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Use of cyclodextrins as scavengers of inhibitory photo-products in light controlled *in vitro* synthesis of RNA

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ABSTRACT

We recently reported on the use of caged nucleotides to attain full control of enzymatic polymerization of RNA solely by light. In the absence of light no RNA formation was possible due to the efficient caging by the coumarin moiety; after irradiation, caged ATP was released with quantitative precision and RNA polymerization was resumed. As photolabile protecting group [7-(diethylamino)coumarin-4-yl]methyl] (DEACM) was used due to its high absorbance in the visible region of the spectrum, fast deprotection kinetics and absence of radical intermediates. However, the 7-diethylamino-4-hydroxymethylcoumarin photo-product (DEACM-OH) was shown to inhibit the transcription reaction for concentrations higher than 30 μM [5]. This inhibition has been associated with poor water solubility, which is commonly dealt with via cumbersome chemical modifications of the protecting moiety. To overcome inhibition, we evaluated the use of molecular scavengers to sequester DEACM-OH formed after irradiation. Determination of association constants of coumarin with β-cyclodextrins allowed the assessment of its capability to remove free coumarin molecules from solution. The influence of β-cyclodextrin in transcription reaction was also assessed. Results show that β-cyclodextrin can be successfully used as scavenger as it increases the DEACM-OH threshold concentration for inhibition, amplifying the efficiency of light controlled *in vitro* transcription.

1. Introduction

Controlled temporal and spatial release of biomolecules from photolabile precursors, commonly known as *caged* molecules, is of extreme relevance as a tool for bio-molecular studies [1–4]. The cage (chemical modification with a photolabile protecting group) renders the molecule of interest biologically inactive; upon irradiation with light of a suitable wavelength, the biologically active molecule is released, generating a time-controlled burst in concentration with tight spatial control. For this purpose, the selected cage molecule ought to fulfill several critical conditions: (i) the efficiency of uncaging should be high in order to avoid long irradiation times and deleterious effects to the biological samples; (ii) the rate of uncaging must be faster than the process under study, i.e. the rate at which the biomolecule is released should not be the rate limiting step; (iii) easy to synthesize; and (iv) the resulting by-products should not hamper the desired reaction.

We have previously described the use of ATP nucleotides caged with [7-(diethylamino)coumarin-4-yl]methyl (DEACM-ATP) for light-controlled *in vitro* transcription reactions, where the quantity of RNA being polymerized could be controlled through DEACM-ATP irradiation [5]. Coumarin derivatives present high molar absorption coefficients, high photochemical quantum yields and absorptions in the visible region of the spectrum (<420 nm), with the advantage of fast photocleavage kinetics and wavelength tunability through changes in the position and/or nature of the chemical residues attached to the coumarin moiety [6–9]. In most aspects, coumarin cages, and in particular DEACM, follow the above mentioned selection requirements. However, following irradiation, high concentrations of photo-by-product 7-diethylamino-4-hydroxymethylcoumarin (DEACM-OH) inhibited the transcription reaction [5]. This effect was attributed to the poor coumarin solubility in water leading to the partitioning of the hydrophobic DEACM-OH molecule to the T7 RNA Polymerase, causing inhibition. To circumvent the solubility issue, hydroxyl or acetate groups can be added to the coumarin moiety that due to their anionic characteristics at physiological pH increases considerably the coumarin water solubility [10,11]. Although it might constitute a robust process of reducing the inhibition, it requires additional synthesis steps, on top of an already complex synthetic route.

Here, we present an alternative supramolecular approach to decrease the inhibitory effect of water insoluble DEACM photo-by-products in enzymatic reactions – using β-cyclodextrin molecules

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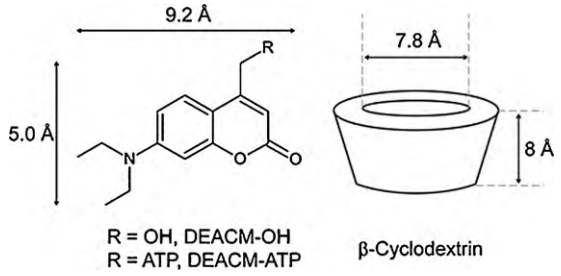

Fig. 1. DEACM photo-by-products (DEACM-OH and DEACM-ATP) and β-cyclodextrin. Potentially, the β-cyclodextrin has the ability to include molecules of organic compounds, such as the coumarin rings of DEACM products, into its hydrophobic cavity. The β-cyclodextrin cavity [12,13] has a 7.8 Å diameter that is suitable to accommodate the complexation of the DEACM along its long axis (diameter 5 Å). Due to the larger long axis size of the coumarin ring (9.2 Å *vs.* 8 Å), and the presence of the –OH or –ATP moieties, it is expected the formation of a 1:1 complex where the 7-diethylamino benzene moiety is inside the cyclodextrin cavity.

as molecular scavengers (see Fig. 1). β-Cyclodextrin is a cyclic oligosaccharide composed by 7 α-D-glucopyranose units, forming a hydrophobic cavity that is suitable for complexation of hydrophobic small molecules [12].

Using cyclodextrins in the reaction mixture, we were able to increase the concentration of released substrate (ATP), while scavenging the DEACM-OH photo-product generated after DEACM-ATP photocleavage. When added to the light-controlled transcription reaction, it was possible to channel the partitioning of DEACM-OH molecules into the formation of coumarin–cyclodextrin complex, thus reducing inhibition. We believe that this strategy can be easily extended to other coumarin derivatives, and any other similar compound for that matter, for *in vitro* reactions, i.e. reducing photo-product inhibition due to poor water solubility without further synthesis steps.

2. Materials and methods

2.1. General information

All chemicals were purchased from Sigma–Aldrich in the highest purity available and used without further purification. T7 RNA Polymerase was purchased from Fermentas (Vilnius, Lithuania). All oligonucleotides were purchased from STAB Vida (Lisbon, Portugal). Irradiations were performed in a monochromated (model 1681 0.22 m monochromator included in a SPEX Fluorolog spectrofluorimeter) 150 W Xe lamp at 390 nm, with a bandpass of 15 nm. A Hitachi-Merck HPLC L6200A Pump with an L-4500 Diode Array Detector using a RP-18 end-capped (Purospher Star, Merck) analytical column (4.6 mm × 15 mm, 5 μm) was employed for DEACM-ATP and DEACM-OH detection in photolysis and photochemical quantum yield determinations. Eluent A was triethylammonium acetate buffer in water, 5 mM, pH 6.9; eluent B was methanol. The gradient used started with 35% of B in A, from 0 min to 3 min; with an increase to 100% B after 6 min, and finished after 15 min at 100% of B. Separations were run at a flow rate of 0.9 mL/min and the column temperature was 35 °C. All spectroscopic measurements and irradiations were performed in 3 mL quartz fluorescence cuvettes (1 cm optical path) at 21 °C. Absorption spectra were recorded on a Varian Cary Bio 100 UV-Visible spectrophotometer. Fluorescence measurements of aerated solutions were performed on a Horiba-Jobin-Yvon SPEX Fluorolog 3.22 spectrofluorimeter. All emission spectra were collected with 1.5 nm slit bandwidth for excitation and emission, with correction files.

β-Cyclodextrin was chosen due to the proximity between its internal cavity diameter (7.8 Å internal diameter; 8 Å height [12,13]) and the dimensions of the coumarin (5.0 Å short axis, 9.2 Å long axis), allowing the formation of a 1:1 complex (Fig. 1).

2.2. DEACM derivatives: synthesis and purification

P3-[7-(diethylamino)coumarin-4-yl]methyl adenosine 5′-triphosphate trisodium salt (DEACM-ATP) was synthesized as described by Geiβler et al., method B [7]. 7-Diethylamino-4-hydroxymethylcoumarin (DEACM-OH) was synthesized and purified as described by Schönleber et al. [14]. A Hitachi-Merck HPLC L6200A Pump with an L-4500 Diode Array Detector using a Polystyrene-Divinylbenzene (PLRP-S, Polymer Labs, Germany) semi-preparative column (7.4 mm × 15 mm, 8 μm, 300 Å) was employed for separation and purification of DEACM-ATP. Eluent A was triethylammonium acetate buffer in water, 5 mM, pH 6.9; eluent B was methanol. Gradient started with 20 min at 30% of B in A; with an increase to 100% B after 21 min, and finished after 26 min at 100% of B. Separations were run at a flow rate of 3 mL/min and the column temperature was 35 °C. After peak separation and collection, samples were lyophilized, re-suspended in water and stored in the dark at −20 °C. A purity of >95% was determined by HPLC. All solutions were protected from light and DEACM-ATP manipulations were made in a dark chamber under red-light illumination.

2.3. Association constant determinations

The absorption and emission spectra of a 30 μM DEACM-OH solution in transcription buffer (50 mM Tris–HCl, 6 mM $MgCl_2$, 10 mM Dithiothreitol (DTT), 30 mM NaCl and 2 mM spermidine) were measured after the addition of increasing β-cyclodextrin quantity at 37 °C. A 10 min equilibration period was used between each cyclodextrin addition and measurement cycle. Emission at 570 nm was corrected for absorption/dilution variation and plotted as function of β-cyclodextrin concentration. Emission at 570 nm was chosen to minimize interference from the DEACM-OH/cyclodextrin complex, i.e. at this wavelength emission is almost only due to the free DEACM-OH. Using Valeur's model [15] and a non-linear least square fit, the association constant was determined (see Section 3 for details).

2.4. In vitro transcription reactions

In vitro RNA synthesis was performed using 400 ng of a 130 bp DNA template and 10 U of T7 RNA Polymerase (Fermentas) according to the manufacturer's protocol. In brief, reactions were carried out in a volume of 25 μl containing *in vitro* transcription buffer (50 mM Tris–HCl, 6 mM $MgCl_2$, 10 mM DTT, 30 mM NaCl and 2 mM spermidine), 50 μM of each NTP, template DNA and T7 RNA polymerase. As DNA template a T7 promoter coupled to the human p53—exon 7 was used [5]. The reaction mixtures were incubated at 37 °C for 60 min, followed by heat inactivation of the enzyme for 15 min, at 75 °C. For DEACM-OH inhibition of transcription, increasing concentrations of DEACM-OH (water solution) were used to induce decrease of transcription. For DEACM-OH inhibition suppression, *in vitro* transcription reactions were performed with increasing amounts of DEACM-OH, in the presence of 500 μM of β-cyclodextrin.

All transcription reaction products were analyzed in 3% agarose gels (TBE) with GelRed™ (Biotium, Hayward, CA, USA) staining. Determination of the 130 bp transcription product quantity was performed by pixel intensity/counting using Adobe Photoshop™ imaging software.

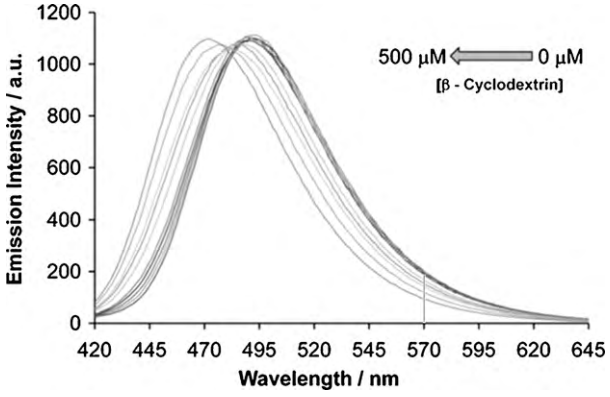

Fig. 2. Emission spectra (excitation at 385 nm) of DEACM-OH, 30 μM, in transcription buffer (50 mM Tris–HCl, 6 mM $MgCl_2$, 10 mM DTT, 30 mM NaCl and 2 mM spermidine) with increasing β-cyclodextrin concentration (0, 5, 10, 15, 20, 50, 75, 100, 200 and 500 μM). As scavenger concentration is increased, a blue-shift in emission is observed as a consequence of complexation between DEACM-OH and β-cyclodextrin, without significant change in fluorescence quantum yield. Dashed line marks 570 nm.

3. Results and discussion

3.1. Association between DEACM-OH and β-cyclodextrin

Addition of β-cyclodextrin to the aqueous buffered solution (50 mM Tris–HCl, 6 mM $MgCl_2$, 10 mM DTT, 30 mM NaCl and 2 mM spermidine) containing 30 μM of DEACM-OH led to a blue-shift in the coumarin emission maximum without a significant change in the fluorescence quantum yield (Fig. 2). It has been observed that solvent polarity significantly affects coumarin relative ground and excited state energies, leading to changes in absorption and emission spectra—solvatochromic effect [16]. The closely related Coumarin 1 (7-diethylamino-4-methylcoumarin) is known to present solvatochromism due to a charge transfer between the 7-amine non-bonding electrons and the coumarin ring. Switching to a less polar solvent medium is characterized by a blue shift in absorption and emission due to destabilization of highly polarized excited state. Thus, the blue-shift in emission observed is associated to the formation of a DEACM-OH/cyclodextrin complex, where the insertion of DEACM-OH into the cyclodextrins' cavity presents DEACM-OH with a medium of lower polarity than bulk water. This solvatochromic effect in DEACM-OH due to partitioning to hydrophobic organized medium has been previously observed in the presence of T7 RNA Polymerase [5].

The relative concentrations of free and associated DEACM-OH can be monitored through fluorescence spectroscopy, and thus the association constant of cyclodextrin/DEACM-OH formation (defined by Eq. (1)) can be determined experimentally through fitting of the equation developed by Valeur and co-workers [15] for a 1:1 complex (Eq. (2)).

$$K_a = \frac{[HG]}{[H][G]} \tag{1}$$

where [H] is the cyclodextrin host concentration, [G] is DEACM-OH guest concentration, [HG] is the DEACM-OH/cyclodextrin complex concentration and K_a the association constant in M^{-1}. The Valeur model is based on a 1:1 host–guest (HG) association in which an intensity signal (in this case, fluorescence emission) is proportional to the free guest species. Plotting DEACM-OH emission intensity at 570 nm (at which the fluorescence is solely due to free DEACM-OH species emission) as function of β-cyclodextrin host concentration and fitting Eq. (2) through a non-linear least squares analysis, the association constant can be calculated (Fig. 3).

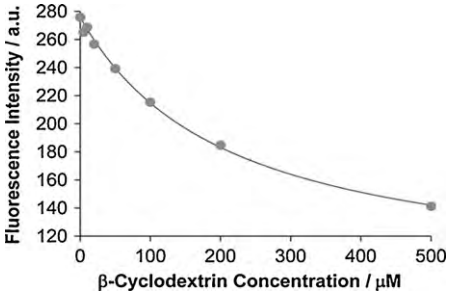

Fig. 3. (•) DEACM-OH fluorescence emission intensity at 570 nm as function of β-cyclodextrin concentration. (black line) Valeur model curve (Eq. (2)) fitted by non-linear least square method ($r^2 = 0.996$), with $K_a = 5000 \pm 500\ M^{-1}$ and $I_{lim} = 88$. It is estimated that in the presence of 500 μM of β-cyclodextrin, 79% of DEACM-OH is in the complexed form.

$$I = I_0 + (I_{lim} - I_0) \times \frac{\left([G]_0 + [H]_0(1/K_a)\right) - \sqrt{\left([G]_0 + [H]_0(1/K_a)\right)^2 - 4 \times [G]_0 \times [H]_0}}{2[G]_0} \tag{2}$$

The experimental points are well fitted by Eq. (2), which corroborates the formation of a 1:1 complex. The K_a value of $5 \times 10^3\ M^{-1}$ for the association constant between DEACM-OH and β-cyclodextrin, is comparable with values found in the literature for the association of β-cyclodextrin with several drugs (10^2–$10^4\ M^{-1}$) [17], much higher than 4-trifluoromethyl-7-diethylaminocoumarin ($10^1\ M^{-1}$) [18], half of that for the closely related Coumarin 153 ($10^4\ M^{-1}$) [13] and similar to 3-carboxyl-7-diethylaminocoumarin ($5 \times 10^3\ M^{-1}$) [19].

The light-controlled *in vitro* transcription reaction is based on the use of DEACM-ATP to release ATP through light irradiation. Averting inhibition due to the formation of DEACM-OH photoproduct requires that the cyclodextrin scavenger is present in solution prior to ATP release. Since DEACM-ATP is the sole DEACM species present in solution before irradiation, this poses two additional questions: (i) is DEACM-ATP able to form a complex with β-cyclodextrin, and (ii) if it does, is there any change in photochemical properties that might affect effective release of the substrate?

At the transcription buffer pH value (pH 8.0), the ATP moiety is fully deprotonated [20] with a net charge of −3, while DEACM-OH is neutral. Since the β-cyclodextrin's core is hydrophobic, both the ATP negative charge and the introduction of a bulky substituent (ATP *vs.* OH) would be expected to impair association of DEACM-ATP and the cyclodextrin. The association constant of β-cyclodextrin with DEACM-ATP was determined and a value of $9 \times 10^3\ M^{-1}$ was obtained, which was surprisingly similar (slightly higher) to that found for DEACM-OH/cyclodextrin complex. This seems to confirm our hypothesis that association is achieved through the coumarin hydrophobic part (7-diethylamino group and benzene ring) and driven solely by hydrophobic aspects (entropy increase due to the release of water from the cyclodextrin cavity).

Photochemical quantum yields for DEACM-ATP alone or in association with β-cyclodextrin were determined and results show similar yields for both species within experimental error: $(10 \pm 1) \times 10^{-3}$ and $(9.4 \pm 0.3) \times 10^{-3}$, respectively. It should be pointed out that the DEACM-ATP/β-cyclodextrin photochemical quantum yield indicated is a mixture of both complexed and free DEACM-ATP as, due to complete absorption spectra overlap of the two species, both forms were simultaneously irradiated. For a DEACM-ATP concentration of 30 μM and in the presence of 500 μM of β-cyclodextrin (which are approximately the same conditions used for *in vitro* transcription reactions), 81% of DEACM-ATP is in

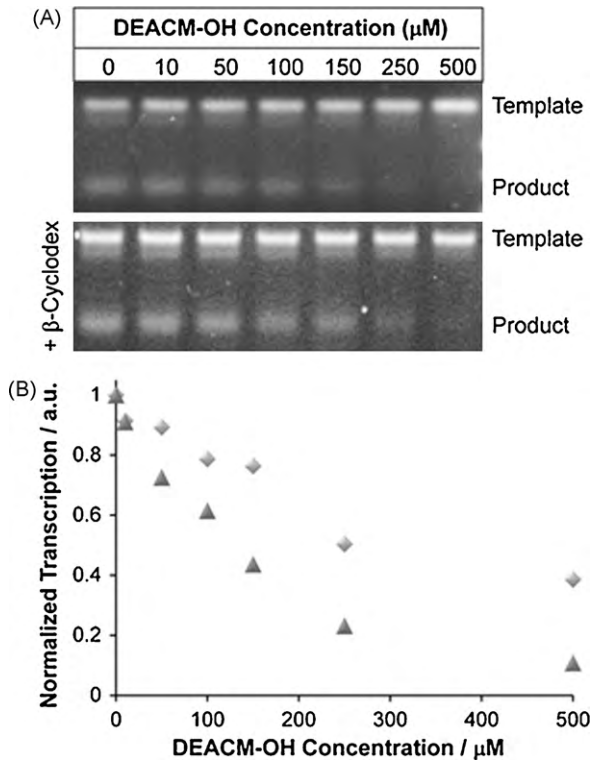

Fig. 4. (A) (top) DEACM-OH inhibition of transcription. *In vitro* transcription reaction in the presence of increasing DEACM-OH concentration. Higher concentrations of DEACM-OH lead to a decrease in transcription product quantity. (bottom) DEACM-OH inhibition suppression. *In vitro* transcription reaction with increasing amounts of DEACM-OH, in the presence of 500 μM of β-cyclodextrin. Transcription products can be observed at higher DEACM-OH concentrations, indicating that inhibition effect was reduced. In both gels, DNA template is observed as the upper band, and it was used as internal standard for each sample in transcription product (lower band) quantification. The band observed slightly below the template corresponds to DNA–RNA hybrids as determined by RNase and DNase digestion experiments (data not shown). (B) Transcription levels as function of DEACM-OH concentration, in the absence (▲) and presence (♦) of β-cyclodextrin. Addition of β-cyclodextrin decreases the inhibitory effect of DEACM-OH by half, when compared to the inhibitory effect in the absence of scavenger.

the complexed form. Furthermore, no secondary photo-products were detected by HPLC after irradiation.

3.2. Effect of β-cyclodextrin on DEACM-OH scavenging for in vitro transcription reactions

To assess the interference of β-cyclodextrin presence in RNA polymerization, increasing scavenger amounts were added to an *in vitro* transcription reaction. Due to the dynamic nature of the association equilibrium, the fraction of DEACM-OH scavenged is dependent on the total β-cyclodextrin concentration present in solution (Eq. (1)). Therefore, it is desirable that a large excess of β-cyclodextrin is present to drive the equilibrium into the formation of the complex rather than DEACM-OH partitioning into the RNA polymerase. The observed 18% decrease in transcription level in presence of 500 μM of β-cyclodextrin, albeit undesirable, is still considerably less than what is observed for DEACM-OH (>90% decrease for the same concentration). The presence of scavenger had no effect on transcript size (130 bp). It should be noted that the band observed slightly below the template corresponds to DNA–RNA hybrids as determined by RNase and DNase digestion experiments (data not shown), which are difficult to resolve and quantify. Nevertheless, it can be observed that DEACM-OH also inhibits formation of the hybrid, i.e. reduction of RNA being synthesized. This way, 500 μM of β-cyclodextrin were used as scavenger in transcription reactions with increasing amounts of DEACM-OH. Fig. 4 shows that a significant decrease of transcription levels for DEACM-OH concentrations above 50 μM is observed, which is in clear agreement to what had been as previously observed [5]. Inhibition linearly increases up to 250 μM of DEACM-OH, and at 500 μM only 11% of transcription product is obtained when compared to transcription with no DEACM-OH. Addition of β-cyclodextrin (Fig. 4) strongly quenches the inhibitory effect of DEACM-OH, and inhibition is approximately reduced by half.

These results seem to indicate that DEACM-OH is being channeled into the β-cyclodextrin complex, thus removing the inhibitor from solution. Thus, twice the previously allowed amount of DEACM-ATP can now be used in transcription. The need to use caged nucleotides in concentrations much lower than those commonly used in standard transcription reactions can now be effectively improved by means of the proposed supramolecular strategy. We believe that the application of coumarin caged compounds associated with cyclodextrin may also circumvent solubility problems found in other applications of these compounds.

4. Conclusions

Many enzymatic reactions that use a substrate generated *in loco* from a given precursor can be inhibited by increasing concentrations of undesirable by-products. In the case of photo-caged substrates, attempts to circumvent this issue have gone through inclusion of additional synthetic steps, that through addition of anionic groups such as hydroxyl or acetate groups increase the cage solubility in water [10,11]. When using DEACM caged ATP for *in vitro* transcription reactions, undesirable inhibition was observed due to the photo-by-product DEACM-OH low water solubility. The results here presented show a supramolecular approach to overcome the intrinsic solubility problems of commercially available coumarin derivatives.

Addition of β-cyclodextrin to the light-controlled *in vitro* transcription reaction was able to reduce the inhibition associated with DEACM-OH by half. This new strategy prevents the need of further complex synthesis steps for coumarin caged-nucleotides, or possibly other biomolecules. This approach can easily be extended to other coumarins and screening of different cyclodextrin derivatives might obtain higher association constants for coumarin–cyclodextrin complexes, further reducing inhibition. Also, the use of cyclodextrins can be a straightforward solution for other reactions that require caged compounds that face similar inhibition. As an example of a possible application area, current DNA sequencing by synthesis techniques [21,22] often use photo-removable protecting groups that could profit from the supramolecular strategy here presented, allowing extension of the sequencing range by suppressing photo-products inhibitory effects, with no need of expensive and time-consuming new molecule design.

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