STRUCTURAL AND FUNCTIONAL ANALYSIS OF BIOPOLYMERS AND THEIR COMPLEXES

SUBSTRATE EFFICIENCY OF Cy5-MODIFIED DERIVATIVES OF DEOXYURIDINE AND DEOXYCYTIDINE IN THE ROLLING CIRCLE AMPLIFICATION

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Received April 24, 2024

Revised June 06, 2024

Accepted June 10, 2024

Abstract. The kinetics of amplification and the features of individual and simultaneous incorporation of modified deoxynucleoside triphosphates in DNA during rolling circle amplification (RCA) have been studied. The study was carried out for six pairs of Sy5-labeled triphosphates of deoxyuridine (dU) and deoxycytidine (dC) previously synthesized with similar fluorescent substituents inside the pair. The effect of the linker length between the fluorophore and the pyrimidine base on the incorporation density was determined: nucleotides with a linker length of six carbon atoms are embedded in a growing DNA chain better than with three carbon atoms. It was found that the combined introduction of triphosphates into the reaction in an equivalent total concentration does not enhance the inhibitory effect, which gives grounds for a more detailed study of the simultaneous use of labeled dU and dC.

Keywords: *rolling circle amplification, fluorescently labeled dNTPs*

DOI: 10.31857/S00268984250108e3

INTRODUCTION

Nucleic acid amplification is a fundamental method of molecular biology and genetic research. This method can be used to accumulate the amount of DNA or RNA fragments required for further analysis, such as sequencing, hybridization, etc. One of the most promising areas for obtaining an analyzed DNA sample is isothermal amplification, which does not require a thermal cycler [1], including the rolling circle amplification (RCA) reaction [2].

The use of fluorescent tags with high quantum yields and the possibility of their introduction into DNA directly in the process of amplification allow increasing the sensitivity and reliability of diagnostic systems based on hybridisation analysis or amplification on solid substrates (biological microarrays). One of the most sought-after classes of fluorophores for insertion of tags into DNA are fluorescent cyanine dyes of the Cy5 series [3]. Cy5-pyrimidine nucleotides are able to efficiently incorporate into the forming DNA strand during amplification. This allows real-time visualisation and measurement of the amplification level, which is important for monitoring and optimising the process [4]. However, there is a problem of inhibiting the reaction, which can lead to the formation of artefacts and distortion of amplification results. A possible solution to increase the sensitivity of the assay may be the parallel introduction of differently named Cy5-triphosphates into the reaction without increasing their total concentration in the reaction solution.

In the present work, a comprehensive study of the substrate efficiency of six pairs of deoxyuridine and deoxycytidine triphosphates synthesized by us was conducted in the isothermal RCA amplification variant using BST 3.0 polymerase. This work extends our previous studies of two pairs of modified deoxyuridine triphosphates [5]. The kinetics of amplification was studied with determination of RCA efficiency (E_t) and the incorporation density of each labeled deoxynucleoside triphosphate into the growing DNA chain (K). The influence of the fluorophore structure and the nature of the triphosphate itself (dU or dC) on the degree of reaction inhibition was studied. As a model template for amplification, we used the whole-genome DNA of one of the important pneumonia pathogens - Staphylococcus aureus *Staphylococcus aureus*.

EXPERIMENTAL SECTION

Strains. Genomic DNA of *S. aureus* (ATCC 25923) was isolated and decontaminated at the State Research Center for Applied Microbiology and Biotechnology (Obolensk, Russia) according to [6].

Primers and other oligonucleotides. Primers for RCA amplification were designed using the Integrated DNA Technologies web resource (idtdna.com), and specificity was analyzed using the BLAST algorithm (NIH, USA). Two primers were used: forward - 5'-CGATAGACGATACCTGCCATCG-3' and reverse - 5'-ATCGTCATCTGCATTCCATC-3'.

To create a circular species-specific oligonucleotide, a linear 90-mer oligonucleotide was synthesized, Ph-TTAGAGGCATGTGGTTATGCTAGCAACAGATAGACAAGATGGAATGCAGATGACGATAGACGATACCTGCCATCGTTTTGGCTTGCATTA-3', phosphorylated at the 5' end, where Ph is phosphate.

Solid-phase synthesis of oligonucleotides was performed using an automatic synthesizer ABI 394 DNA/RNA ("Applied Biosystems", USA) according to standard protocol and purified on a BDS Hypersil C18 column ("Thermo", USA).

Real-time RCA with fluorescently labeled triphosphates.

To create a circularized 90-mer oligonucleotide, T4 ligase ("NEB", USA) and genomic DNA of *S. aureus* were used. The resulting circular oligonucleotide, after purification on microcolumns from the GeneGET Purification kit ("Thermo") and concentration measurement, was used as a template in RCA.

The reaction mixture contained natural dNTPs at a concentration of 0.2 mM, 1.6 units of BST 3.0 DNA polymerase ("New England Biolabs", USA) and its corresponding reaction buffer in the amount recommended by the manufacturer; various labeled dUTP and dCTP [7-9] at a concentration of 32 μM for individual introduction or 16 μM each when combined; circular template (10^4 copies) and specific primers at a concentration of 5 pmol per 20 μl reaction volume. The reaction was carried out on a Gentier 96E DNA amplifier (" Tianlong ", China) in real-time mode according to the following program: 50 min at 65°C with fluorescence signal recording once per minute, followed by storage mode (10°C). The accumulation of the reaction product was visualized using the intercalating dye EvaGreen ("Biotium", USA).

Purification of reaction products. RCA products were freed from primers and deoxynucleoside triphosphates (including labeled ones) using the GeneGET Purification kit ("Thermo") according to the manufacturer's instructions. The resulting product in a reaction volume of 20 μl was washed from the GeneGET column with an equal volume of Milli-Q water. The degree of purification from fluorescently labeled dNTPs was controlled by agarose gel

electrophoresis and fluorescence excitation at 630 nm to exclude their influence in subsequent spectrophotometric determination of the amount of label incorporated into DNA.

Agarose gel electrophoresis. The products obtained during isothermal amplification were separated in a 4% agarose gel (Agarose LE, "Helicon", Russia) for 40 min at 10 V/cm, then stained for 10 min using SYBR Green I ("Molecular Probes", USA) in 1 × TBE buffer ("Helicon", Russia). For visualization, the ChemiScope 6200 Touch gel documentation system ("Clinx Science Instruments", China) was used. Total DNA detection was performed using built-in LED lights and "Green light excitation/emission" filters and SYBR Green I staining; selective detection of oligonucleotides labeled with Cy5c series fluorescent dyes was performed using built-in LED lights and "Red light excitation/emission" filters.

Spectrophotometry. Spectrophotometric analysis was carried out on a NanoPhotometer NP80 ("Implen", Germany) at wavelengths of 260 and 647 nm to calculate the yields of total DNA and incorporated fluorescent label, respectively.

RESULTS AND DISCUSSION

In this work, six pairs of modified derivatives of deoxyuridine and deoxycytidine with similar fluorescent substituents within the pair were studied, differing in the length of the linker between the fluorophore and the nitrogenous base, as well as the length of the linker between the second heterocycle of the fluorophore and the quaternary ammonium group that is part of the dye. As a control, we selected a pair of dU and dC in which the Cy5 dye is linked to the nitrogenous base via a linker through a sulfo group (U_k and C_k). Currently, the labeled deoxyuridine triphosphate U_k is widely used in gel microchip technology [6, 8]. The structural formulas of labeled deoxynucleotides are shown in Fig. 1, the structures $R_{1–4}$ are indicated in Table 1.

Fig. 1. Structural formulas of nitrogenous bases and fluorophore. *a* – Fluorescently labeled 5-allylamine-2'-deoxyuridine-5'-triphosphate **(** dUTP-Dye); *b* – fluorescently labeled 5-allylamine-2'-deoxycytidine-5'-triphosphate **(** dCTP-Dye); *c* – fluorescent dye (Dye). $R_{1–4}$ are shown in Table 1.

Table 1. Fluorescently labeled nucleoside triphosphates $R_{1–4}$

Nucleoside triphosphate triphosphate	No. synthesis	R_1	R_2	R_3	R_4
dU_k	49	C_2H_5	$SO_2NHCO(CH_2)_6CONHCH_2(CH)_2$ dUTP	SO_3^C	C_2H_5
dC_k	129	C_2H_5	$SO_2NHCO(CH_2)_6CONHCH_2(CH)_2$ dCTP	SO_3^C	C_2H_5
dU_1	2	$(CH_2)_5CONHCH_2(CH)_2$ dUTP	H	SO_3^C	C_2H_5
dC_1	128	$(CH_2)_5CONHCH_2(CH)_2$ dCTP	H	SO_3^C	C_2H_5
dU_2	79	$(CH_2)_5CONHCH_2(CH)_2$ dUTP	SO_3^{SO}	SO_3 (CH	$(CH_2)_3N(CH_3)_3^+$
dC_2	128	$(CH_2)_5CONHCH_2(CH)_2$ dCTP	SO_3^{SO}	SO_3 (CH	$(CH_2)_3N(CH_3)_3^+$
dU_3	81	$(CH_2)_5CONHCH_2(CH)_2$ dUTP	SO_3^{SO}	SO_3 (CH	$(CH_2)_5N(CH_3)_3^+$
dC_3	128	$(CH_2)_5CONHCH_2(CH)_2$ dCTP	SO_3^{SO}	SO_3 (CH	$(CH_2)_5N(CH_3)_3^+$
dU_4	80	$(CH_2)_5CONH(CH_2)_5CONHCH_2(CH)_2$ dUTP	SO_3^{SO}	SO_3 (CH	$(CH_2)_3N(CH_3)_3^+$
dC_4	128	$(CH_2)_5CONH(CH_2)_5CONHCH_2(CH)_2$ dCTP	SO_3^{SO}	SO_3 (CH	$(CH_2)_3N(CH_3)_3^+$
dU_5	82	$(CH_2)_5CONH(CH_2)_5CONHCH_2(CH)_2$ dUTP	SO_3^{SO}	SO_3 (CH	$(CH_2)_5N(CH_3)_3^+$

dC $_5$	128	$(CH_2)_5CONH(CH_2)_5$ $CONHCH_2(CH)_2$dCTP	SO_3^{SO}	SO_3 (CH	$(CH_2)_5$ $N(CH_3)_3^+$

We determined substrate efficiency parameters of modified dU and dC, such as E_t (amplification efficiency), which allows evaluating the degree of RCA inhibition; target product yield; incorporation coefficient " K ". To increase the accuracy of determining the inhibitory effect, the algorithm for calculating E_t presented in [5] was adjusted: we only considered the time of signal accumulation from the beginning of the exponential growth of the signal accumulation curve until reaching the plateau, instead of the total reaction time. The data are presented in Table 2.

Table 2. Summary table of substrate efficiency indicators for Cy5-labeled dU and dC

dNTP, μM	Product yield $\pm \sigma$, μg	$E_t \pm \sigma$	$K \pm \sigma$
$dU_k(32)$	1.88 ± 0.02	1.21 ± 0.05	0.36 ± 0.005
$dC_k(32)$	2.14 ± 0.1	1.30 ± 0.02	0.20 ± 0.02
$dU_k(16) + dC_k(16)$	1.54 ± 0.29	1.24 ± 0.02	0.32 ± 0.01
$dU_1(32)$	0.76 ± 0.02	1.11 ± 0.01	0.19 ± 0.02
$dC_1(32)$	2.09 ± 0.16	1.20 ± 0.01	0.12 ± 0.01
$dU_1(16) + dC_1(16)$	0.96 ± 0.2	1.16 ± 0.02	0.20 ± 0.02
$dU_2(32)$	1.94 ± 0.39	1.34 ± 0.04	0.09 ± 0.02
$dC_2(32)$	2.25 ± 0.31	1.34 ± 0.04	0.07 ± 0.01
$dU_2(16) + dC_2(16)$	2.22 ± 0.42	1.38 ± 0.02	0.11 ± 0.01
$dU_3(32)$	2.36 ± 0.03	1.34 ± 0.03	0.14 ± 0.005
$dC_3(32)$	2.58 ± 0.21	1.35 ± 0.04	0.11 ± 0.02
$dU_3(16) + dC_3(16)$	2.13 ± 0.17	1.39 ± 0.01	0.15 ± 0.01
$dU_4(32)$	2.28 ± 0.17	1.30 ± 0.01	0.24 ± 0.005

dC_4 (32)	2.42 ± 0.05	1.29 ± 0.08	0.18 ± 0.03
dU_4 (16) + dC_4 (16)	2.12 ± 0.24	1.38 ± 0.02	0.20 ± 0.03
dU_5 (32)	2.40 ± 0.13	1.31 ± 0.02	0.20 ± 0.02
dC_5 (32)	2.25 ± 0.22	1.34 ± 0.06	0.10 ± 0.01
dU_5 (16) +dC_5 (16)	1.68 ± 0.3	1.36 ± 0.01	0.18 ± 0.02

The reaction kinetics were studied in RCA using Bst 3.0 polymerase. The concentration of Cy5-dNTP was determined by performing amplification with different concentrations - from 16 to 64 μM. After comparing such indicators as product yield and the degree of reaction inhibition, the optimal concentration of labeled triphosphates for individual incorporation was selected as 32 μM. For simultaneous application, 16 μM each of dU and dC were used, i.e., their total concentration was equal to the optimal concentration for individual introduction into the reaction. The degree of inhibition, indicated by the amplification efficiency E_t, was calculated based on the slope of the linear portion of the accumulation curve presented on a logarithmic scale [10]. The signal accumulation curves are shown in Fig. 2. It can be seen that the simultaneous introduction of both modified triphosphates into the reaction does not lead to a decrease in the rate of product accumulation.

Fig. 2. Kinetics of RCA amplification with individual and combined introduction of labeled deoxynucleoside triphosphates into the reaction with equivalent total concentration (explanations in the text). For clarity, in each group the curves are normalized to the maximum value of the fluorescent signal.

Reaction products were purified on microcolumns with silicon dioxide-based membranes for subsequent measurement of label incorporation density by spectrophotometric method. This made it possible to quantitatively remove unreacted fluorescently labeled triphosphates and thereby ensure that the signal was obtained only from labels that were incorporated into DNA chains during

their growth. Fig. 3 shows an electrophoregram in two-wavelength mode, allowing visual assessment of the degree of purification from free fluorescently labeled Cy5 dNTP.

Fig. 3 . Visualization of the purification degree of reaction products from free fluorescently labeled nucleoside triphosphates in two-channel excitation/detection mode ("green" channel 530/585 nm and "red" channel 630/690 nm). *a* - Electrophoregram of RCA products before purification; *b* - after purification. L - DNA length marker GeneRuler 50 bp; *1* - dU_k ; *2* - dC_k ; *3* - dU_k +dC_k ; *4* - dU_3 ; *5* - dC_3 ; *6* - dU_3 +dC_3 ; *7* - dU_5 ; *8* - dC_5 ; *9* - dU_5 +dC_5 .

The product yield was determined spectrophotometrically at a wavelength of 260 nm. To assess the label incorporation density, the incorporation coefficient " *K* " was used - the ratio of sample absorption at a wavelength of 647 nm (label) to 260 nm (DNA).

The influence of the linker length between the fluorophore and the pyrimidine base on the incorporation density of deoxynucleoside triphosphates was detected. Extending the linker from three to six carbon atoms increased the ability of polymerase to recognize labeled nucleoside triphosphates as substrates. Figure 4 shows three electropherograms: "green channel", SYBR staining, visualization of the total reaction product (*a*); "red" channel, displaying the fluorescence of Cy5-labeled triphosphates (*b*); dual-wavelength mode, containing signals from both channels (*c*). It can be seen that in each pair of labeled dU and dC with similar fluorophore and linker, the uridine derivative has a higher incorporation density. In Fig. 4 *b* , the effect of linker extension on incorporation density can be visually assessed. However, the combined use of dU and dC with a short linker showed a higher incorporation density than individual use. This may indicate the absence of a "total concentration" effect for such nucleoside triphosphates.

Fig. 4. Effect of linker length on incorporation density of Cy5-nucleotides in RCA. *a* - Visualization of RCA on the "green" channel 530/585 nm; *b* - on the "red" channel 630/690 nm; *c* - in dual-channel excitation and detection mode. L - dsDNA length marker GeneRuler 50 bp; *1* - dU_k ; *2* - dC_k ; *3* - dU_k + dC_k ; *4* - dU_3 ; *5* - dC_3 ; *6* - dU_3 + dC_3 ; *7* - dU_5 ; *8* - dC_5 ; *9* - dU_5 + dC_5 .

Lengthening the carbon chain between the quaternary ammonium group in the dye and the corresponding (second) heterocyclic fragment of the fluorophore in pairs with a short linker allowed for a higher density of incorporation of labeled triphosphates, both when introduced together and individually. At the same time, in pairs with a long linker, the same lengthening reduced the incorporation density. The length of the linker between the nucleotide base and the fluorophore has a significantly greater impact on substrate efficiency.

CONCLUSION

Testing the degree of simultaneous and individual incorporation of fluorescently labeled triphosphates, taking into account the product yield in the RCA reaction, showed that the highest substrate efficiency, necessary for increasing the sensitivity of analysis, is achieved when deoxyuridines with a long linker between the nucleotide base and the fluorophore are introduced into the reaction. The simultaneous introduction of dU and dC appears promising for ensuring the versatility of diagnostic systems in the simultaneous analysis of several DNA templates with different GC compositions.

FUNDING

This research was supported by the Russian Science Foundation grant No. 22-14-00257.

ETHICS DECLARATION

This article does not contain any studies involving humans or animals as objects.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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