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Controlled Pore Glass-based oligonucleotide affinity support: towards High Throughput Screening methods for the identification of conformation-selective G-quadruplex ligands

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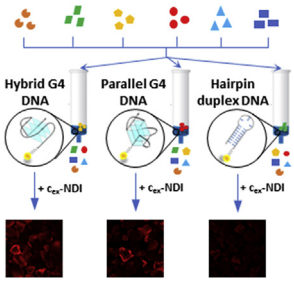
HIGHLIGHTS

- A functionalized Controlled Pore Glass incorporating hexaethylene glycol was developed.
- G-quadruplex-forming oligonucleotides were synthesized on this novel solid support.
- These oligonucleotide-bound supports allowed affinity chromatography-based screenings.
- Putative G-quadruplex ligands were analyzed by a rapid, easy spectrophotometric test.
- Fluorescence confocal microscopy discriminated G-quadruplex vs. duplex DNA on CPG.

GRAPHICAL ABSTRACT



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ABSTRACT

Target selectivity is one of the main challenges in the search for small molecules able to act as effective and non-toxic anticancer and/or antiviral drugs. To achieve this goal, handy, rapid and reliable High Throughput Screening methodologies are needed. We here describe a novel functionalization for the solid phase synthesis of oligonucleotides on Controlled Pore Glass, including a flexible hexaethylene glycol spacer linking the first nucleoside through the nucleobase via a covalent bond stable to the final deprotection step. This allowed us preparing fully deprotected oligonucleotides still covalently attached to their supports. In detail, on this support we performed both the on-line synthesis of different secondary structure-forming oligonucleotides and the affinity chromatography-based screenings of conformation-selective G-quadruplex ligands. By using a fluorescent core-extended naphthalene diimide with different emitting response upon binding to sequences folding into G-quadruplexes of different topologies, we have been able to discriminate not only G-quadruplex vs. duplex DNA structures, but also different G-quadruplex conformations on the glass beads by confocal microscopy.

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1. Introduction

In the quest for innovative and effective anticancer/antiviral drugs [1–4], G-quadruplexes (G4s) have emerged as appealing therapeutic targets for their crucial roles in human telomeres and in genomic regions associated with human oncogene and viral gene regulation [5–7]. In addition to their increasing significance as drug targets, G4 structures are gaining interest as privileged scaffolds to produce therapeutic agents in anticancer or antiviral aptamer-based treatments [8–10] and/or diagnostic tools for bioanalytical applications [11,12].

From a structural point of view, G4s are non-canonical nucleic acid secondary structures, stabilized by cations located within the central channel formed upon stacking of two or more G-tetrads, i.e. cyclic planar arrangements of four guanines linked through Hoogsteen hydrogen bonds [13]. G4s exhibit a marked structural polymorphism, which depends on strands stoichiometry and orientation, type of linking loops and guanine residues conformation. In particular, G4s can be arranged in parallel, antiparallel or hybrid-type conformations depending on the relative orientation of the G4-forming strands [14]. To achieve an optimal recognition selectivity, which is the prerequisite to erase the off-target drug toxicity, most research efforts have been directed to identify structure-selective ligands, able to discriminate G4 vs. duplex DNA [15]. One additional, major challenge is the discovery of conformation-selective ligands, i.e. small molecules able to selectively recognize specific G4 conformations over others, for which only rare examples are currently known [16,17].

In this context, we recently developed an experimental assay, named G-quadruplex on Oligo Affinity Support (G4-OAS), based on affinity chromatography for the screening of libraries of putative G4 binders [18,19]. It consists in flowing solutions of potential ligands through a polystyrene resin functionalized with a G4-forming oligonucleotide. The compounds with high affinity for the G4 are retained, while those with low or no affinity are eluted and quantified by UV measurements. Though rapid and simple, two main limitations emerged on using this method: i) unspecific interactions with the polystyrene resin, observed with ligands featuring large aromatic cores and low hydrophilicity [19], and ii) absence of direct information about the effective conformation adopted by the G-rich sequences immobilized on the solid support.

With the aim of addressing these general issues and obtaining a universal support for effective screenings of putative conformation-selective G4 ligands, we here describe a novel functionalization for Controlled Pore Glass (CPG) allowing both the on-line synthesis of fully deprotected, support-bound oligonucleotides and subsequent affinity chromatography-based binding assays [20]. For its intrinsic chemical inertness and peculiar structure, which determines e.g. higher hydrophilicity and inability to give stacking interactions with aromatic putative ligands, this solid support is expected to overcome the drawbacks (essentially, unspecific ligand binding) associated with the use of polystyrenic Oligo Affinity Support [18–21]. Additional prerequisite for an affinity chromatography-based screening to be effective is that the oligonucleotide linked to the support must maintain its native conformation, as in solution. To get information about the effective conformation adopted by the G-rich sequences immobilized on the solid support, we have employed a fluorescent core-extended naphthalene diimide [22] which, when bound to the oligonucleotide-linking glass beads, allowed discriminating different topologies of DNA structures by fluorescence confocal microscopy.

2. Experimental section

2.1. Functionalization of Long Chain AlkylAmine-CPG (LCAA-CPG) with 5′-O-DMT, 3′-O-acetyl-thymidine through a hexaethylene glycol spacer

2.1.1. Synthesis of 4,4′-dimethoxytrityl-hexaethylene glycol-COOH (DMT-HEG-COOH)

DMT-HEG-OH (450 mg, 0.77 mmol, commercially available from Berry & Associates) was reacted with a mixture of 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO, 19 mg, 0.12 mmol), KBr (20 mg, 0.17 mmol), tetrabutylammonium chloride (46 mg, 0.17 mmol), $NaHCO_3$ (1.0 mL of an aq. satd. solution), NaClO (2.5 mL) and NaCl (1.0 mL of an aq. satd. solution) at r.t. for 4 h. The resulting product (DMT-HEG-COOH) was purified by flash chromatography on a silica gel column eluted with increasing amounts of CH_3OH in CH_2Cl_2, from 5% to 10%, containing 1% of triethylamine (recovered yield = 62%).

The identity of the purified product was confirmed by ^{1}H NMR (C_6D_6, 400 MHz): δ 8.11 (br, 1H, COOH), 7.43–6.48 (complex signals, 13H, DMT aromatic protons), 3.88 (s, 2H, CH_2CO), 3.34–3.06 (complex signals, 20H, 5 × OCH_2CH_2), 3.21 (s, 6H, 2 × OCH_3).

2.1.2. Functionalization of LCAA-CPG with the hexaethylene glycol spacer DMT-HEG-COOH

LCAA-CPG 1000 Å (155 mg, 0.069 meq g^{-1}, 10.7 μmol; Link Technologies, Bellshill, UK) [23] was left in contact with a mixture of DMT-HEG-COOH (64 mg, 107 μmol), *N*,*N*′-dicyclohexylcarbodiimide (DCC, 22 mg, 107 μmol) and 1-hydroxybenzotriazole (HOBt, 14.5 mg, 107 μmol) in anhydrous DMF (500 μL) at r.t. overnight. After removing the excess of the reactants from the resin by exhaustive washings with DMF, CH_3OH, AcOEt, *n*-hexane and CH_2Cl_2, the support was taken to dryness under reduced pressure. DMT tests performed on dried and weighed samples of the resulting support allowed determining the incorporation yield of DMT-HEG-COOH onto the CPG support, which resulted to be 74% (0.051 meq g^{-1}). The solid support was then treated with a capping solution, consisting of a mixture of acetic anhydride/pyridine (1:1, v/v), at r.t. for 4 h to block the unreacted amino groups, followed by exhaustive washings with CH_2Cl_2, CH_3OH, and again CH_2Cl_2. Finally, the DMT protecting group was removed by treatment with 3% trichloroacetic acid (TCA) in CH_2Cl_2 giving support **1** (Scheme 1). The DMT cation collected after this acidic treatment and quantified by UV measurements gave a functionalization of 0.051 meq g^{-1}, in agreement with the previous value.

2.1.3. Synthesis of 5′-O-DMT, 3′-O-acetyl-thymidine

5′-O-DMT-thymidine (94 mg, 0.17 mmol, Sigma-Aldrich) was reacted with 1 mL of a mixture of acetic anhydride/pyridine (1:1, v/v) at r.t. for 2 h. The resulting product was taken to dryness under reduced pressure, dissolved in CH_2Cl_2 and purified by water/CH_2Cl_2 extraction, finally giving pure 5′-O-DMT, 3′-O-acetyl-thymidine (87 mg, 0.15 mmol). The identity of the purified product was confirmed by ^{1}H NMR (C_6D_6, 400 MHz): δ 8.81 (br, 1H, NH thymine residue), 7.62–6.82 (complex signals, 13H, DMT aromatic protons), 7.51 (s, 1H, H-6 thymine residue), 6.64 (t, 1H, H-1′), 5.42 (s, 1H, H-4′), 4.01 (s, 1H, H-3′), 3.54 (s, 2H, H-5′), 3.36 (s, 6H, 2 × OCH_3), 2.18 (d, 2H, H-2′), 1.59 (s, 6H, thymine CH_3-5 and CH_3COO).

2.1.4. Functionalization of support **1** with 5′-O-DMT, 3′-O-acetyl-thymidine

Diethyl azodicarboxylate (DEAD, 118 μL, 0.75 mmol) was added

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