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ORIGINAL ARTICLE

Effective staining of tumor cells by coumarin-6 depends on the stoichiometry of cyclodextrin complex formation

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Abstract In a comprehensive picture of inclusion complex formation of the highly fluorescent dye coumarin-6 (C6) and betacyclodextrin (beta-CD), which was obtained using various fluorescence spectroscopic methods, it was demonstrated that up to three beta-CD rings can thread on the rod like dye molecule. Interaction of coumarins and modified coumarins with cellular organelles or proteins has been reported in several publications. Especially 7-amino-coumarins are characterized by unique properties like high fluorescence quantum yield and are thus already used successfully in different areas, like staining of fluorescent nanoparticles. We could show that Coumarin-6 made soluble by complexation with beta-cyclodextrin is able to stain eukaryotic cells specifically dependent on their origin and cellular behaviour. The staining reaction is independent from pH, is photo stable, and shows no cross talk with proteins in the cytoplasm and other staining procedures or erythrocytes. Staining with coumarin 6/cyclodextrin complexes can thus be used for fast discrimination of different cell types. Importantly, it could be shown that the ideal staining reaction is dependent on the stoichiometry of the complex-formation.

Keywords Cyclodextrines · Coumarin-6 · Fluorescence correlation spectroscopy (FCS) · Cell staining · Fluorescence microscopy

Introduction

Self-assembling phenomena attract considerable interest as basic issues for supramolecular chemistry, molecular biology and fields like nanotechnology and nanofabrication [1]. A specific example for molecular self-assembly is the formation of host–guest complexes where an active small guest molecule binds into a cavity of a macromolecular host. Complexation due to the hydrophobic effect is a widely found phenomenon in chemistry and can be applied to increase the solubility of hydrophobic molecules in water [2, 3]. Cyclodextrins are well known hydrophilic host molecules, consisting of six (alpha-cyclodextrin), seven (beta-cyclodextrin) or eight (gamma-cyclodextrin) α-1,4-glycosidically linked glucopyranose subunits providing a hydrophobic cavity. They are known to complex a variety of guest molecules unspecifically [4, 5] providing a hydrophilic envelope for a hydrophobic guest in water.

Fluorescent molecules play an important role in the development of biosensors, photo catalytic systems, opto-electronic devices and for staining reactions of specific compartments in living cells to make them discernable in microscopic imaging. Especially the staining of hydrophobic compartments in cells like membranes needs hydrophobic molecules which are generally not soluble in an aqueous environment. Using organic co-solvents to increase their solubility interferes, however, with cellular function. This limitation could be overcome using cyclodextrins to solubilize the dyes, as they can be used at rather low concentration.

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One example we used recently is coumarin-6 complexed with β-cyclodextrins, which has been shown to stain cancer cells specifically [6]. Commercially available systems to detect cancer-markers on the cell surface make use of specific monoclonal and fluorescent labeled antibodies, which have a wide range of sensitivity, high selectivity but are very expensive and can often be used only for to detect specific cancer cells [7]. Despite significant advances in treatment, bladder cancer is a common disease with a high mortality rate but intensive screenings have shown to decrease mortality rates [8]. Therefore, for an optimized treatment and more important early diagnosis it is essential to have a fast, reliable and high quality diagnostic tool, which allows detecting cancer cells or other cells characteristic for a disease directly from urine.

In this paper we show that specific staining of cancer cells in urine is highly dependent of the stoichiometry of the coumarin-6 complexes with β-cyclodextrin, which depends strongly on the β-cyclodextrin concentration in relation to the dye concentration.

Materials and methods

Preparation of β-cyclodextrin solution

A nearly saturated 10^{-2} M stock solution of β-cyclodextrin was obtained by adding 10 mg/mL β-cyclodextrin to purified water followed by 15 min sonication at 65 °C. This procedure gave a clear and stable solution.

Preparation of coumarin-6 solution

A 60 μM coumarin-6 stock solution was obtained by dissolving coumarin-6 in absolute ethanol resulting in a clear, green fluorescent solution.

Complex formation between coumarin-6 and β-cyclodextrin

Ethanolic coumarin-6 and aqueous β-cyclodextrin was mixed in varying amounts to obtain the desired dye/β-cyclodextrin concentration ratios. This mixture was stirred for 10 min at room temperature and this procedure gave a clear, highly green fluorescent solution in the case the β-cyclodextrin concentration exceeded 20 μM. To stain cells this solution was added to the growing buffer for a final concentration of 30 nM for the dye.

Fluorescence Correlation Spectroscopy (FCS) measurements

A confocal microscope (Confocor1-Carl Zeiss, Jena, Germany) was used to measure dynamics of complex formation by fluorescence correlation spectroscopy (FCS). An argon ion laser supplied 488 nm light for excitation of coumarin-6, filtered using a 488 ± 10 nm interference filter. The excitation light was focused by a water immersion objective (C-Apochromat 63×, 1.2 NA) in the solution and the fluorescence light was collected through the same objective and passed through a broad band filter (520–560 nm) before passing the confocal pinhole with a diameter of 45 μm. The instrument was calibrated using a 10 nM aqueous solution of rhodamine 6G. FCS measurements were performed in aqua bidest or PBS respectively. The experiments were analyzed with the FCS ACCESS software package obtained from Zeiss/EVOTEC, using a multicomponent fit model. Analysis was performed for a fixed structural parameter defining the ratio between the height and the width of the detection volume. This parameter was also obtained from the calibration procedure.

Epifluorescent images

Bladder cancer cells were grown in chambered cover slides in MEM medium. For imaging the medium was replaced by phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) containing the coumarin-6/β-cyclodextrin complex at a final concentration of 30 nM for coumarin-6. Epifluorescence images were taken after 10 min incubation using a Zeiss AXIOVERT S100TV, appropriate filter settings and ×10 magnifications. For quantification of the images they were analyzed using the cell counting module of ImageJ (free image processing software).

Theoretical methods

The quantum chemical calculations were performed using the GAUSSIAN09 program package [9]. The structures of coumarin-6 and β-cyclodextrin were fully optimized by HF with 3-21G basis set. Complexes between C coumarin-6 and one β-cyclodextrins were built and were optimized by HF/3-21G.

Results

FCS measurements of complex formation

The mean diffusion times for coumarin-6/β-cyclodextrin complexes were measured for 30 nM coumarin-6 and varying the β-cyclodextrin concentrations, in between relative concentrations of the two partners of 1: 300 up to 1:150.000 (coumarin-6/β-cyclodextrin), by FCS. The autocorrelation function ($G_{(t)}$) was measured and fitted to the following model [10]:

$$G(t) = 1 + \frac{1}{N}\left[\frac{1}{1+\frac{4Dt}{\omega_1^2}}\sqrt{\frac{1}{1+\frac{4Dt}{\omega_2^2}}}\right]$$

where N is the number of fluorescent molecules in the confocal volume, D the diffusion coefficient, ω_1 the radius of the confocal ellipsoid horizontally and ω_2 its length. The mean diffusion time was obtained from the fit procedure and its mean values are shown in Fig. 1 as a function of the relative concentrations of coumarin-6 and β-cyclodextrin.

Adding less than 20 μM β-cyclodextrin to 30 nM aqueous coumarin-6 gives mean diffusion times near to that typical for the free dye, however, with a large statistical error. This is most likely due to the low solubility of coumarin-6 in water and hence aggregation of the dye. Small dye aggregates were also denoted by the observation of particles moving through the observation volume of higher brightness and longer diffusion time than a single dye molecule. In comparison the complex with cyclodextrins has the same brightness but a long diffusion time as it consists only of one dye molecule. Increasing the β-cyclodextrin concentration above ca. 50 μM the particle distribution becomes more homogeneous as no dye aggregates are observed and the statistical error of the mean diffusion time decreased significantly. Self-aggregation of cyclodextrins should not contribute at these low concentrations. These results indicate, however, coumarin-6/β-cyclodextrin complex formation even in concentration range below 20 μM. Increasing the β-cyclodextrin concentration the mean diffusion time increased and becomes nearly 37 μs near to 150 μM host, which is near to an estimated diffusion time for the 1:1 coumarin-6/β-cyclodextrin complexes. At even higher β-cyclodextrin concentrations larger complexes are formed. Around 900 μM β-cyclodextrin complexes of a 1:2 stoichiometry dominate. Formation of even 1:3 coumarin-6/β-cyclodextrin complexes is indicated by the value of the mean diffusion time when the host concentration becomes larger than 1 mM. These data indicate that coumarin-6 forms strong inclusion complexes with β-cyclodextrin with complexation constants near to 10,000 M^{-1} for the formation of the 1:1 complex, about 2,000–3,000 M^{-1} for the formation of the 1:2 complex from the 1:1 complex and about 1,000 M^{-1} for further addition of an cyclodextrin-molecule.

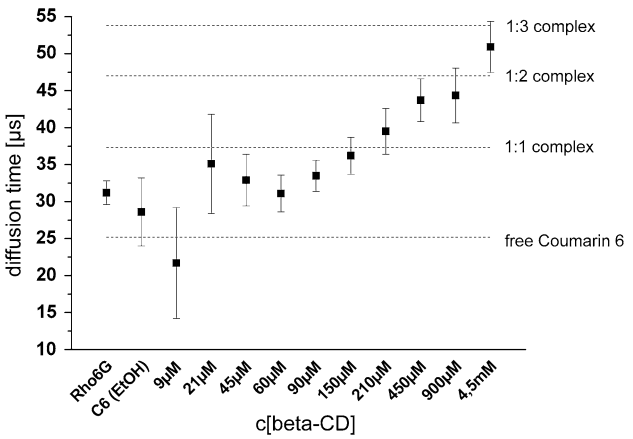

Fig. 1 Results of the FCS measurements of different stoichiometric mixtures of coumarin-6 (concentration 30 nM) and β-cyclodextrin (final concentrations between 9 μM and 4.5 mM). *Dashed lines* represent estimated diffusion times of free coumarin-6, and 1:1-, 1:2- and 1:3 complexes with β-cyclodextrin. Measurements for coumarin-6 in ethanol are also shown (C6-EtOH). The *error bars* were obtained from the subsequent repetition of the measurements

Influence of complex formation on staining reaction

In a further series of experiments the staining efficiency of living cells when complexes of different stoichiometry are used. The results of these experiments are summarized in Fig. 2. It has already been shown that different cell types show significantly different fluorescence patterns after staining with coumarin-6/β-cyclodextrin. Images of cells obtained with ×10 magnification can be used to characterize the different fluorescence distribution in the cells [6]. From these images a mean brightness of the stained cells could be calculated for given cell type by integrating the intensity pattern over the area of the cell. This defines the staining capability of the dye. Representative results obtained for bladder tumor cells are presented in Fig. 2. The results show clearly that it is very important to use mainly 1:1 coumarin-6/β-cyclodextrin complexes, i.e. to apply a ca. 100 μM β-cyclodextrin solution, to assure an intensive staining reaction without a significant background (see Fig. 2a). Cells stained at these conditions show a mean intensity of about 27 a.u. Using primarily uncomplexed dye for staining, i.e. at a cyclodextrin concentration below 45 μM (see Fig. 2b for staining of the same cells with 21 μM β-cyclodextrin) or stable 1:2 complexes (see Fig. 2b for staining of the same cells with 900 μM β-cyclodextrin) yield much less fluorescence intensity under identical condition and background fluorescence becomes much more intensive. The overall fluorescence intensity drops below 10 a.u. in both cases, which compares to nearly 1/3 of the intensity that is obtained using preferentially 1:1 complexes. It should be mentioned that these intensity values are corrected for background fluorescence, which becomes increasingly important when even higher β-cyclodextrin concentrations are applied corresponding to the predominant formation of 1:3 complexes. In that case the dye is not significantly incorporated into the cells and most of the Coumarin fluorescence is found in the medium outside the cells (see Fig. 3a). Cells stained with 1:3 complexes show

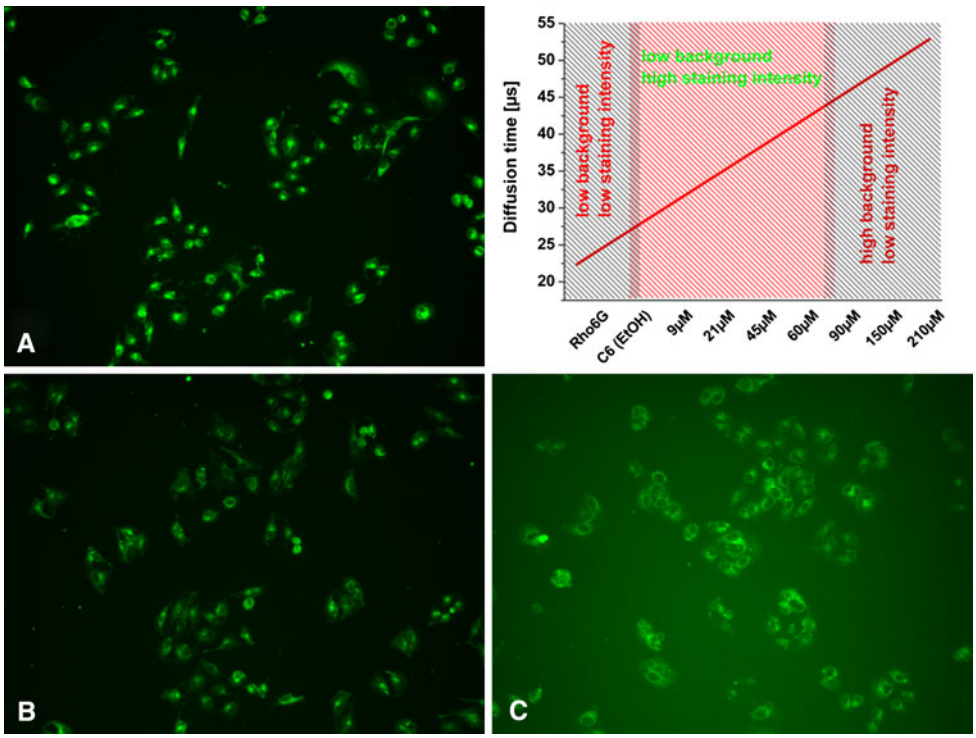

Fig. 2 Human bladder tumor cells stained with coumarin-6 (30 nM)/β-cyclodextrin complexes of different stoichiometrical composition. Cells are stained using a 1:3.000 (**a**), 1:750 (**b**) and 1:30.000 (**c**) coumarin-6 to β-cyclodextrin mixture. *Upper-right panel*: comparison between complex stoichiometry and staining intensity. The *red line* indicates a linear regression of the mean diffusion time caused in dependence of an increased concentration of β-cyclodextrin. The *black boxes* indicate the mean intensity of the stained cells using different concentration of β-cyclodextrin. Generally a final concentration of 30 nM coumarin-6 was applied in all cases

almost the same total intensity as cells stained with pure coumarin-6 solution (see Fig. 3b) with the main difference that the background is much less intense most likely due to quenching by aggregation.

The results indicate clearly the importance of the complex stoichiometry for the interaction with life cells and the uptake of the dye.

Model calculations

An ab initio model of the 1:1 complex of dye and β-cyclodextrin was calculated and is shown in Fig. 4 as these complexes are the most efficient structures to stain the cells. The figure shows clearly that at least two further cyclodextrin rings fit to the size of the dye molecule and this supports the interpretation of the FCS measurements. The position of the cyclodextrin ring threaded on the dye molecule suggests a slow dissociation of the complex, which could explain the high association constant found for the 1:1 complex. On the other hand side, the two additional rings in 1:2 and 1:3 guest to host complexes associate only with free end of the dye molecule and this accounts for the lower binding constants for association of an additional sugar molecule.

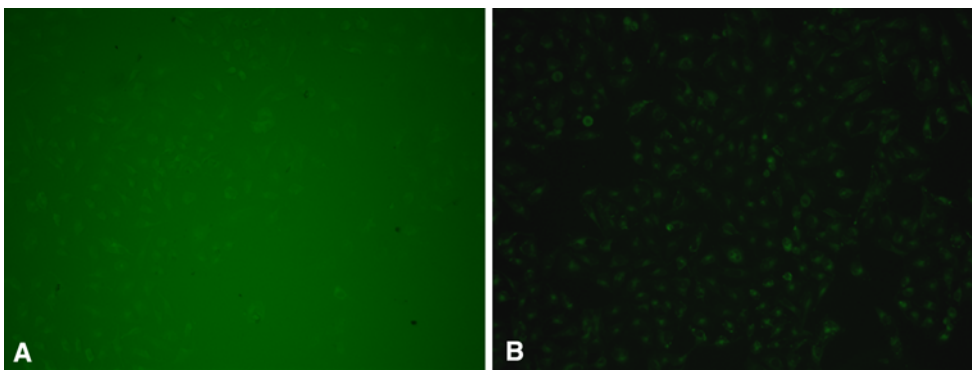

Fig. 3 *Left*: human bladder tumor cells stained with coumarin-6 (30 nM)/β-cyclodextrin (4.5 mM). The cells do not show a significant fluorescent signal and the fluorescence originating from the cells is hidden by the background fluorescence. *Right*: human bladder tumor cells incubated with coumarin-6 without cyclodextrins added. Although a fluorescent pattern can be observed, the fluorescence of stained cells is much weaker, compared to the fluorescence of cells incubated using a cyclodextrin concentration around 100 µM

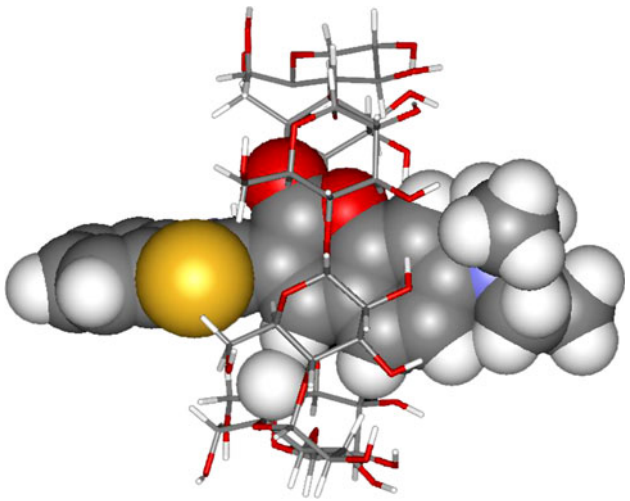

Fig. 4 A typical geometry of a complex between coumarin-6 and β-cyclodextrin, fully optimized at HF/3-21G level

Summary

Coumarin-6 is an amphipathic dye with high fluorescence in the green spectral region and high photochemical stability. The amphipathic character of the dye and its lipid like structure allows specific staining of lipid structures in eukaryotic cells. As it has, however, only low solubility in aqueous solution delivery of the dye to the cell membrane is an important issue. It was demonstrated that threading of β-cyclodextrins on the rod like molecule accounts for increases the staining capability of dye efficiently when complexes of a low 1:1 stoichiometry are used. Higher complex structures like 1:2 or 1:3 coumarin-6 per β-cyclodextrin molecules reduces the uptake of the dye by the cells efficiently. The mechanisms and the thermodynamics governing this behavior is currently studied in detail, as this is an important issue for understanding of drug delivery to cells using cyclodextrins. The results could be used for the development of effective drug delivery systems pharmacological applications of cyclodextrins.

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