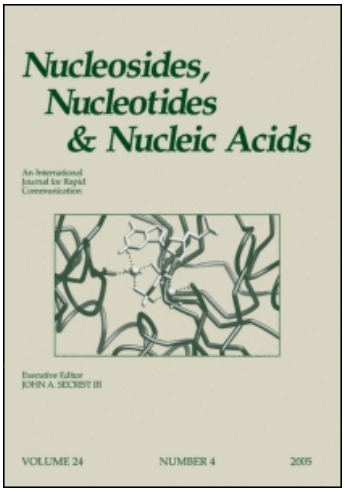

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Maksim V. Kvach[a]; Igor A. Prokhorenko[b]; Alexey V. Ustinov[b]; Sergei V. Gontarev[a]; Vladimir A. Korshun[b]; Vadim V. Shmanai[a]
[a] Institute of Physical Organic Chemistry NAS, Minsk, Belarus [b] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moskow, Russia

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REAGENTS FOR THE SELECTIVE IMMOBILIZATION OF OLIGONUCLEOTIDES ON SOLID SUPPORTS

Maksim V. Kvach □ *Institute of Physical Organic Chemistry NAS, Minsk, Belarus*

Igor A. Prokhorenko and Alexey V. Ustinov □ *Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moskow, Russia*

Sergei V. Gontarev □ *Institute of Physical Organic Chemistry NAS, Minsk, Belarus*

Vladimir A. Korshun □ *Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moskow, Russia*

Vadim V. Shmanai □ *Institute of Physical Organic Chemistry NAS, Minsk, Belarus*

□ *New reagents (CPGs and phosphoramidites) for automatic solid phase synthesis of modified oligonucleotides were designed. Three oligonucleotides carrying fluorescent label at the 5′-terminus and an anchor group at the 3′-terminus were prepared and their immobilization in orthogonal conditions on solid supports was studied.*

Keywords Oligonucleotides; immobilization; click chemistry; streptavidin-biotin

In the last two decades microarray use became widespread in different fields of science and diagnostics, e.g., for detecting hundreds of mutations and diseases.[1,2] At present, several methods for producing oligonucleotide microarrays are known.[3] The immobilization of oligonucleotides carrying functional groups is superior to on-chip synthesis. It can be performed using noncovalent interactions (biotin-avidin), covalent binding (e.g., amide bond formation), or self-assembled monolayer (SAM) technique. The aim of the present work is to develop a technique allowing the selective immobilization on solid surface of one oligonucleotide from the mixture. The procedure could be used to produce microarrays or affinity sorbents.

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Address correspondence to Vadim V. Shmanai, Institute of Physical Organic Chemistry NAS, Surganova 13, Minsk 22072, Belarus. E-mail: shmanai@ifoch.bas-net.by

TABLE 1 Structure of double-labeled oligonucleotides

Name	Composition (5′ → 3′)
FAM-NH_2	FAM-TTTTTTTTTTTTTTT-NH_2
Cy3.5-Yn	Cy3.5-TTTTTTTTTTTTTTT-Alkyne
Cy5-Biotin	Cy5-TTTTTTTTTTTTTTT-Biotin

Three different types of interactions between modified oligonucleotides and solid phases were selected: aminogroup—activated hydroxyl (results in carbamate bond formation), biotin—streptavidin (strong noncovalent interaction), and terminal alkyne—azide (in the presence of catalysts a cycloaddition reaction occurs).[4–6] To get a vivid proof of the principle of selective immobilization, three oligonucleotides containing anchor group at 3′-terminus and fluorescent dye at 5′-terminus were synthesized. A fluorescent label at 5′-terminus was introduced to determine whether immobilization of oligonucleotide is successful. If an anchor group of any double-labeled modified oligonucleotide reacts with a solid support, the bright fluorescent signal appears on the surface.

Modifying reagents were synthesized from hydroxyprolinol **1**. It was first acylated with an activated ester of appropriate acid, treated with dimethoxytrithyl chloride in pyridine, and then attached to solid phase or phosphitylated with bis(diisopropylamino)-2-cyanethoxy phosphine to give supports **2-4** or phosphoramidites **5–7** (Figure 1).

Using reagents **2–7** we synthesized three double-labeled oligonucleotides (Table 1) according to the standard protocols.

Three agarose-based solid-phase supports were also prepared (Figure 2). We chose agarose beads because this sorbent is easy to activate and modify. On the other hand, this sorbent is highly hydrophilic and the degree of nonspecific binding of biomacromolecules is negligible.

Every of these sorbents have to react with the appropriate anchor group attached to the oligonucleotides: NHS-sepharose—with aminogroup, N_3-sepharose—with alkyne, streptavidin-sepharose—with biotin moiety. To be sure that these reactions occur with selected pairs of sepharose-oligonucleotide we carried out cross-reactions between every sepharose and every oligonucleotide (Table 2).

TABLE 2 Cross-reactions of oligonucleotides with different sepharose-sorbents

	FAM-NH_2	Cy3.5-Yn	Cy5-Biotin
NHS-sepharose	+	−	−
N_3-sepharose	−	+	−
Strept.-sepharose	−	−	+

1 **2-7**

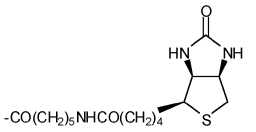
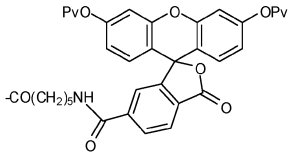
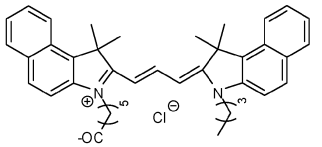
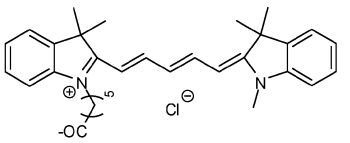
#	R^1	R^2
2	$-CO(CH_2)_5NHCOCF_3$	$-CO(CH_2)_2CO$-LCAA-CPG
3	$-CO(CH_2)_3C{\equiv}CH$	$-CO(CH_2)_2CO$-LCAA-CPG
4		$-CO(CH_2)_2CO$-LCAA-CPG
5		$-P(NPr^i_2)O(CH_2)_2CN$
6		$-P(NPr^i_2)O(CH_2)_2CN$
7		$-P(NPr^i_2)O(CH_2)_2CN$

FIGURE 1 Synthesis of modified reagents based on hydroxyprolinol structure.

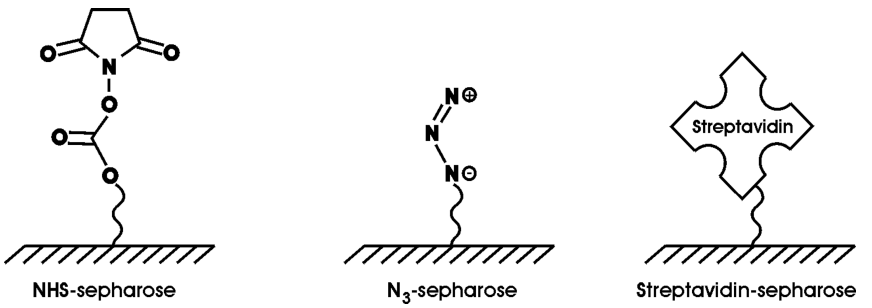


FIGURE 2 Three agarose-based solid surfaces containing: a) active ester; b) azido-group; c) streptavidine protein.

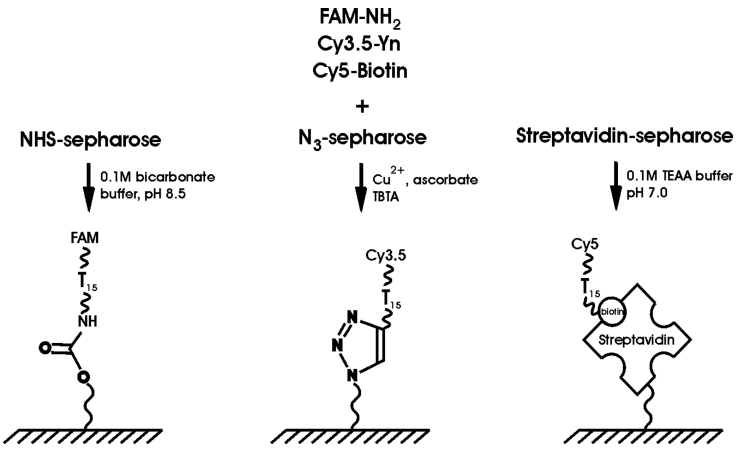


FIGURE 3 Mixture of oligonucleotides carrying different anchor groups reacts only with appropriate surface to form an explicit fluorescent signal.

Oligonucleotide **FAM-NH_2** was incubated with every sepharose in 0.1M bicarbonate buffer (pH 8.5). In the case of **Cy3.5-Yn** click catalysts (Cu^{2+}, ascorbate, TBTA) were added and a reaction was performed in 0.1M TEAA buffer (pH 7.0). Biotinylated oligonucleotide **Cy5-Biotin** was incubated with sepharoses in 0.1M TEAA buffer (pH 7.0). After an overnight incubation at room temperature the supernatant was removed and the sorbent was washed twice with the appropriate buffer. Fluorescence of every sample was visualized with UV-lamp (excitation at 364 nm). As expected, in the case of **FAM-NH_2** only NHS-sepharose produced green fluorescence; the **Cy3.5-Yn** oligonucleotide gave red fluorescence on the N_3-sepharose and the **Cy5-Biotin** conjugate was bound to streptavidin-sepharose to produce a violet signal in a UV lamp. In all other cases, the agarose beads were not colored (they had no absorbtion of the fluorophore) and had no fluorescence. That was why we concluded that immobilization of the oligonucleotides had not occurred.

To conclude, we developed reliable procedure for the selective immobilization of one from the mixture of three functionalized oligonucleotides (Figure 3). The conditions are rather similar, and now the work is in progress to elaborate universal conditions for three reactions to proceed simultaneously to make the reactions perfectly orthogonal.

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