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Nanostructured Polyelectrolyte-based System as a Toolbox for Metal Ions Detection

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Abstract The capability of certain heavy metal ions to induce fluorescence decrease by a quenching mechanism suggested us to design and build a sensor potentially tunable for different ions at different concentrations. We propose a quenching-based sensor exploiting a nanostructured architecture in which fluorescent molecules (the sensing probe) are entrapped to recognize a specific analyte (heavy metal ions) through an optical transduction. The polyelectrolyte nanostructured system, named nanocapsule, improves the fluorophore-ion quenching sensitivity allowing a micromolar detection. Furthermore we couple our sensor with an electrical device in order to refine the sensing procedure: the electric field created allows a metal ions spatial gradient, necessary to detect a specific element on a single sample solution, avoiding a comparative analysis with an intensity reference value. Results obtained will show the advantages and the potentialities of our system as a smart toolbox for metal ions detection.

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Introduction

Heavy metal ions are essential elements for life, for biological metabolism and biosynthesis, but they become extremely toxic for the organism when reaching abnormal concentrations [1]. Furthermore, several heavy metal ions are involved in the physiopathology of many diseases, in particular neurodegenerative as Alzheimer (Cu^{2+}, Fe^{2+}) [2], amyotrophic lateral sclerosis, prion diseases (Cu^{2+}, Zn^{2+}), cataracts, mitochondrial disorders, Creutzfeldt–Jakob disease, Parkinson's disease, Menkes disease and Wilson disease (Cu^{2+}) [3, 4].

The great interest on toxic effect has lead, in the last decades, the development of analytical techniques to monitor heavy metal ions. Within this framework, optical-fluorosensors are important investigation tools [5], keeping the selectivity and sensitivity detection requirements and offering the possibility to have a "sterile sensing" suitable for *in vivo* analysis [6]. A wide variety of fluorescent sensors currently available exploits quenching mechanism, a phenomenon based on the interaction between fluorophores and specific quencher elements, like heavy atoms, whose effect results in a decrease of fluorescence [7, 8]. Quenching-based systems can thus be used to measure precise analyte concentrations monitoring variations induced by a specific analyte by means of an optical transduction process.

We present a novel quenching-based sensing device able to detect micromolar heavy metal ion concentrations through a Light Scanning Microscope analysis. This device employs the characteristics of a polyelectrolyte nanostruc-

tured system, named nanocapsule, which offers the possibility of entrapping a proper fluorescent molecule sensitive to a specific metal ion, in a nano-controlled way. Within this work we investigate the interaction between nanocapsules labeled with cyanine fluorescent molecules (Cy3) and copper ions. The importance of studying copper ions is related to their impact on health, in particular to the damage for respiratory, haematological, endocrinal, hepatic and ocular system [9].

Finally we coupled the nanocapsule sensing tool with an electrical device able to drive the analyte ions in solution: the idea is to follow fluorescence variations in real time inducing a space-controlled quenching effect. The electric field allows ions migration towards one electrode which is translated into a quenching–dequenching mechanism. The main advantage achieved is the possibility to measure, directly from the same sample solution, either the quenched signal, due to quencher ions, and the reference one (0 M ions).

Results obtained will show the advantages to employ this nanostructured system, coupled with the electrical driving device, as an environment sensing tool able to follow precise fluorescence variations related to the spatial micromolar concentrations of metal ions.

Experimental section

Sensing system structure

A polyelectrolyte nano-system, named nanocapsule, is used as sensing structure to entrap the sensitive probe (cyanine fluorescent molecules) for the analyte detection (copper ions). Nanocapsules are built through a layer-by-layer deposition technique of two oppositely charged polyelectrolyte (PE) chains sequentially adsorbed on a crystal core [10]. A monodispersed $CaCO_3$ cubic crystals (diagonal of nearly 5–10 μm) solution is obtained quickly adding a $CaCl_2$ (0.05 M) solution in a Na_2CO_3 (0.05 M) solution. The crystals are separated from the solution by centrifugation, washed with Milli-Q-grade water (Millipore GmbH) with a specific resistance of 18.2 MΩ/cm, and then dried at 55 °C.

The PEs, prepared in a 0.1 M NaCl salt solution, 2 mg/ml, are: poly(allylamide hidrocloride) (PAH, MW 15,000 Da, Aldrich) as polycation and poly(stirene sulfonate) (PSS, MW 70,000 Da, Aldrich), as polyanion. This procedure leads to the formation of a PE random coil structure whose tangling depends on the number of compensated charges conferring variable layer permeability to different ions [11]. It is worth noting that the resulting nanocapsules are biocompatible [12]. In present work, at 0.1 M NaCl, the layer thickness is estimated to be nearly 20 Å [13].

The capsules are usually 6–8 layers, starting with PAH adsorbed on the $CaCO_3$ core; the fluorescent layer (5th) is obtained through a covalent binding of PAH to the fluorescent cyanine molecule, Cy3 (CyDye mono reactive NHS Ester, Amersham Biosciences).

Analyte sample solution

Copper ions are obtained preparing $CuCl_2$ solutions in water milli-Q at different micromolar concentrations. Measurements are performed on solutions containing a constant concentration of nanocapsules labelled with Cy3 and different $CuCl_2$ concentrations (1, 7.5, 10, 15, 20, 25, 30, 40, 50, 60 μM) and on solutions with pure free Cy3, at constant concentration, with $CuCl_2$ at 10 μM, 50 μM 500 μM, 10 mM and 50 mM.

Imaging techniques

Fluorescence Imaging measurements are performed, through a confocal microscope system (Leica TCS SP5 AOBS, 63× N.A. 1.4, Oil objective, Leica Microsystem, Germany; Nikon Confocal C1-SI, 60× N.A. 1.4, Oil objective, Nikon, Japan), on solutions containing nanocapsules labelled with Cy3, NaCl (0.1 M) and $CuCl_2$ (at the concentrations previously described). A statistical analysis on several populations of nanocapsules (25 nanocapsules each $CuCl_2$ concentration) is performed.

On these samples, an intensity analysis is carried out observing several identical square regions (Fig. 1) along the whole perimeter of the nanocapsule (20 regions each nanocapsule). The fluorescence intensity of each nanocapsule is assumed as the mean intensity value of all square regions. Cy3 molecules are excited with the He–Ne (1, 2 mW) laser (543 nm laser line) and the acquisition is performed in the 550–650 nm spectral range. All measurements are performed in the very same imaging acquisition conditions: image size 512×512, pixel size 26.75× 26.75 μm; line scan rate 400 Hz, frame time 1.310 s; laser power 0.15 mW.

Cy3 emission spectra are recorded between 550 and 650 nm with a bandwidth of 5 nm, through the Leica TCS SP5 AOBS microscope system. Absorption spectra are collected with a spectrophotometer (V-530 UV/Vis Spectrophotometer, Jasco, Japan) across the wavelength region from 450 to 600 nm (bandwidth 1 nm, scan speed 100 nm/min).

A spectrofluorometer analysis (Fluorolog-3 spectrofluorometer, Horiba Jobin Yvon, USA) on free Cy3 in $CuCl_2$ solution is performed under 543 nm excitation (550–650 nm spectral range, bandwidth 0.5 nm).

Life-time measurements are carried out with a frequency domain approach [14] using a time resolved spectrofluorometer (Chronos, ISS, USA) set for excitation 470 nm, acquisition 560 nm and as reference fluorophore Rodhamine 6G in H_2O, (τ_0=4 ns).

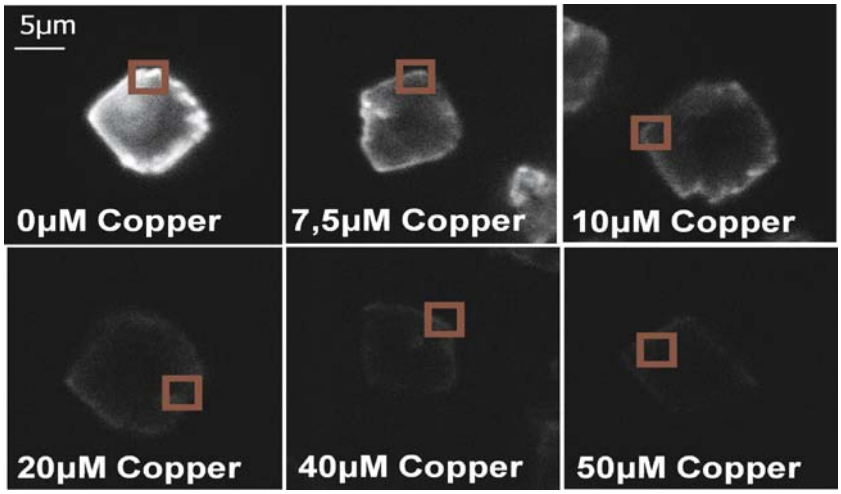

Fig. 1 Cy3 fluorescence signal collected from nanocapsules at different copper ion concentrations

Electric field creation

An electrical device, suitable for fluorescence microscope analysis, is designed to produce a uniform electric field (Fig. 2). The electric field is used to induce a controlled analyte elements migration (ensuring no capsules mobility). The electrical device is formed by a couple of metal strips (3 cm×35 μm×35 μm), as electrodes, in a distance of around 300 μm between which 100 μl of sample solution is introduced (Fig. 3). We designed two kinds of electrical device: one with copper electrodes to validate the system capability, one with platinum electrode, as a first sensor prototype, to perform quantitative analysis. Measurements are carried out applying a potential difference between the two electrodes in the range of 1 V in DC (higher voltages induce a nanocapsules migration and heating effects, lower voltages do not induce fluorescence variations).

Results and discussion

In order to study the quenching mechanism occurring between Cy3 molecules and copper ions, we performed fluorescence analysis on Cy3-nanocapsules at different copper ions concentrations as described in materials and methods (Fig. 1 shows the quenched signal recorded in a micromolar copper concentrations range). The ratio between the reference intensity value (F_0 at 0 M $CuCl_2$) and the quenched signal (F) is plotted versus copper ion concentrations, exhibiting a linear relationship. Figure 4 analysis demonstrates the system capability to be employed as a sensing system able to associate a specific fluorescence decrease to a precise copper concentration. Only Cu^{2+} ions are responsible for these variations, even if Cl^- are present in solution. In fact, nanocapsules are prepared in a 0.1 M NaCl solution containing Cl^- in the millimolar range and no significant alteration on Cy3 fluorescence signal is observed. This means that, within the device operating conditions, i.e. micromolar ion concentration range, chloride ions quenching effect can be neglected.

Such a linear behaviour, shown in Fig. 4, can be associated both to a pure collisional and a pure static quenching effect [15]. Now, supposing a pure collisional quenching, the F_0/F-copper dependence of Fig. 4 should be described through the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] \quad (1)$$

where k_q represents the bimolecular quenching constant and τ_0 is the fluorophore lifetime in the absence of the quencher.

Fig. 2 Device set-up on the microscope stage, enlarged view of the active area and details of the electrodes in transmission light

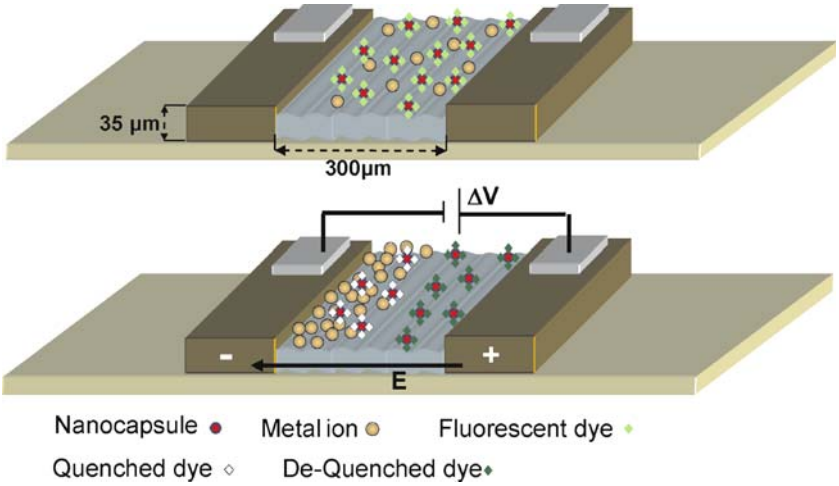

Fig. 3 Device scheme representing copper ions migration associated to a quenching–dequenching effect, induced by the electric field

Fitting the experimental data of Fig. 4 a k_q value of nearly 6, 5×10^{14} M^{-1} s^{-1} is evaluated ($y = 1 + k_D x$, $k_D = k_q \tau_0$, $\tau_0 = 0.3 \times 10^{-9}$s, $k_D = (0.197 \pm 0.011) \times 10^6 M^{-1}$, correlation coefficient 0.974). For a pure diffusion-controlled mechanism a theoretical quenching constant k_0 of 2×10^{10} M^{-1} s^{-1} is obtained through the Smoluchowski equation [15]. k_0 is linearly related to the bimolecular quenching constant k_q through the quenching efficiency f_q. Therefore, for $f_q=1$, the maximum value accessible to k_q is in the order of 10^{10} M^{-1} s^{-1}: the experimental value overcomes this limit of four orders of magnitude.

These results suggest to reason on a pure static quenching. The linearity shown in Fig. 4, thus can be described by:

$$\frac{F_0}{F} = 1 + K_S[Q] \tag{2}$$

giving a K_S (the association constant for complex formation) value of $(0.197\pm0.011)\times10^6$ M^{-1}. Such a value indicates a quite high interaction rate, especially considering that Cu^{2+} ions are interacting with a generic fluorophore like Cy3: in fact, Cy3 is a common dye which has not been modified to selectively interact with a specific ion.

In order to get a deeper insight into the quenching mechanism we performed spectral and lifetime analysis.

Emission spectra of Fig. 5 show an intensity decrease proportional to copper ions concentration without any modifications in the spectral shape and without observing new long wavelength bands.

We also observed no modifications in the absorption spectral shape (Fig. 6), frequently associated to a ground-state complex formation between the quencher and the fluorophore. Since a static quenching can involve excited-state complex formation without affecting the shape of the absorption spectrum, further photophysical information are needed. To this end, lifetime measurements can provide a definitive proof towards static quenching hypothesis. In fact, under static quenching condition lifetime values do not change. This is our case, since pure Cy3 molecules at

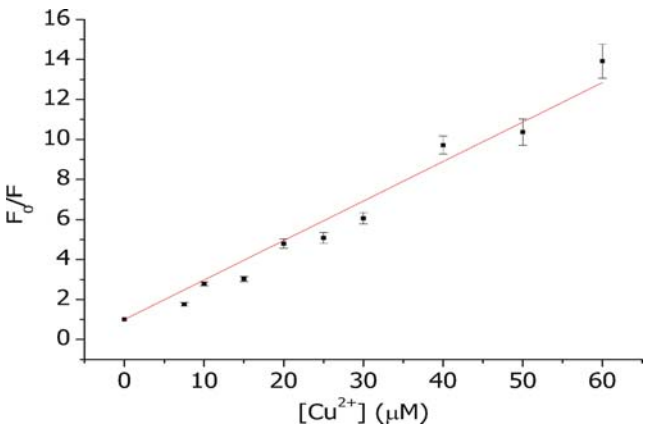

Fig. 4 Fluorescence decrease (F_0/F) at different copper ion concentrations. The error bars are evaluated as the standard error. The *line* represents the linear fitting of the experimental data

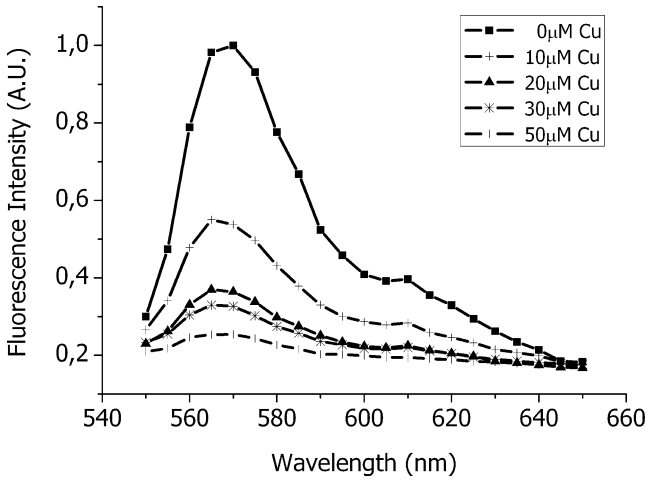

Fig. 5 Cy3-nanocapsule emission spectra at different copper ion concentrations. Lines to guide the eyes

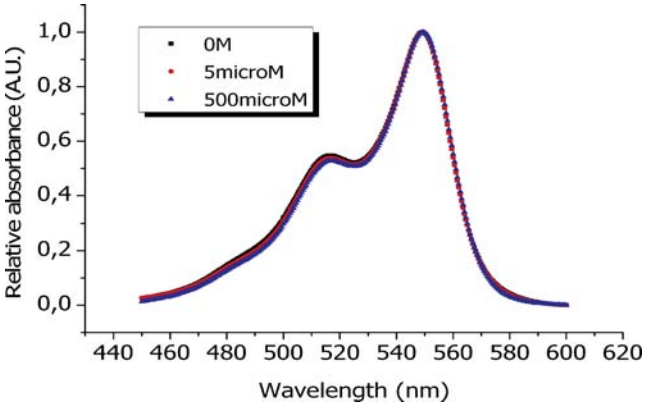

Fig. 6 Cy3-nanocapsule absorption spectra at different copper ion concentrations: 0 M (*square*), 5 μM (*circle*), 500 μM (*triangle*)

different $CuCl_2$ solutions (0 M, 10 μM, 10 mM, 50 mM) exhibit the same lifetime value (0.35±0.04)ns.

In order to investigate a possible influence of the polyelectrolyte in the quenching mechanism between Cy3-nanocapsules and copper ions, a spectrofluorometric analysis on free Cy3 in $CuCl_2$ solutions (50 μM, 500 μM and 50 mM) is performed. Since Fig. 7 shows that there is a detectable quenching effect for free Cy3 solution only at millimolar copper concentrations, indicating a static quenching constant in the order of around 20 M^{-1}, we can conclude that the high K_S value obtained with Cy3-nanocapsules (nearly 2×10^6 M^{-1}) is related to the polyeletrolyte nanostructured system.

So far, we claim that the polyelectrolyte architecture induces charge effect allowing a high quencher density in the vicinity of the fluorophore, thus increasing the complex formation probability during the lifetime of the excited state. The increase in the local copper ion density could be ascribed to the random coil structure which allows the formation of charged cavities surrounding the fluorophore.

Results obtained show the advantages to employ this nanostructured system as an environment sensing tool able to follow precise fluorescence variations related to the spatial micromolar concentrations of quencher ions. Moreover, assuming that a polyelectrolyte charge effect is involved in the extent of the quenching phenomenon, it would be tempting to conclude that a sensitivity improvement of any fluorescent sensitive probe to a charged quencher analyte could be obtained in the very same way.

Measurements performed to study quenching mechanism and to characterize our sensing system are based on the comparison of the fluorescence signal recorded from two identical solutions: a working solution with copper ions and a reference one without copper ions. A reference measurement, performed in the very same conditions of the working one, can not be simply obtained in practical situations, where the metal ion species to be detected is dispersed in a mixture of unknown elements. Therefore, a system able to analyze a single sample solution, avoiding a comparative analysis with an intensity reference value, is of great impact.

Hence, we coupled the nanocapsule sensing tool with an electrical device able to induce spatially controlled concentration of analyte ions. The goal is to be able to follow fluorescence variations in real time inducing a space-controlled quenching effect (Fig. 3). The electric field allows an ions drift towards one electrode which is translated into a quenching–dequenching mechanism. This effect allows to monitor from the very same solution either the quenched and the non-quenched fluorescence signal avoiding the need of a separate reference measurement (0 M Cu^{2+}).

Our preliminary purpose was to check the possibility to follow copper ions migration through the consequent quenching/dequenching effect with the designed electrical device: the simplest way was to perform qualitative measurements using oxidized copper electrodes which allow an immediate release of copper ions in the nanocapsule sample solution, as described by:

$$2Cu^0 + O_2 + 2H_2O \rightarrow 2Cu^{2+} + 4(OH)^-$$

The experiments run as it follows. At the beginning, the fluorescence signal recorded is homogeneously quenched through all the samples because of the copper ions already released in solution. Applying a potential difference, a fast fluorescence recovery (dequenching) is observed in the proximity of the positive electrode as copper ions are carried away from it; conversely, switching off the electric field, a slower copper ions free diffusion in solution leads to a fluorescence quenching, at the positive electrode, towards the initial intensity value. The signal, recorded in real time during the on/off switching intervals of the electric field, shows a reproducible behaviour (Fig. 8).

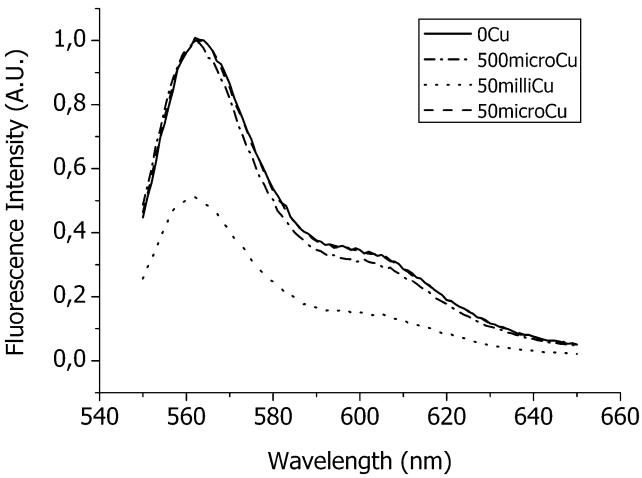

Fig. 7 Emission spectra of pure Cy3 free in solution at different copper ion concentrations

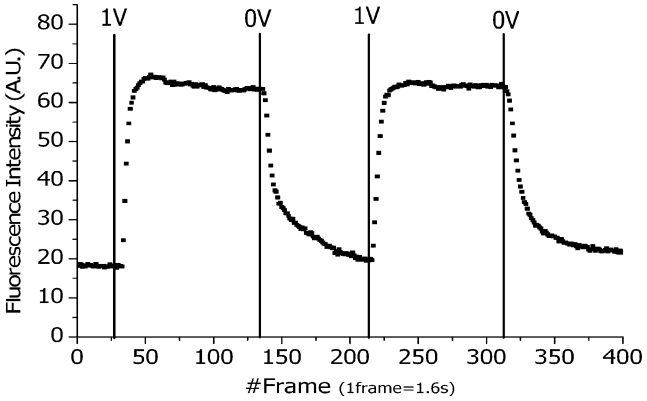

Fig. 8 Fluorescence course in the vicinity of the positive electrode switching on/off a potential difference of 1 V in DC (1 frame=1.6 s) with the oxidized copper device

This indicates the potential of exploiting such fluorescence variations as the transductional signal of our sensing system. In order to perform quantitative measurements, some improvements are necessary: first of all, an inert electrical device (i.e. platinum electrodes) is required to prevent the copper ions release directly from the electrodes, in order to quantify the real sample copper ions concentrations; secondly, a more rigorous calibration is necessary for studying the accuracy and the detection limits of the sensor. Furthermore, in order to establish the sensor selectivity to copper ions, the sensing system should be tested adding different metal ions in solution. Since, in general, metal ions are sensitive to the electric field, an appropriate choice of the fluorescent probe quenchable by a precise metal ion, will provide the sensor specificity.

Results obtained using the platinum electrical device are shown in Fig. 9.

The sample solution is made by nanocapsules and NaCl (0.1 M) as for the oxidized copper device previously seen, but in the present case copper ions are provided inserting a known concentration of $CuCl_2$ (10 μM). A potential difference is applied in order to induce the space-controlled ions migration. Figure 9a shows a similar course to the one shown in Fig. 8: also in this case a fluorescence dequenching can be observed at the positive electrode, but now the fluorescence variation can be associated to a fixed copper ions concentration. Moreover, the fluorescence course has been followed alternatively switching the polarity of the analyzed electrode: in this way it has been possible to follow a dequenching–quenching effect applying +1 V/−1 V respectively.

Figure 9b shows the results obtained performing the same experiment on a solution without copper ions: as expected a constant fluorescence signal is registered, ensuring no effect on the Cy3 fluorescence emission induced by the applied electric field, hence indicating that such a system can be really employed as a metal ion sensing system. Moreover, the constant fluorescence signal, arising from nanocapsules in a NaCl (0.1 M) solution, confirms there is no significant effect of chloride ions.

Concluding, a metal ion sensing system, based on a polyelectrolytes nanostructure coupled to an electrical driving device able to detect micromolar concentration, is here presented.

Since the nanocapsule structure improves the interaction sensitivity of the fluorophore-quencher ion couple (here shown for Cy3–Cu^{2+} couple) its utilization could be extended to a variety of fluororphore–quencher ion couples.

This makes such a device a powerful and useful toolbox to explore and sense the degree of metal ions contaminations in the microenvironment.

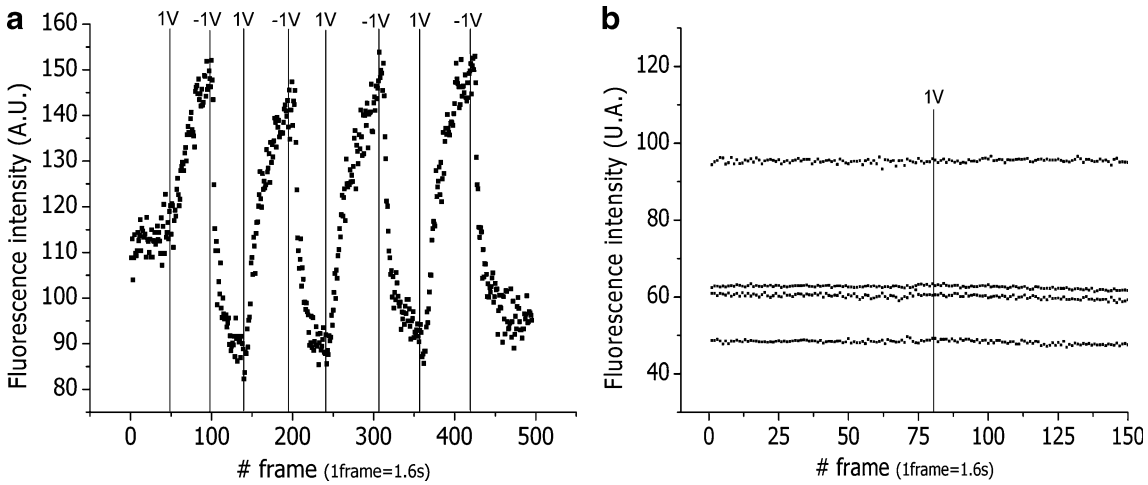

Fig. 9 Fluorescence course with (**a**) and without (**b**) copper ions, switching on/off the electrical field with the platinum electrical device (1 frame=1.6 s) The different constant flourescence intensity courses in (**b**) correspond to different size square regions from which the flourescence signal is acquired

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