated. The use of such nanosensors constitutes a noteworthy contribution for the success and efficiency of the proposed methodology regarding other available alternatives. Due to the extended range of variables to be optimized, the multipumping flow methodology allowed a simplification of the entire process and the gathering of the ideal conditions in a simple and fast way. The pulsed flowing stream generated by micro-pump actuation not only permitted the attainment of very stable flow rates but it also imparted a fast homogeneous mixing which contributed to improve the interactions development. Furthermore, the use of the merging zones strategy was of great benefit as it enabled the reduction of the time required for the analysis as well as the reagent consumption.

Additionally, in comparison with the reference procedure, the proposed system proved to fill many of the gaps left by those former methodologies which always require high volume of reagents, often hazardous for the environment, expensive equipment and time-consuming sample handling operations. Good repeatability, a wide linear working range, low limits of detection and quantification, low consumption of reagents and a high sampling frequency were easily achieved with the proposed method. These results point out the potential use of water-soluble CdTe QDs as pharmaceutical drugs probes by pH modulation of the analyte solutions. In conclusion, the effect of luminescence enhancement of CdTe nanoparticles when NAC is added in a slight acidic medium was much more pronounced for the QDs of smallest size. Due to the their reduced size, a great number of local surface-trap states become more accessible to coordination by the thiol groups of NAC as proved by FT-IR measurements.

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