Talanta 124 (2014) 67–70

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Antibody mediated fluorescence enhancement of nucleoside analogue 1,3-diaza-2-oxophenoxazine (tC°)

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ARTICLE INFO

Article history:
Received 4 November 2013
Received in revised form
14 February 2014
Accepted 20 February 2014
Available online 28 February 2014

Keywords:
Fluorescent nucleoside analogue
1,3-Diaza-2-oxophenoxazine (tC°)
Monoclonal antibody
Fluorescence enhancement
Homogeneous immunoassay
Hypsochromic shift

ABSTRACT

We report on the generation and analytical application of the monoclonal antibody G93-ED2 raised against the tricyclic fluorescent nucleoside analogue 1,3-diaza-2-oxophenoxazine (tC°). G93-ED2 is specifically binding this deoxycytidine analogue and was found to raise its fluorescence intensity by a factor of 5. This unique feature makes it a valuable tool in fluorescence dependent immunoassays. G93-ED2 was successfully applied in a homogeneous fluorescence quenching immunoassay (DNA-Q) for the sequence specific determination of DNA.

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1. Introduction

The binding of monoclonal antibodies directed against fluorophores, often leads to dramatic changes in the fluorescence properties of the bound fluorophore. Decades ago, Voss et al. found quenching effects and bathochromic shifts for polyclonal and monoclonal antibodies to fluorescein [1–3]. In our group, we also found such effects for another fluorescein antibody [4] and its recombinant scFv fragment [5,6]. Fluorescence quenching was also described for an anti-TAMRA antibody [7] and even for time-resolved fluorescence of Eu-kryptate antibodies [8].

Antibody mediated fluorescence quenching is caused by static quenching. Amino acid side chains of antigen binding site and fluorophore form a nonfluorescent complex. This is a unique property of an individual monoclonal antibody, meaning not all antibodies binding the fluorophore necessarily alter its fluorescence.

Surprisingly, the opposite effect (fluorescence enhancement and hypsochromic shift) is rarely described, mostly for fluorophore conjugates [9,10].

One group of fluorophores are fluorescent nucleoside analogues, which replace normal nucleobases inside the DNA/RNA duplex and aims at minimal perturbation of the natural properties of the nucleic acid system under investigation [11–13]. These are applied in photo-physical studies of DNA interacting with macromolecules (proteins) or for determining size and shape of DNA tertiary structure. Usually, fluorescent base analogues, such as 2-aminopurine and pyrrolo-C are highly sensitive to their microenvironment [12,14,15].

The tricyclic fluorescent nucleoside analogues, 1,3-diaza-2-oxophenothiazine (tC), 1,3-diaza-2-oxophenoxazine (tC°) and 7-nitro-1,3-diaza-2-oxophenothiazine (tC_{nitro}) are a family of deoxycytidine analogues that have been shown to base pair faithfully with dG with virtually no disruption of the normal duplex structure [16–18]. This means that the stability of the DNA duplex is not compromised as compared to the control regardless of DNA sequence. The fluorescence quantum yield of tC is essentially unchanged between single- and double-stranded DNA - 0.21 for single stranded DNA and 0.19 for duplex DNA. Also, the fluorescence characteristics of tC are not sensitive to neighbouring base combinations [19]. tC° has been shown to be the brightest fluorescent nucleoside analogue in duplex context reported so far and even retains the majority of its fluorescence when surrounded by guanine residues. Indeed, tC° has been reported to be 25–50 times brighter than 2-aminopurine [16] (Fig. 1).

The tC-family has already been utilized in several biophysical, biochemical and biological investigations. In an anisotropy study, it was shown that tC and tC° work excellently as probes for directly

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http://dx.doi.org/10.1016/j.talanta.2014.02.046
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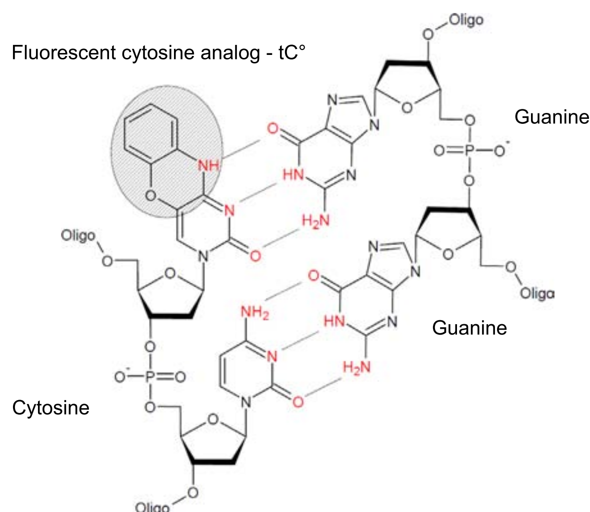


Fig. 1. Tricyclic cytosine analogue structure. Guanine is shown base paired with fluorescent cytosine analogue tC° and cytosine, respectively. The differences between cytosine and tC° were highlighted.

monitoring motion of nucleic acid helical structures, rather than a combination of motion of the overall structure and the probe itself, as is the case for most of the currently available fluorescent base analogues [16]. Furthermore, tC has been utilized as a FRET-donor in pair with rhodamine in a PNA-DNA-hybrid [20] and in pair with Alexa-555 in a study of conformational dynamics of DNA polymerase [21]. Importantly, tC° and tC_{nitro} were recently developed to be the first nucleic acid base analogue FRET-pair and shown to successfully monitor both distance and geometry changes within natural duplex systems [18].

The 5'-triphosphates of tC and tC° have also been synthesized and been shown in several polymerase studies to be efficiently incorporated into DNA although with increased frequency of mutations [22,23].

Here we present the generation of a novel monoclonal antibody binding the fluorescent nucleoside analogue 1,3-diaza-2-oxophenoxazine (tC°). This antibody dramatically increased the fluorescence of its antigen and induced a hypsochromic shift. We were able to apply this monoclonal antibody G93-ED2 successfully in a DNA-Quenching immunoassay (DNA-Q) for the sequence specific determination of oligonucleotide DNA.

This assay format was recently published (Sellrie et al. [7]). In this work we applied 5-TAMRA as fluorophore and the 5-TAMRA binding and quenching monoclonal antibody G71-DC7. DNA-Quenching assay (DNA-Q) bases on our finding that an antibody sensitive fluorophore gets protected from fluorescence quenching caused by an anti-fluorophore antibody, if the fluorophore replaces one of the internal nucleotides of one of the strands of the DNA duplex. In the DNA-Q assay the fluorescence probe is annealed to single stranded analyte DNA in the sample (for example denatured or asymmetric PCR amplicon). Then fluorescence quenching antibody is added and the change in fluorescence is detected. If the sample contains the probe target sequence, the probe hybridizes with the target. That escapes the fluorophore from antibody quenching and results in a positive fluorescence signal.

2. Experimental

2.1. Generation of fluorescence enhancing antibodies

Monoclonal tC° specific antibodies were generated by hybridoma technology [24]. For this purpose Balb/c mice were immunized three times with a BSA-tC° conjugate (1,3-diaza-2-oxophenoxazine–bovine serum albumin). Fluorophore conjugates were synthesized by emp Biotech GmbH (Germany). Immunization started with 100 μg conjugate using Freund's complete adjuvant. Booster immunizations were carried out six and eight weeks after the first immunization using 50 μg conjugate without adjuvant. Four days after the final booster immunization electrofusion of spleen cells with myeloma cells (P3 × 63Ag8.653, ATCC CRL-1580) in the presence of polyethylene glycol 8000 was performed as described [25]. Selected hybrids were cultivated in RPMI 1640 medium (containing 10% FCS, 2 mM glutamine and 50 mM β-mercaptoethanaol) and subcloned by limiting dilution on mouse peritoneal feeder cells. Culture supernatants of clones and subclones were tested in an enzyme immunoassay (ELISA) for antigen binding to a Biotin (3'-terminal) and tC° (internal) modified oligonucleotide, adsorbed to streptavidin coated microtiter plates. The class and subclass of monoclonal antibodies were determined as described [25]. Purification of antibody from culture supernatant was performed by protein A affinity chromatography [26].

2.2. Competitive ELISA for tC° and tC binding

Microtiter plates (Greiner Bio-One GmbH-Germany) were coated with BSA-tC° conjugate (incubation overnight with 50 μl per well containing 5 μg/ml in PBS), washed with tap water and blocked with 60 μl PBS-NCS (neonatal calf serum) per well for 40 min at room temperature. The wells were then incubated for 2 h with 50 μl of a mixture (incubated in advance for 30 min) of antibody G93-ED2 (0.5 μg/ml) and different tC° and tC concentrations. Microtiter plates were washed and incubated (50 μl per well; 1 h) with goat anti-mouse IgG-HRP conjugate (Sigma Aldrich-Germany). Plates were washed, and substrate reaction using TMB (3,3',5,5'-tetramethylbenzidine) performed. Reaction was stopped after 10 min and absorption measured at 450 nm.

2.3. Fluorescence enhancement experiments

Excitation and emission spectra of fluorophores tC° and tC were examined for the free fluorophores and in the presence of antibody G93-ED2 at equimolar concentrations of 0.2 μM.

BSA-tC° conjugate and tC° binding antibody G93-ED2 or control antibody G63-EC9 [27] were mixed (conjugate and antibody 0.2 μM in PBS) and fluorescence measured (Ex. 360 nm, Em. 432 nm).

Further fluorescence enhancement experiments were performed in the presence of 80% neonatal bovine serum (Biochrom GmbH, Germany) and 1 mg/ml DNA from salmon testis (Sigma-Aldrich, Germany). Concentration of fluorescence probe (see Section 2.4) and antibody G93-ED2 was 0.2 μM.

2.4. Oligonucleotide determination by DNA-quenching assay

tC°-CE phoshoramidite [5'-*O*-(4,4'-Dimethoxytrityl)-1'-(1,3-diaza-2-oxophenoxazin-1-yl)-2'-deoxy-B-D-ribofuranosyl-3'-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite] was purchased from Glen Research Corporation (USA). Fluorescence probe synthesis was performed by IBA GmbH (Germany). Its sequence (5'-GGT TTT GTT GTX TTC TCT ATT-3'; X=tC°) was chosen from *Salmonella enterica* invA gene.

A template oligonucleotide complementary to fluorescence probe was synthesized by metabion GmbH (Germany) (5'-GGT GAC AAT AGA GAA GAC AAC AAA ACC CAC-3'). The DNA-Quenching assay was performed in PBS. Fluorescence probe (40 μl of 0.3 μM solution) and template oligonucleotide (40 μl, varying concentrations) were mixed and hybridized for 10 minutes at 40 °C. Antibody G93-ED2 (40 μl of 0.3 μM) was added to get the final 0.1 μM concentration of antibody and probe. Fluorescence measurement (Ex. 360 nm, Em. 432 nm) was carried out after 15 minutes of incubation.

2.5. Fluorescence measurement

Fluorescence measurement was performed on an Edinburgh Instruments (UK) F900 spectrometer. The excitation wavelength for tC° was 360 nm. Fluorescence emission after antibody binding was detected at 432 nm.

3. Results and discussion

We performed hybridoma technology to raise monoclonal antibodies binding the tricyclic fluorescent deoxycytidine analogue tC° (1,3-diaza-2-oxophenoxazine). These experiments yielded the monoclonal antibody G93-ED2. The isotype of the antibody was determined as IgG1. G93-ED2 was tested to bind tC° and tC in competitive ELISA. The cytidine analogue tC° was bound with higher affinity then tC. The half maximal inhibitory concentration (IC50) of both antigen molecules differs by a factor of 6 with 0.09 μM for tC° and 0.56 μM for tC (Fig. 2). This fact is emphasising the high binding specificity of G93-ED2 as the molecule structures of tC° and tC vary in one single position (tC°—oxygen, tC—sulphur) (Fig. 1).

The antibody G93-ED2 bound to either tC° or tC was found to alter the fluorescence properties of its antigen (Fig. 3). In a more thorough study of the influence of G93-ED2 on tC° the antibody was shown to induce a hypsochromic shift and a raise in fluorescence by a factor of 5 (Fig. 4).

Fluorescence enhancement by a factor of 2.7 was observed even in the presence of 80% bovine serum. Antibody binding in the presence of DNA from salmon testis [1 mg/ml] raised the fluorescence by a factor of 4.6 (data not shown).

The observed effects are most probably caused by a change in microenvironment of the antigen when bound to and surrounded by the antigen binding site of the antibody. A solvatochromic effect (fluorescence dependence with solvent polarity) is the most likely reason. But this cannot be concluded by the data presented so far and is subject to further investigation.

The fluorescence modulating properties of G93-ED2 were applied in a variation of a homogeneous fluorescence quenching immunoassay (DNA-Q) recently published (Sellrie et al. [7]). Homogeneous immunoassays are simple mix and measure tests avoiding tedious and time consuming wash- and separation-steps. The sample (containing the analyte) is added to the test system and directly generating signal. The sensitivity is comparable with that of real time PCR (Sellrie et al. [7]). The assay principle is shown in Fig. 5.

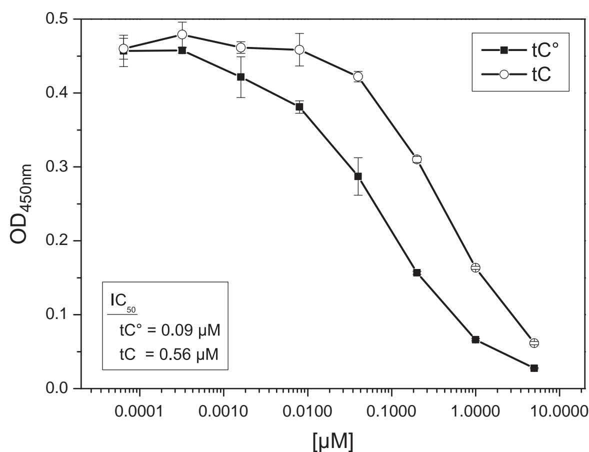


Fig. 2. Competitive immunoassay for cytosine analogue binding. Binding of antibody G93-ED2 to free tC and tC° was determined in a competitive immunoassay with BSA-tC° at the solid phase. tC° was bound with higher affinity then tC.

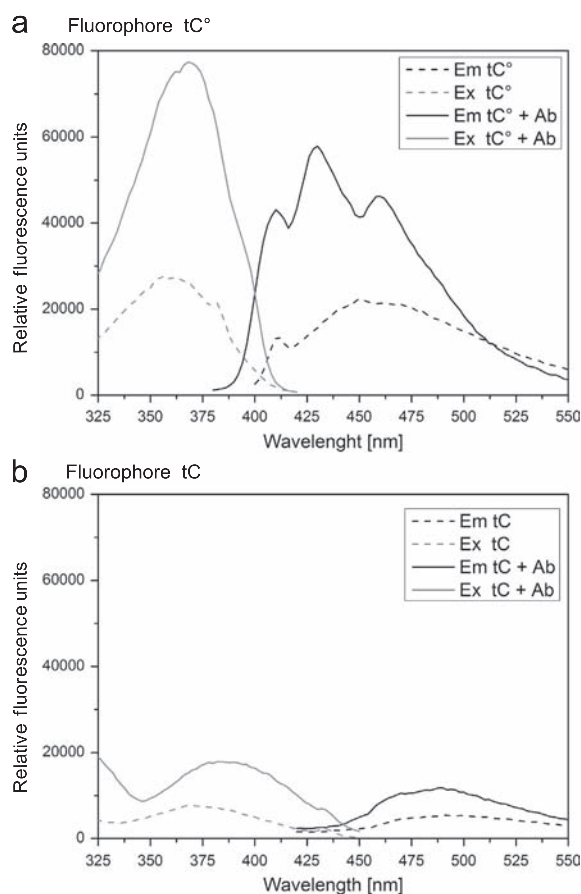
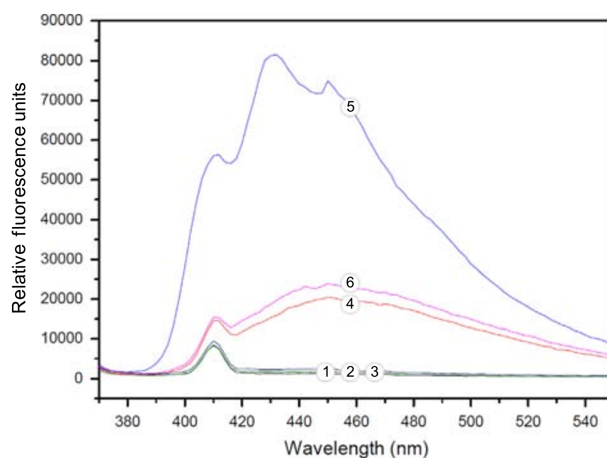


Fig. 3. Spectral properties of cytosine analogues. The excitation (Ex) and fluorescence (Em) properties of fluorescent cytosine analogues tC° (a) and tC (b) are presented for the free fluorophore and bound to the antibody G93-ED2 (Ab), respectively.



	1	2	3	4	5	6
BSA - tC° tC° - Antigen	-	-	-	+	+	+
G93-ED2 tC° - Antibody	-	+	-	-	+	-
G63-EC9 Control - Antibody	-	-	+	-	-	+

Fig. 4. Antibody mediated fluorescence enhancement. Antibody G93-ED2 binding to BSA-tC° causes a hypsochromic shift of the emission and raises the fluorescence intensity by a factor of 5.

The assay was applied for the sequence specific determination of oligonucleotide DNA. Hybridisation of target DNA to an internal tC° labelled fluorescence probe was protecting tC° from antibody

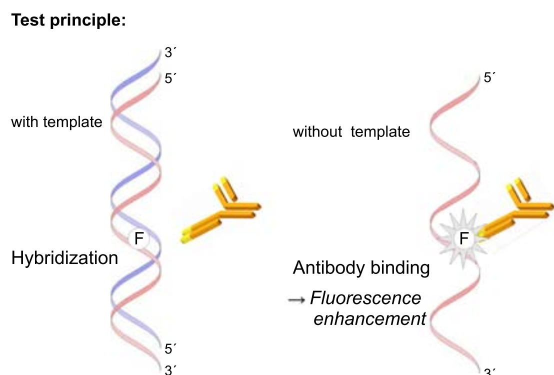


Fig. 5. Schematic presentation of assay principle. The assay consists of three components: a template DNA molecule (single-stranded or denaturated), a fluorescence probe and a fluorescence enhancing antibody. The fluorescence probe is added to the sample and hybridized specifically to the template. Hybridization of the fluorescence probe and template protects the fluorophore (*F*) from antibody binding leaving its fluorescence unaffected. Fluorophores of non-hybridized probes are bound by the antibody and their fluorescence enhanced.

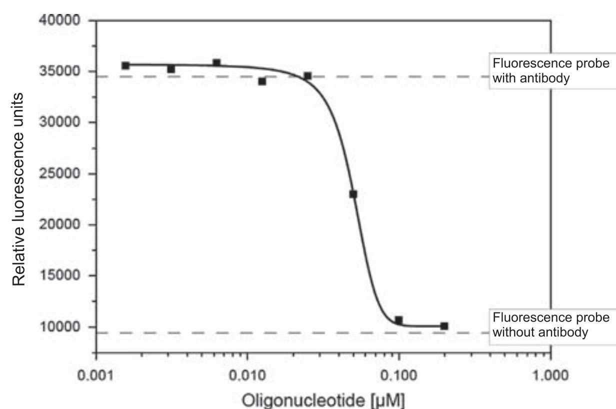


Fig. 6. Oligonucleotide detection applying the tC° binding and fluorescence enhancing antibody G93-ED2. With increasing amount of oligonucleotide template a larger fraction of fluorescent probe (from the total of 0.1 µM) gets protected from antibody mediated fluorescence enhancement due to hybridization to the oligonucleotide resulting in a fluorescence decline. Negative control (fluorescence probe with antibody) and positive control (fluorescence probe without antibody) were inserted as dashed lines.

binding leaving its fluorescence unchanged. Non-hybridised fluorescence probes were bound and its fluorescence significantly enhanced. Thus the observed fluorescence was indirectly dependent on the concentration of hybridised target DNA. An addition of target DNA at equimolar concentration (0.1 µM) reduced the fluorescence probe signal to background level (Fig. 6).

Fluorescence enhancing antibodies are in principle suitable for the development of homogeneous DNA-detecting immunoassays of the DNA-Q format. But for practical considerations the application of fluorescence quenching antibodies is preferred. Fluorescence enhancing antibodies lack the advantage of direct dependency between target concentration and fluorescence signal without providing an additional benefit. Nevertheless fluorescence enhancing antibodies can be considered as valuable tools in immunoassay applications not only for DNA detection but also for the determination of low molecular weight analyts.

4. Conclusion

We were able to generate a murine monoclonal antibody specifically binding and altering the fluorescence properties of the tricyclic fluorescent deoxycytidine analogue tC°. The observed fluorescence enhancement can be applied for the development of fast and sensitive homogeneous immunoassays as demonstrated for DNA detection in a DNA-Q assay format. According to our experimental experience we expect fluorescence modulating antibodies (quenching or enhancing) to have a great potential in analytical applications.

Acknowledgement

We thank Dr. Geßner and his group (Fraunhofer Institute for Applied Polymer Research - NanoPolyPhotonik) for technical assistance concerning fluorescence measurement and gratefully acknowledge the support of F. Ebert. We also thank Dr. Graser (AJ Innuscreen GmbH) for their support in design of fluorescence probe.

This work was supported by a grant from the Federal Ministry of Economics and Technology (ZIM KF2058407SK9).

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