Tetrahedron 67 (2011) 5956–5963

Contents lists available at ScienceDirect

Tetrahedron

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

Synthesis of new azaphthalocyanine dark quencher and evaluation of its quenching efficiency with different fluorophores

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ARTICLE INFO

Article history:
Received 7 January 2011
Received in revised form 24 May 2011
Accepted 13 June 2011
Available online 17 June 2011

Keywords:
Phthalocyanine
Tetrapyrazinoporphyrazine
Dark quencher
Hybridization
Fluorescence
Click chemistry

ABSTRACT

New unsymmetrical zinc azaphthalocyanine (AzaPc) was synthesized using statistical condensation of two precursors. Postsynthetic modifications led to incorporation of azide group that efficiently underwent Cu(I)-catalyzed azide/alkyne 1,3-dipolar cycloaddition with terminal alkyne on a solid phase. The modified solid phase was then used for synthesis of oligodeoxyribonucletides labeled with AzaPc. DNA hybridization assays confirmed high quenching efficiency (*QE*>96%) of zinc AzaPc quencher with six different fluorophores ranging in emission maxima from 517 nm to 701 nm (FAM, HEX, Cy3, Cy3.5, Cy5, and Cy5.5).

1. Introduction

Azaphthalocyanines (AzaPc) are analogues of well known and widely investigated synthetic dyes phthalocyanines (Pc) where some of the methine groups in benzene rings are replaced with nitrogens. Due to their promising spectral, photochemical, photophysical, and electrochemical properties, they were investigated in different areas (e.g., as photosensitizers in photodynamic therapy,[1–3] for non-linear optical properties[4,5] or as pH sensitive dyes[6]). Our recent efforts were directed toward their application in quenching fluorescence in DNA hybridization assays.[7] This method is widely used in genetic analysis for detection and quantification of DNA targets as well as for monitoring of real-time polymerase chain reaction. The method utilizes two dyes—a fluorophore (reporter) and a quencher. After appropriate approximation, the quencher is able to quench fluorescence of the fluorophore leading thus to changes in intensity of fluorescence signal. Modern types of the quenchers, so called 'dark quenchers' have no intrinsic fluorescence that increases sensitivity of the test and simplifies the hybridization assay. Despite a large number of different available fluorophores, the quenchers are recruiting only from few structural types. That is why, enlargement of a family of the dark quenchers by new compounds is highly desirable.

Recently, metal-free alkylamino substituted AzaPc has been studied as promising dark quenchers of a new structural type.[7] They have no fluorescence due to a unique deactivation of the excited states by intramolecular charge transfer (ICT)[8] and proved to quench fluorescence of two fluorophores in hybridization assays.[7] An efficient quenching, however, should be confirmed for wider range of fluorophores routinely used. Thus this work is focused on investigation of new alkylamino AzaPc and determination of their quenching efficiencies with several fluorophores emitting at different wavelengths (Fig. 1). The work deals also with an improved method of binding AzaPc to a solid phase as this is usually a crucial point of synthesis of modified oligodeoxyribonucleotides (ODN).

2. Results and discussion

2.1. Synthesis azaphthalocyanines

An AzaPc quencher must be based on the following requirements to be suitable: the peripheral substituents should be bulky in order to decrease aggregation, which is typical for planar macrocyclic systems, such as AzaPc and decreases their solubility as well as complicates their isolation and purification. They must also

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doi:10.1016/j.tet.2011.06.038

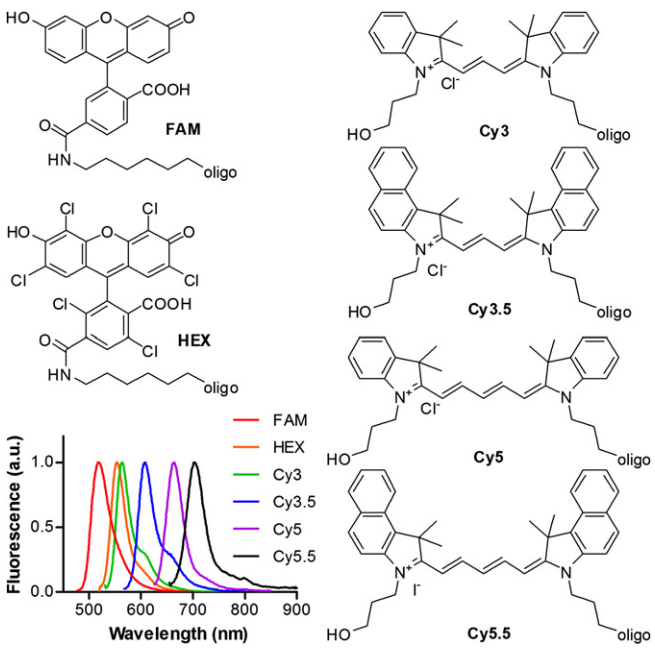


Fig. 1. Structures and emission spectra of fluorophores in the study.

be attached to a macrocyclic core through nitrogen linkage to allow the ultrafast intramolecular charge transfer (ICT) responsible for deactivation of excited states through a non-radiative pathway.[8] Such an AzaPc has no intrinsic fluorescence and becomes a 'dark quencher'. Diethylamino substituents meet both the above-mentioned requirements. Also, AzaPc must contain a hydroxy group to enable standard phosphoramidite oligonucleotide synthesis and another functional group suitable for binding to the solid phase.

The synthetic strategy of AzaPc binding to the solid phase was based on a Cu(I)-catalyzed azide/alkyne 1,3-dipolar cycloaddition ('click' chemistry), which is known for its selectivity and high yields.[9,10] For this reason, an alkyne modified solid phase and azide substituted AzaPc were prepared (Scheme 1). The synthesis of AzaPc started from preparation of their precursors, suitably substituted pyrazine-2,3-dicarbonitriles. Compound **1** undergoes nucleophilic substitutions due to the presence of strongly electrondeficient carbon atoms in positions 5 and 6.[11–13] That is why, treatment of **1** with 4-(methylamino)butanoic acid in THF at room temperature gave selectively **2** in excellent yield, as well as its subsequent reaction with 2-(piperidin-4-yl)ethanol in THF afforded **3**. 4-(Methylamino)butanoic acid was obtained by basic hydrolysis of *N*-methylpyrrolidin-2-on using barium hydroxide.[14] Equimolar condensation of pyrazine-2,3-dicarbonitriles **3** and **4** (generally A and B) yielded a mixture of six different AzaPc (AAAA, AAAB, ABAB, AABB, ABBB, and BBBB type). The yield of the desired ABBB congener (compound **5**) was increased to 16% by using a molar ratio of starting materials of 1:3. Although several selective methods were developed for synthesis of ABBB type derivatives of Pc and AzaPc,[15,16] the statistical condensation is still very reliable method, particularly when AzaPc or Pc are not aggregating[17] as in the case of compound **5**. The desired congener was easily isolated due to very good solubility in organic solvents, no tailing on silica and different R_f values allowing excellent separation of all congeners in the statistical mixture.

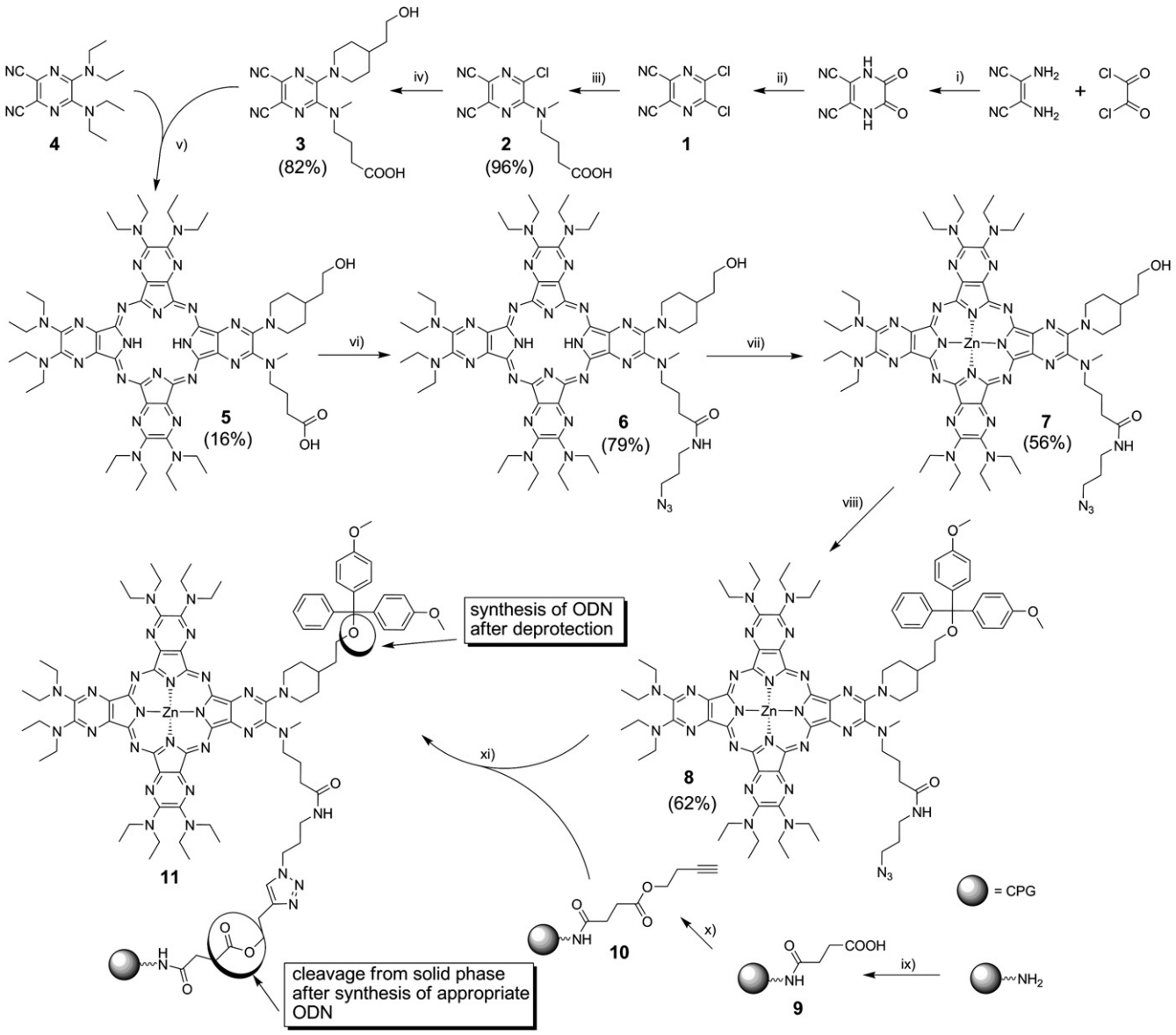
AzaPc **5** was further modified with linker bearing azide (**6**) and zinc was chelated into the center of the macrocyclic core (**7**). Previously, only metal-free AzaPc has been investigated as dark quenchers.[7] However, the recent work of Mammana et al.[18] showed that metal-free porphyrins conjugated with ODN may efficiently incorporate Cu(II) and Zn(II) cations that are present as trace amounts in solvents (e.g., in ammonia). Our observations (unpublished results) indicated very similar behavior also for metal-free AzaPc. Furthermore, click chemistry utilizes Cu(I) cations that, if oxidized, may be chelated to the center of the AzaPc core as well. Both these reasons led us to incorporate a central cation (zinc) in order to avoid abovementioned side reactions that might complicate composition of synthesized ODNs. As a final step of AzaPc synthesis, the hydroxy group of **7** was protected with a 4,4′-dimethoxytrityl (DMTr) group (**8**). Compounds **7** and **8** repeatedly did not give satisfactory elemental analysis most likely due to the presence of labile DMTr protecting group (**8**) and azide function, which might be unstable at high temperatures used during elemental analysis. However, their mass spectra and NMR spectra fully confirmed the right composition (see Supplementary data).

Long chain amino alkyl (lcaa) solid phase (controlled pore glass, CPG) was reacted with succinic anhydride (**9**) in order to introduce a carboxy group that allows incorporation of terminal alkyne (but-3-yn-1-ol) through an ester bond (**10**). Several conditions were subsequently tested for click reaction between AzaPc **8** and the modified solid phase **10** including different combinations of solvents (THF, DMF, DMSO) and bases (diisopropylethylamine (*i*-Pr_2NEt), triethylamine, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine). The highest loading on the solid phase was obtained in THF with *i*-Pr_2NEt and CuI, actually similar conditions that were previously used for click reaction of AzaPc with mestranol in solution.[19] The final construct **11** contained two important functional groups. The ester bond was used for cleavage of the modified ODN from the solid phase after synthesis in DNA/RNA synthesizer. The DMTr protecting group was removed before start of ODN synthesis and vacant hydroxy group was used for attaching the first nucleic base in DNA/RNA synthesizer. DMTr was also used for quantification of AzaPc loading on the solid phase **11**. The loading was ranging between 50 and 55 μmol/g for different batches. For comparison, the reported loading of AzaPc to similar lcaa solid phase utilizing formation of amide bond reached approximately 30 μmol/g.[7] It seems therefore that newly developed azide/alkyne cycloaddition approach is almost twice as efficient as the previously reported method.

2.2. Synthesis and characterization of oligodeoxyribonucleotides

The modified solid phase **11** was introduced into DNA/RNA synthesizer and oligodeoxyribonucleotides **ODN1** and **ODN2** (Fig. 2) were synthesized using a standard phosphoramidite synthetic protocol. After cleavage from the solid phase and deprotection of oligonucleotides with ammonia, the dark blue solution was passed through a hydrated gel column to remove low molecular weight impurities and then analyzed and purified with reverse phase HPLC. The analysis of unpurified AzaPc/ODN conjugates revealed three major peaks in retention times 1.40 min, 13.87 min and 24.66 min (see Supplementary data). Based on absorption spectra analysis and retention features of standards, the peaks corresponded to unmodified ODN, AzaPc/ODN conjugate and unbound AzaPc, respectively. HPLC-purified ODNs were free of any unbound AzaPc (Fig. 3) and only a small amount of unmodified ODN was detected not exceeding 3% of total ODNs in the sample (based on AUC calculations at 254 nm). **ODN1** and **ODN2** were also analyzed using MALDI-TOF mass spectrometry that confirmed the right mass to charge ratio (see Supplementary data).

For comparative purposes in hybridization assays, **ODN1** and **ODN2** labeled with commercially available quenchers BHQ-1,



Scheme 1. Reaction conditions: (i) dioxane, 60 °C; (ii) $SOCl_2$/DMF, dioxane, 60 °C; (iii) 4-(methylamino)butanoic acid, THF, rt; (iv) 2-(piperidin-4-yl)ethanol, THF, rt; (v) Li, *n*-butanol, reflux; (vi) HBTU, 3-azidopropan-1-amine, *i*-Pr_2NEt, DMF, rt; (vii) $Zn(CH_3COO)_2$, DMF, reflux; (viii) DMTrCl, pyridine, rt; (ix) succinic anhydride, DMAP, pyridine, rt; (x) HBTU, DMAP, 3-butyn-1-ol, DMF, rt; (xi) CuI, *i*-Pr_2NEt, THF, rt.

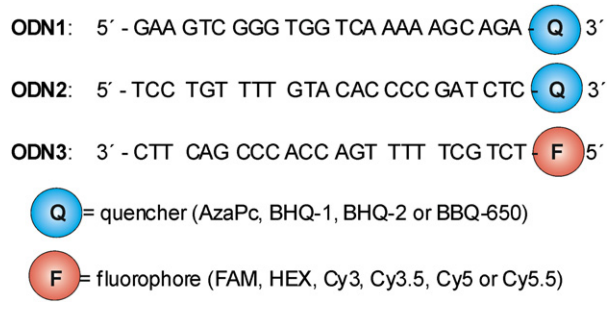


Fig. 2. Sequences of modified ODNs.

BHQ-2, and BBQ-650 were prepared and purified according to the suppliers' recommendations. Similarly, **ODN3** (complementary to **ODN1**) labeled with different fluorophores on 5′-end (FAM, HEX, Cy3, Cy3.5, Cy5, Cy5.5) were also synthesized according to supplier's recommendations.

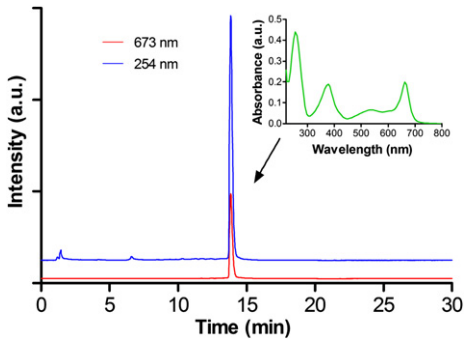


Fig. 3. HPLC chromatograms of HPLC-purified AzaPc/**ODN1** conjugate monitored at 254 nm and 673 nm. Chromatogram at 254 nm was offset. Inset: absorption spectrum taken in t_R=13.87 min.

2.3. Spectral, photophysical, and photochemical properties

The absorption spectra of zinc AzaPc **7** in THF showed a shape typical for metal complexes of AzaPc and Pc in monomeric form with B-band at 373 nm and Q-band at 655 nm (Fig. 4). An additional band arising from an $n-\pi^*$ transition from lone pairs of peripheral diethylamino substituents was detected around 505 nm. The shape of the spectrum and extinction coefficients did not change in a wide range of concentration (from 0.05 to 50 μM) indicating that no aggregates are formed in organic solution. This was most likely a consequence of bulky diethylamino substituents preventing efficiently aggregation. The spectra of AzaPc/ODN conjugates contained features characteristic of both moieties. Besides abovementioned bands typical for AzaPc, a new band due to ODNs absorption appeared between 254 nm and 260 nm. The shape of the AzaPc spectra in the **ODN1** and **ODN2** conjugates in hybridization buffer indicated significant amounts of both monomeric and aggregated forms as assumed from Q-band broadening and decreased extinction coefficient (Fig. 4). Similar, but stronger aggregation was described for buffer solutions of various Pc/ODN conjugates in literature.[20] Although similar changes in the shape of Q-band could be attributed also to protonation of peripheral diethylamino substituents in acidic solution.[21] The shape of spectra did not vary in buffers ranging from pH 5 to 10 excluding thus possible influence of pH. Furthermore, the addition of organic solvent (THF or acetonitrile) into the buffer solution of AzaPc/ODN conjugates led to a restoration of the sharp Q-band and the spectra gained the shape corresponding to monomeric form of **7** in THF (see Fig. 4 and Supplementary data).

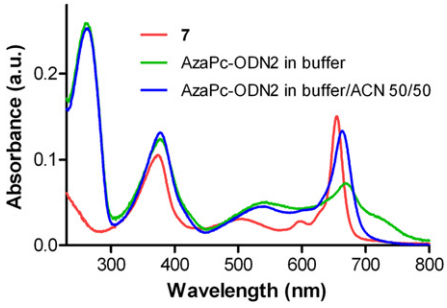


Fig. 4. Absorption spectra of compound **7** in THF and AzaPc/**ODN2** conjugate in hybridization buffer and buffer/ACN (50/50). Concentration of all solutions was 1 μM.

The photophysical and photochemical properties of AzaPc macrocycle were tested in THF on compound **7**. Dark quenchers should be lacking any intrinsic fluorescence that may interfere with the signal of the reporter and their singlet oxygen quantum yields (Φ_Δ) should be as low as possible. Singlet oxygen is a highly reactive species, that is, known to destroy ODNs.[22] In the previous work,[7] optimal photophysical and photochemical properties of metal-free alkylamino AzaPc were confirmed. However, incorporation of zinc into macrocyclic core is known to alter significantly the excited states relaxation pathways. Although the AzaPc, particularly their zinc complexes, are known by high singlet oxygen production,[23,24] a Φ_Δ value of **7** was found to be only 0.025. This is a consequence of unique relaxation of the excited states by ICT.[8] For comparison, the Φ_Δ value of fluorescein (FAM), a widely used dye in hybridization assays is reported to range 0.03–0.10.[25] Concerning fluorescence, no signal was detected either for compound **7** in THF or for **ODN1** and **ODN2** modified with AzaPc in hybridization buffer. Excitation at both B-band and Q-band was tested. Both photochemical and photophysical parameters of zinc complex **7** and its ODN conjugate showed high promise of these derivatives for use in fluorescence quenching in DNA hybridization probes.

2.4. Quenching efficiency

Modified ODNs were introduced into hybridization tests in order to determine the quenching efficiency of new zinc AzaPc dark quenchers. The method was adopted from Marras et al.[26] A series of six different fluorophores was chosen to cover the whole spectrum of currently routinely used fluorophores from FAM on lower end to Cy5.5 on the upper end (Fig. 1). As a positive control, three highly efficient dark quenchers were chosen and introduced into the tests (BHQ-1, BHQ-2, BBQ-650).

Complementary **ODN1** and **ODN3** were mixed together and fluorescence was measured before and after mixing. A marked decrease of the signal was always detected (Fig. 5) and quenching efficiency (*QE*) was calculated. Data are summarized in Table 1. From the obtained results, it is obvious that all quenchers were highly efficient with *QE* over 94%. It is noteworthy that AzaPc always showed the highest *QE* for each fluorophore, in some cases the signal almost completely disappeared (Fig. 5, inset). The only exception was quenching of HEX where AzaPc was only comparable with other quenchers. In order to prove origin of the quenching, non-complementary **ODN2** bearing AzaPc quencher was also tested. No significant decrease of fluorescence was observed after mixing **ODN2** and **ODN3** (Table 1) confirming that no non-specific quenching occurred.

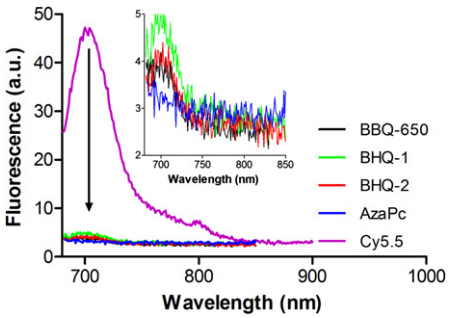


Fig. 5. Quenching of Cy5.5 fluorescence (**ODN3**) before (Cy5.5) and after hybridization with **ODN1** labeled with different quenchers. Inset: enlarged area.

The mechanism of quenching in case of duplex of two complementary ODNs (e.g., duplex of **ODN1**/**ODN3**) is most likely static.[26] Static quenching is an important mechanism utilized e.g., in molecular beacons.[27] When the fluorophore and the quencher are placed in close proximity in space, they form a non-fluorescent ground-state complex that absorbs energy and immediately returns to the ground state without emission of a photon.[28] The ground-state complex has a different absorption spectrum than a simple sum of absorption spectra of both dyes involved.[26,29] Therefore measurements of the absorption spectra of duplexes may elucidate the quenching mechanism and confirm formation of the ground-state complex.

The spectra of duplexes of **ODN1** labeled with AzaPc and **ODN3** labeled with different fluorophores are presented in Fig. 6. Further spectra can be found in the Supplementary data. The spectra of the duplexes did not correspond to a simple sum showing the ground-state complex formation. This finding indicates that bulky peripheral diethylamino substituents, despite their ability to inhibit efficiently aggregation of AzaPc molecules in organic solvents, do not preclude approximation of the quencher and the fluorophore to proper distance and allow formation of the ground-state complex important for static quenching. The absorption spectra were also measured after mixing equimolar amounts of non-complementary **ODN2** and **ODN3**. In all cases, the spectra corresponded well with the sum of absorption spectra of the dyes alone confirming that no

Table 1
Quenching efficiencies (*QE*) in % for different combinations of quenchers and fluorophores[a]

	ODN3/FAM	**ODN3**/HEX	**ODN3**/Cy3	**ODN3**/Cy3.5	**ODN3**/Cy5	**ODN3**/Cy5.5
ODN1/AzaPc	98.42±0.02	96.62±0.10	99.06±0.15	99.73±0.09	96.56±0.19	99.45±0.70
ODN1/BHQ-1	97.76±0.03	96.87±0.05	98.32±0.12	98.87±0.04	94.56±0.05	94.34±0.42
ODN1/BHQ-2	97.95±0.02	97.17±0.05	98.28±0.11	98.73±0.07	95.33±0.12	96.05±0.37
ODN1/BBQ-650	97.53±0.04	96.74±0.10	96.28±0.06	98.62±0.05	94.99±0.10	96.90±0.73
ODN2/AzaPc	0.62±0.47	1.56±1.01	1.90±2.21	2.78±1.55	3.05±1.36	−1.71±0.94

[a] Mean of three independent measurements±SD.

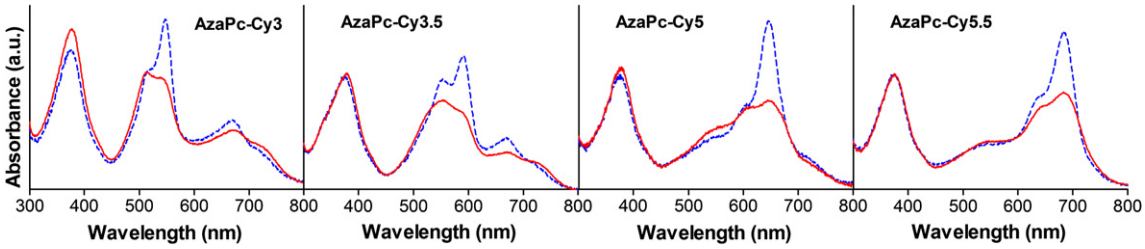


Fig. 6. Absorption spectra of duplexes **ODN1** (labeled with AzaPc) and **ODN3** labeled with different fluorophores (red/full line) and a simple sum of absorption spectra of corresponding labeled ODNs without hybridization (blue/dashed line).

ground-state complex is formed on the basis of non-specific interactions (e.g., hydrophobic π−π interactions) between the fluorophore and the quencher.

3. Conclusion

In this work, a new unsymmetrical zinc AzaPc was synthesized using the statistical condensation of two precursors. Postsynthetic modifications led to incorporation of an azide group that efficiently underwent Cu(I)-catalyzed azide/alkyne 1,3-dipolar cycloaddition with a terminal alkyne on the solid phase. Binding of AzaPc to the solid phase using click chemistry was almost twice as efficient as the reported approach utilizing formation of amide bond. The modified solid phase was used for synthesis of ODNs labeled at 3′-end with AzaPc quencher. Photophysical and photochemical properties of the synthesized zinc AzaPc indicated good promise as a dark quencher. Subsequent hybridization assays confirmed the high quenching efficiency (*QE*>96%) of zinc AzaPc quencher with six different fluorophores ranging in emission maxima from 517 nm to 703 nm (from FAM to Cy5.5). Measurements of the absorption spectra of duplexes indicated the formation of ground-state complex between zinc AzaPc and all tested fluorophores.

4. Experimental section

4.1. General

All organic solvents were of analytical grade. Anhydrous butanol was stored over magnesium and distilled prior to use. Anhydrous pyridine was distilled from potassium hydroxide and stored over molecular sieves. All chemicals for synthesis were obtained from established suppliers (Aldrich, Acros, Merck, TCI Europe) and used as received. TLC was performed on Merck aluminum sheets with silica gel 60 F_{254}. Merck Kieselgel 60 (0.040−0.063 mm) was used for column chromatography. Long chain amino alkyl (lcaa) solid phase (500 Å controlled pore glass, CPG, NH_2 loading 172 μmol/g) was obtained from ChemGenes Corporation (Wilmington, MA, USA). All HPLC chromatographic separations were performed on a Shimadzu chromatography system consisted of a communication bus module CBM 20A, a diode array detector SPD-M20A, two pumps LC-20AD, an autoinjector SIL-20AC, a column compartment CTO-20AC, a degasser DGU-$20A_3$ and a computer-based chromatographic software LC solution, Shimadzu (Tokyo, Japan). The infrared spectra were measured on an IR-Spectrometer Nicolet Impact 400 in ATR modes. The ^1H and ^{13}C NMR spectra were recorded on a Varian Mercury—Vx BB 300 (299.95 MHz—^1H and 75.43 MHz—^{13}C). The reported chemical shifts are relative to TMS. The UV/Vis spectra were recorded using a Shimadzu UV-2401PC spectrophotometer. The fluorescence spectra were obtained by an AMINCO-Bowman Series 2 luminescence spectrometer. The elemental analysis was carried out on Automatic Microanalyser EA1110CE (Fisons Instruments S.p.A., Milano, Italy). AzaPc always contained several molecules of water in their solid form, which is a typical observation for this type of compounds.[30,31] The MALDI-TOF mass spectra of compounds **5**–**8** were collected by a Voyager-DE STR mass spectrometer (Applied Biosystems) in α-cyano-4-hydroxycinnamic acid or *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]-malononitrile as the matrix. High resolution mass spectra (HRMS) of compounds **5**–**8** were obtained using the same instrument but each spot was further calibrated internally after addition of metal-free azaphthalocyanine with peripheral camphorquinone units[32] to the mass of its monomer (*m*/*z* 954.5030 $[M]^+$) and a dimer that appeared in the mass spectrum as well (*m*/*z* 1909.0061 $[2M]^+$). HRMS of compound **3** was obtained on Q-TOF Ultima API (Waters, UK). Mass spectra of oligodeoxyribonucleotide probes were obtained using MALDI-TOF Bruker Autoflex II mass spectrometer. Compound **1** was obtained from TCI Europe, compound **4** was prepared according to previously published procedure.[33] 3′-BHQ-1 CPG, 3′-BHQ-2 CPG, 5′-Fluorescein (FAM) Phosphoramidite, 5′-Hexachloro-Fluorescein (HEX) Phosphoramidite, Cy3™ Phosphoramidite, Cy3.5™ Phosphoramidite, Cy5™ Phosphoramidite and Cy5.5™ Phosphoramidite were supplied by Glen Research, Sterling, VA, USA. 3′-BBQ-650 CPG and was supplied by Berry & Associates, Inc., Dexter, MI, USA. Hybridization buffer used was 10X TrueStart *Taq* buffer from Fermentas, St. Leon-Rot, Germany.

4.2. Synthesis

*4.2.1. (4-(3-Chloro-5,6-dicyanopyrazin-2-yl)(methyl)amino)butanoic acid (**2**).* A mixture of **1** (1.0 g, 5 mmol) and 4-(methylamino) butanoic acid (1.25 g, 10.7 mmol) in THF (50 mL) was stirred at room temperature for 3 days. The reaction mixture was filtered and the solvent was evaporated under reduced pressure to give crude product. This was purified by column chromatography (silica) using hexane/ethyl acetate/acetic acid (6:3:1) as an eluent to yield slowly

crystallizing pale oil (1.34 g, 96%). [Found: C, 47.33; H, 3.76; N, 25.15. $C_{11}H_{10}N_5O_2Cl$ requires C, 47.24; H, 3.60; N, 25.04%]; R_f (hexane/ethyl acetate/acetic acid 6:3:1) 0.35; ν_{max} (ATR) 2928, 2357, 2341, 2226, 1709, 1555, 1420, 1389, 1352, 1291, 1263, 1197, 1120, 1045, 1022, 909 cm^{-1}; δ_H (300 MHz, $CDCl_3$) 3.84–3.76 (m, 2H, NCH_2), 3.39 (s, 3H, NCH_3), 2.45 (t, 2H, *J* 7 Hz, CH_2COOH), 2.06 (p, 2H, *J* 7 Hz, CH_2CH_2COOH); δ_C (75 MHz, $CDCl_3$) 178.5, 152.1, 135.5, 129.2, 118.3, 113.1, 112.8, 52.1, 39.7, 30.8, 21.9.

*4.2.2. 4-((5,6-Dicyano-3-(4-(2-hydroxyethyl)piperidin-1-yl)pyrazin-2-yl)(methyl)amino)butanoic acid (**3**).* A mixture of **2** (653 mg, 2.35 mmol) and 2-(piperidin-4-yl)ethanol (907 mg, 7.03 mmol) in THF (20 mL) was stirred at room temperature for 3 h. Then, the solvent was evaporated under reduced pressure to give crude product. This was purified by column chromatography (silica) using ethyl acetate/acetic acid (10:1) as an eluent to yield viscous pale yellow oil (713 mg, 82%). R_f (ethyl acetate/acetic acid 10:1) 0.37; ν_{max} (ATR) 2929, 2853, 2225, 1706, 1517, 1491, 1434, 1406, 137, 1240, 1089, 1051, 985 cm^{-1}; δ_H (300 MHz, acetone-d_6) 5.12 (br s, 1H, OH), 4.05 (2H, d, *J* 13 Hz, piper·NCH_2), 3.76–3.65 (m, 2H, NCH_2), 3.61 (2H, t, *J* 7 Hz, CH_2OH), 3.13 (3H, s, NCH_3), 2.84 (t, 2H, *J* 13 Hz, piper·CH_2), 2.22 (2H, t, *J* 7 Hz, CH_2COOH), 1.91–1.66 (m, 5H, CH+CH_2CH_2COOH+piper·NCH_2), 1.47 (q, 2H, *J* 7 Hz, CH_2CH_2OH), 1.42–1.26 (m, 2H, piper·CH_2); δ_C (75 MHz, acetone-d_6) 174.1, 148.2, 147.7, 121.4, 120.0, 116.0, 115.9, 59.2, 47.9, 47.7, 40.0, 37.0, 33.2, 32.4, 31.3, 22.8. HRMS (EI): M+H^+, found 373.1970, $C_{18}H_{25}N_6O_3$ requires 373.1988.

*4.2.3. 2-[(4-Carboxypropyl)methylamino]-3-[(4-2-hydroxyethyl)-piperidin-1-yl]-9,10,16,17,23,24-hexakis(diethylamino)-1,4,8,11,15,18,22,25-(octaaza)phthalocyanine (**5**).* A mixture of **3** (259 mg, 0.69 mmol) and **4** (568 mg, 2.09 mmol) in dry *n*-butanol (10 mL) was heated to reflux and metal lithium (102 mg, 14.6 mmol) was added. The reaction mixture was heated to reflux for 3 h. The solvent was evaporated under reduced pressure, diluted acetic acid (50% v/v, 100 mL) was added and the suspension was stirred at room temperature for 30 min. The crude product was filtered and washed thoroughly with water. The resulting mixture of congeners was separated by column chromatography (silica) with dichloromethane/acetone/methanol 10:1:1 as mobile phase. The second intense purple fraction (compound **5**) was purified on silica once more with dichloromethane/acetone/methanol 15:1:1. Finally, the pure compound **5** was dissolved in 5 mL of dichloromethane, dropped into 200 mL of hexane and cooled to −30 °C overnight. The precipitated fine suspension was filtered and washed with hexane to give a purple solid (135 mg, 16%), mp >300 °C. [Found: C, 58.46; H, 7.64; N, 27.12. $C_{60}H_{86}N_{24}O_3+2H_2O$ requires C, 58.71; H, 7.39; N, 27.39%]; R_f (dichloromethane/acetone/methanol 15:1:1) 0.24; ν_{max} (ATR) 3300, 2967, 2930, 1726, 1641, 1423, 1376, 1340, 1283, 1250, 1159, 1133, 1058, 1018 cm^{-1}; δ_H (300 MHz, pyridine-d_5) 13.72 (s, 2H, centr·NH), 4.49 (2H, d, *J* 12 Hz, piper·NCH_2), 4.21 (t, 2H, *J* 7 Hz, NCH_2), 3.98 (2H, t, *J* 7 Hz, CH_2OH), 3.95–3.83 (m, 24H, NCH_2), 3.46 (3H, s, NCH_3), 3.03 (t, 2H, *J* 12 Hz, piper·CH_2), 2.69 (2H, t, *J* 7 Hz, CH_2COOH), 2.36–2.34 (m, 2H, CH_2CH_2COOH), 2.01–1.81 (m, 3H, CH+piper·NCH_2), 1.80–1.71 (m, 2H, CH_2CH_2OH), 1.69–1.53 (m, 2H, piper·CH_2), 1.27–1.13 (m, 36H, NCH_3). δ_C (75 MHz, pyridine-d_5) 175.4, 151.4, 151.2, 150.72, 150.68, 149.2, 147.6 (broad), 142.1, 141.3, 140.6, 59.6, 50.4, 48.4, 43.0, 42.8, 40.4, 37.6, 33.3, 32.7, 32.5, 30.2, 23.4, 13.2, 13.1; UV/vis (THF): λ_{max} (log ε) 367 (4.98), 512 (4.73), 649 (4.78), 679 nm (4.91 M^{-1} cm^{-1}); MS (MALDI-TOF, [M^+]) calcd for $C_{60}H_{86}N_{24}O_3$, 1190.7; found, 1190.7; HRMS (MALDI): M+Na^+, found 1213.7206, $C_{60}H_{86}N_{24}NaO_3$ requires 1213.7212.

*4.2.4. 22-{[(N-3-Azidopropyl)-4-carboxamidopropyl]methylamino}-3-[4(-2-hydroxyethyl)-piperidin-1-yl]-9,10,16,17,23,24-hexakis(diethylamino)-1,4,8,11,15,18,22,25-(octaaza)phthalocyanine (**6**).* *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) (152 mg, 0.4 mmol) in dry DMF (5 mL) was added to a solution of **5** (300 mg, 0.25 mmol) in dry DMF (20 mL) followed by addition of 3-azidopropan-1-amine[19] (160 mg, 1.6 mmol) in dry DMF (5 mL) and *i*-Pr_2NEt (0.2 mL). The mixture was stirred at room temperature for 1 h. Solvent was evaporated under reduced pressure and the crude product was suspended in water and filtered. The product was purified by column chromatography (silica) using dichloromethane/acetone/methanol (20:1:1) as eluent. Finally, the product was dissolved in dichloromethane (5 mL), dropped into hexane (200 mL) and cooled to −30 °C overnight. The precipitated fine suspension was filtered and washed with hexane to give a purple solid (250 mg, 79%), mp >300 °C. [Found: C, 56.88; H, 7.53; N, 29.39. $C_{63}H_{92}N_{28}O_2+3H_2O$ requires C, 56.99; H, 7.44; N, 29.54%]; R_f (dichloromethane/acetone/methanol 20:1:1) 0.30; ν_{max} (ATR) 3301, 2967, 2929, 2871, 2093, 1640, 1422, 1341, 1283, 1158, 1132, 1057, 1017 cm^{-1}; δ_H (300 MHz, pyridine-d_5) 13.73 (s, 2H, centr·NH), 8.65 (br s, 1H, CONH), 4.46 (d, 2H, *J* 12 Hz, piper·NCH_2), 4.18 (t, 2H, *J* 7 Hz, NCH_2), 4.00 (t, 2H, *J* 7 Hz, CH_2OH), 3.96–3.79 (m, 24H, NCH_2), 3.48 (q, 2H, *J* 7 Hz, $NHCH_2$), 3.43 (3H, s, NCH_3), 3.24 (t, 2H, *J* 7 Hz, CH_2N_3), 3.01 (t, 2H, *J* 12 Hz, piper·CH_2), 2.56 (t, 2H, *J* 7 Hz, CH_2CONH), 2.56–2.40 (m, 2H, CH_2CH_2CONH), 2.02–1.82 (m, 3H, CH+piper·NCH_2), 1.81–1.51 (m, 6H, CH_2CH_2OH+piper·CH_2+$CH_2CH_2N_3$), 1.26–1.12 (m, 36H, NCH_3). δ_C (75 MHz, pyridine-d_5) 172.5, 151.3, 151.0, 150.7, 150.6, 149.2, 147.6 (broad), 141.9, 141.2, 140.4, 59.6, 50.5, 49.3, 48.4, 43.0, 42.9, 40.3, 37.5, 37.0, 34.2, 33.3, 32.6, 29.4, 23.9, 13.1; UV/vis (THF): λ_{max} (log ε) 368 (5.00), 515 (4.76), 650 (4.80), 680 nm (4.93 M^{-1} cm^{-1}); MS (MALDI-TOF, [M^+]) calcd for $C_{63}H_{92}N_{28}O_2$, 1272.8; found, 1272.7; HRMS (MALDI): M^+, found 1272.7931, $C_{63}H_{92}N_{28}O_2$ requires 1272.7958.

*4.2.5. 2-{[(N-3-Azidopropyl)-4-carboxamidopropyl]methylamino}-3-[4(-2-hydroxyethyl)-piperidin-1-yl]-9,10,16,17,23,24-hexakis(diethylamino)-1,4,8,11,15,18,22,25-(octaaza)phthalocyaninato zinc(II) (**7**).* Compound **6** (100 mg, 0.08 mmol) was dissolved in dry DMF (10 mL) and anhydrous zinc acetate (74 mg, 0.4 mmol) was added. The mixture was heated to reflux for 2 h. The solvent was evaporated under reduced pressure and water was added. The crude product was filtered and washed thoroughly with water. The mixture was purified by column chromatography (silica) using dichloromethane/acetone/methanol (15:1:1) as eluent to give dark blue solid (60 mg, 56%), mp >300 °C. [Found: C, 54.48; H, 7.14; N, 26.85. $C_{63}H_{90}N_{28}O_2Zn+3H_2O$ requires C, 54.40; H, 6.96; N, 28.19%]; R_f (dichloromethane/acetone/methanol 15:1:1) 0.25; ν_{max} (ATR) 3500–3200 (br), 2966, 2929, 2871, 2094, 1642, 1515, 1420, 1318, 1253, 1159, 1109, 1056, 1019 cm^{-1}; δ_H (300 MHz, pyridine-d_5) 8.76 (br s, 1H, CONH), 6.00 (br s, 1H, OH), 4.44 (2H, d, *J* 12 Hz, piper·NCH_2), 4.15 (t, 2H, *J* 6 Hz, NCH_2), 3.99 (t, 2H, *J* 6 Hz, CH_2OH), 3.96–3.77 (m, 24H, NCH_2), 3.50 (q, 2H, *J* 7 Hz, $NHCH_2$), 3.41 (3H, s, NCH_3), 3.21 (t, 2H, *J* 7 Hz, CH_2N_3), 3.00 (t, 2H, *J* 12 Hz, piper·CH_2), 2.71–2.60 (m, 2H, CH_2CONH), 2.40–2.26 (m, 2H, CH_2CH_2CONH), 2.02–1.81 (m, 3H, CH+piper·NCH_2), 1.81–1.47 (m, 6H, CH_2CH_2OH+piper·CH_2+$CH_2CH_2N_3$), 1.35–1.02 (m, 36H, NCH_3); δ_C (75 MHz, pyridine-d_5) 172.7, 151.6, 151.5, 151.46, 151.41, 151.3, 151.1, 150.9, 150.6, 143.6, 143.04, 142.97, 142.90, 142.8, 142.2, 59.7, 50.6, 49.3, 48.4, 43.0, 42.8, 40.4, 37.4, 37.0, 34.5, 33.3, 32.7, 29.4, 23.9, 13.2, 13.13, 13.09; UV/vis (THF): λ_{max} (log ε) 376 (4.99), 507 (4.47), 598 (4.40), 655 nm (5.04 M^{-1} cm^{-1}); MS (MALDI-TOF, [M^+]) calcd for $C_{63}H_{90}N_{28}O_2Zn$, 1334.7; found, 1334.6; HRMS (MALDI): M^+, found 1334.7107, $C_{63}H_{90}N_{28}O_2Zn$ requires 1334.7093.

*4.2.6. 2-{[(N-3-Azidopropyl)-4-carboxamidopropyl]methylamino}-3-[4(-2-dimethoxytrityloxyethyl)-piperidin-1-yl]-9,10,16,17,23,24-hexakis(diethylamino)-1,4,8,11,15,18,22,25-(octaaza)phthalocyaninato zinc(II) (**8**).* Compound **7** (241 mg, 0.18 mmol) was dissolved in dry

pyridine (20 mL) and dimethoxytrityl chloride (DMTrCl) (156 mg, 0.46 mmol) was added. The solution was stirred at room temperature for 22 h and methanol (5 mL) was added. The solvents were evaporated under reduced pressure and the mixture was purified by column chromatography (silica) using toluene/pyridine (50:1) to elute methoxy dimethoxytrityl. Then, the eluent was changed to dichloromethane/pyridine/methanol (75:1:1) to give waxy dark blue solid (180 mg, 62%). Finally, the column was washed with dichloromethane/acetone/methanol (20:1:1) as eluent to give unreacted compound **7** (100 mg). [Found: C, 60.51; H, 7.07; N, 22.83. $C_{84}H_{108}N_{28}O_4Zn+2H_2O$ requires C, 60.22; H, 6.74; N, 23.41%]; R_f (dichloromethane/pyridine/methanol 75:1:1) 0.40; ν_{max} (ATR) 2965, 2929, 2871, 2094, 1671, 1642, 1607, 1509, 1420, 1289, 1251, 1160, 1108, 1057, 1021 cm^{-1}; δ_H (300 MHz, pyridine-d_5) 8.78–8.72 (m, 1H, CONH, overlapped with solvent), 7.81 (d, 2H, *J* 7 Hz, arH), 7.67 (d, 4H, *J* 9 Hz, arH), 7.50 (t, 2H, *J* 7 Hz, arH), 7.35 (t, 1H, *J* 7 Hz, arH), 7.10 (d, 4H, *J* 9 Hz, arH), 4.36 (2H, d, *J* 12 Hz, piper·NCH_2), 4.13 (t, 2H, *J* 7 Hz, NCH_2), 3.97–3.81 (m, 26H, NCH_2+CH_2OH), 3.75 (s, 6H, OCH_3), 3.50 (q, 2H, *J* 7 Hz, $NHCH_2$), 3.40 (3H, s, NCH_3), 3.20 (t, 2H, *J* 7 Hz, CH_2N_3), 2.95 (t, 2H, *J* 12 Hz, piper·CH_2), 2.71–2.60 (m, 2H, CH_2CONH), 2.39–2.24 (m, 2H, CH_2CH_2CONH), 1.86–1,60 (m, 7H, CH+CH_2CH_2OH+piper·NCH_2+$CH_2CH_2N_3$), 1.56–1.38 (m, 2H, piper·CH_2), 1.35–1.03 (m, 36H, NCH_3); δ_C (75 MHz, pyridine-d_5) 172.6, 159.1, 151.63, 151.56, 151.49, 151.43, 151.26, 151.10, 150.84, 150.55, 146.4, 143.7, 143.1, 143.0, 142.9, 142.8, 142.1, 137.3, 130.7, 128.8, 128.3, 127.2, 113.7, 86.4, 61.4, 55.3, 50.6, 49.3, 48.3, 43.0, 42.8, 37.4, 37.0, 34.5, 33.5, 32.6, 29.4, 23.9, 13.19, 13.13, 13.09. UV/vis (THF): λ_{max} (log ε) 234 (4.76), 375 (5.05), 506 (4.53), 599 (4.47), 655 nm (5.11); MS (MALDI-TOF, [M^+]) calcd for $C_{84}H_{108}N_{28}O_4Zn$, 1636.8; found, 1636.8; HRMS (MALDI): M^+, found 1636.8325, $C_{84}H_{108}N_{28}O_4Zn$ requires 1636.8400.

*4.2.7. Succinylated solid phase (**9**).* Succinic anhydride (400 mg, 4 mmol) and 4-*N*,*N*-dimethylaminopyridine (DMAP) (50 mg, 0.4 mmol) were dissolved in dry pyridine (15 mL) and added to lcaa solid phase (2.0 g). Suspension was shaken at room temperature for 38 h. Then, the solid phase was filtered and washed successively with pyridine, THF and diethylether (30 mL each) and dried under vacuum over the P_2O_5. Unreacted aminogroups on the solid phase were subsequently capped at room temperature for 30 min using a mixture of THF/pyridine/acetic anhydride (15 mL, 8:1:1). Then, the solid phase was filtered, washed successively with THF and diethylether (30 mL each) and dried under vacuum over P_2O_5.

*4.2.8. Alkynylated solid phase (**10**).* HBTU (161 mg, 0.425 mmol), DMAP (52 mg, 0.425 mmol), and 3-butyn-1-ol (30 mg, 0.425 mmol) were dissolved in dry DMF (5 mL) and added to the succinylated solid phase **9** (500 mg). The suspension was shaken at room temperature for 18 h. Then, the solid phase was filtered and washed successively with DMF, acetonitrile, THF, and diethylether (30 mL each) and dried under vacuum over P_2O_5.

*4.2.9. Solid phase modified with azaphthalocyanine (**11**).* Azaphthalocyanine **8** (16 mg, 0.01 mmol) and CuI (28 mg, 0.15 mmol) were dissolved in THF/*i*-Pr_2NEt (ratio 2:1, 2 mL) and added to the solid phase **10** (100 mg). The suspension was shaken at room temperature for 20 h. Then, the solid phase was filtered and washed successively with THF, acetonitrile, dichloromethane, and diethylether (30 mL each) and dried under vacuum over the P_2O_5. 4,4′-Dimethoxytrityl (DMTr) loading was determined as follows: approximately 2–3 mg of the support was accurately weighed out directly to test tube, perchloric acid solution (10 mL, 70%$HClO_4$ 51.4 mL+methanol, 46 mL) was added, and the test tube was sealed and shaken. The optical absorbance of the sample, diluted to fit interval 0.2–1.0 AU was measured at 498 nm in 1 cm cuvette. The loading was calculated as given below:

$$\text{Loading}(\mu\text{mol/g}) = (\text{Absorbance at } 498) \times \text{Dilution} \times 143/(\text{weight of support, mg})$$

4.3. Oligodeoxyribonucleotides

Oligodeoxyribonucleotides were synthesized on Perkin–Elmer Applied Biosystems 394 DNA/RNA synthesizer. After synthesis, the AzaPc/modified ODNs were cleaved from the solid phase and deprotected with 32% ammonia (20 h, rt) and passed through hydrated gel filtration column (CentriPure N10, empBIOTECH, Germany) in order to remove low molecular weight impurities. The blue fractions (eluted with water) were evaporated to dryness under high vacuum (4 mbar) and dissolved in water to approximate concentration (based on absorbance of total ODN at 254 nm) 100 μM (for HPLC analysis) or 1 mM (for HPLC purification). The crude mixture was then purified with HPLC (see below). AzaPc/**ODN1**: MS (MALDI-TOF): a broad cluster with peak at *m*/*z* 8970 $[M+H]^+$ and adducts with sodium and potassium; UV/vis (hybridization buffer): λ_{max} (log ε) 256 (5.46), 377 (5.06), 548 (4.67), 670 nm (4.76 M^{-1} cm^{-1}); AzaPc/**ODN2**: MS (MALDI-TOF): a broad cluster with peak at *m*/*z* 8674 $[M+H]^+$ and adducts with sodium and potassium; UV/vis (hybridization buffer): λ_{max} (log ε) 260 (5.35), 377 (5.03), 544 (4.65), 669 nm (4.80 M^{-1} cm^{-1}).

ODNs modified by fluorophores (FAM, HEX, Cy3, Cy3.5, Cy5, and Cy5.5) or commercially available quenchers (BHQ-1, BHQ-2, BBQ-650) were synthesized also on abovementioned DNA/RNA synthesizer using suppliers' recommendations.

4.4. HPLC

The AzaPc/ODN conjugates **ODN1** and **ODN2** were purified on a semipreparative HPLC column. The preparation was done on the Hypersil GOLD column (150×10 mm, 5 μm particle size) using a mobile phase containing acetonitrile and 50 mM triethylammonium acetate. The mobile phase A was consisted of 12% acetonitrile in 50 mM triethylammonium acetate (TEAA), and mobile phase B was pure acetonitrile. The crude samples were purified under isocratic condition A:B=(55:45, v/v) and the flow rate was set at 1.5 mL/min. The fractions corresponding to the conjugates (based on the UV spectra) were collected and the mobile phase was evaporated to dryness under deep vacuum (4 mbar).

The purity of the samples was assessed by HPLC. The separation was performed on a Hypersil BDS C18 column (100×4.6 mm, 3 μm particle size) using mobile phases A and B as determined above. A gradient elution was used. The gradient time program was set as follows: 0–3.5 min 0% B; 3.5–18.5 min 0–85% B; 18–30.5 min 85% B. The column was then equilibrated for 5 min under initial conditions. The column temperature was maintained at 40 °C, and the flow rate was set at 1.0 mL/min. The compounds were analyzed by a diode array detector. Unlabeled oligonucleotide was eluted with t_R of approximately 1.5 min and free dye with t_R of approximately 24.5 min.

4.5. Hybridization assays

4.5.1. Quenching efficiency. The method was adopted from Marras et al.[26] **ODN1** modified with different quenchers was dissolved in hybridization buffer to stock concentration 10 μM. **ODN3** modified with different fluorophores was dissolved in hybridization buffer to concentration 0.05 μM, transferred to cuvette (800 μL) and fluorescence was measured at 519 nm, 554 nm, 564 nm, 607 nm, 664 nm, and 703 nm for FAM, HEX, Cy3. Cy3.5, Cy5, and Cy5.5,

respectively (excitation wavelengths 492 nm, 535 nm, 552 nm, 588 nm, 649 nm, and 675 nm for FAM, HEX, Cy3. Cy3.5, Cy5, and Cy5.5, respectively). Subsequently, **ODN1** with quencher (20 μL of the stock solution, final concentration 0.25 μM) was added, solution was heated to 70 °C for 5 min and allowed to cool to laboratory temperature for 30 min. Thereafter, the fluorescence of duplex was measured. Similar procedure was used for non-complementary **ODN2**. The quenching efficiency (*QE*) was calculated according to following equation.[26]

$$QE = (1 - F_X/F_0) \times 100$$

where F_0 is fluorescence of fluorophore-modified **ODN3** alone and F_X is fluorescence of the duplex. All measurements were performed three times.

4.5.2. Determination of ground-state complex formation. Spectra of duplexes were measured in hybridization buffer. Stock solutions of oligonucleotides **ODN1** or **ODN2** labeled with quenchers AzaPc and **ODN3** labeled with fluorophores were prepared to concentration 100 μM. Hybridization buffer (98 μL) was transferred to a cuvette, stock solution of **ODN3** probe with fluorophore (1 μL) was added and absorption spectrum was measured. Thereafter, stock solution of ODN labeled with quencher (1 μL) was added and solution was heated to 70 °C for 5 min. Absorption spectrum of duplex was measured after 30 min when the sample cooled down to room temperature. Final concentration of both oligonucleotides was 1 μM. Absorption spectra of **ODN1**, **ODN2** were measured also separately after addition of stock solution (1 μL) to hybridization buffer (99 μL).

4.6. Singlet oxygen and fluorescence

Singlet oxygen quantum yields of **7** were determined in THF according to previously published procedure using decomposition of a chemical trap of singlet oxygen 1,3-diphenylisobenzofuran (DPBF).[34] Absorption of the dyes in the Q-band region during measurements was approximately 0.1. Zinc phthalocyanine (ZnPc, Sigma–Aldrich) was used as the reference (Φ_Δ=0.53 in THF[35]).

Fluorescence quantum yields were determined in THF (for **7**) or hybridization buffer (for **AzaPc**/**ODN1** or **AzaPc**/**ODN2** conjugates) by comparative method[28] using ZnPc as reference (Φ_F=0.30 in chloronaphthalene[36]). Absorption of the dyes in the Q-band maximum was approximately 0.05 in order to avoid an inner filter effect. The samples were excited at two wavelengths—366 nm and 610 nm. All measurements (both singlet oxygen and fluorescence) were performed three times and the presented data represent mean of these three experiments.

Acknowledgements

This work was supported by the Grant Agency of the Academy of Sciences of the CR (KJB401100801), Grant Agency of Charles University (57810/2010), and Ministry of Education, Youth and Sports (MSM 0021620822). Authors would like to thank to Jiří Kuneš for measurements of NMR spectra.

Supplementary data

MALDI-TOF mass spectra, ^{1}H NMR spectra, HPLC chromatograms, further spectra of AzaPc/ODN conjugates, absorption spectra of duplexes **ODN1**-**ODN3** labeled with all studied quenchers and fluorophores and absorption spectra of equimolar mixtures **ODN2** and **ODN3** can be found. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tet.2011.06.038.

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