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Synthesis of a multibranched porphyrin–oligonucleotide scaffold for the construction of DNA-based nano-architectures†

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The interest in the functionalization of oligonucleotides with organic molecules has grown considerably over the last decade. In this work, we report on the synthesis and characterization of porphyrin–oligonucleotide hybrids containing one to four DNA strands (P_1–P_4). The hybrid P_4, which inserts one porphyrin and four DNA fragments, was combined with gold nanoparticles and imaged by transmission electron microscopy.

Introduction

The main aim of nanotechnology is the fabrication of functional systems by controlling matter at the nanometer length scale (1–100 nanometers). This approach implies to study, understand and develop nanomanipulation technologies which enable the design and production of well-defined structures at the nanoscale level. Indeed, it is well accepted that the conventional "top-down" approach will face experimental difficulties. At this scale self-assembly, and more generally, "bottom-up" approaches appear to be a more reasonable way to assemble nano-objects into a well-defined two- or three-dimensional (2- or 3-D) layout. Among the new methodologies based on bottom-up approaches, bio-directed assembly of nano-objects is among the most promising ones. Indeed, the nanoscale is the natural scale on which biological systems build up their structural elements and biological molecules have already shown great potential for fabrication and construction of nanostructures and devices.[1–5]

DNA is widely used for the construction of periodic or non-periodic 2- and 3-D structures.[6–15] Initially, the creation of angles and junctions was exclusively achieved by the DNA sequences. This implied the careful design of the sequences in order to obtain the predicted configuration in the resulting material. Another approach was explored more recently: the combination of oligonucleotides with small organic molecules which were used to define the angle in the nanostructures.[16–25] Most of the systems described so far were based on covalent linkage of DNA with planar aromatic molecules. Due to the symmetry of the phenyl ring, the realisation of 2-D structures with angles of 60°, 120° or 180° is easily predictable and only a limited number of nanostructures exhibiting different angles and 3-D topology have been reported so far.[13,16,24–27] One of the major issues when combining organic molecules with DNA is the difference of physicochemical properties between the two moieties. Indeed, DNA is only soluble in water and sometimes in polar organic solvents while organic molecules prefer apolar solvents. In this context, we decided to explore the combination of single-stranded DNA (ssDNA) with organic molecules in a robust and reproducible way. In particular, we used the copper-catalysed *Huisgen* cycloaddition (CuAAC)[28,29] to assemble ssDNA containing a triple bond with tetraphenylporphyrin (TTP) functionalised with azide groups. In a recent review Carell and coworkers[30] showed that this reaction is very efficient in functionalising oligonucleotides and the porphyrin molecule was chosen because it exhibits a planar shape with angles of 90° between the functional groups. In the literature the combination of DNA with organic chromophores and in particular porphyrin has been extensively reported mainly for light harvesting and FRET experiments.[26,31–44] In these reports, suitably modified porphyrin molecules were connected to one

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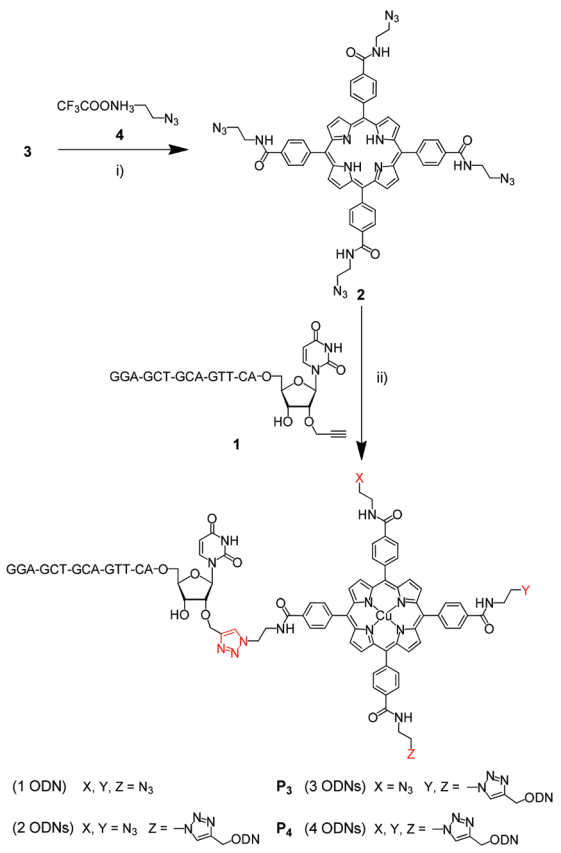
oligonucleotide either in 3′ or 5′ position, to a phosphate between the ribose sugars or to modified nucleobases, and only a limited number of reports have shown the multifunctionalisation of the porphyrin core by DNA strands.[26,37]

In this work we report on the synthesis of porphyrin/oligonucleotide (ODN) polyadducts *via* CuAAC. The hybrids containing one to four ODNs around the porphyrin were purified by RP-HPLC and characterised by absorption spectroscopy, mass spectrometry and gel electrophoresis; the morphology of the tetra-adduct was also studied by transmission electron microscopy (TEM) after reaction with gold nanoparticles.

Results and discussion

The 15-base oligonucleotide sequence **ODN-1** (5′ GGA-GCT-GCA-GTT-CAU-propargyl 3′) was synthesised by a solid phase approach using the phosphoramidite chemistry. At the 3′-end, the sequence contains a uridine modified in position 2′ with a propargyl group.[45,46]

The synthesis of the porphyrin/DNA hybrids $\mathbf{P}_n$ (n = 1–4) is described in Scheme 1. Azido-porphyrin **2** was synthesised by reaction of *meso*-tetra(4-carboxyphenyl)porphyrin **3** with 2-azidoethanamine trifluoroacetic acid salt **4** in the presence of PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate). Azido derivative **4** was synthesised according to the literature[47] in three steps starting from the 2-bromoethylamine hydrobromide. Briefly, the terminal amine group was first protected with a *tert*-butyloxycarbonyl group (Boc) (**5**) and then the azide group was introduced (**6**). The terminal amine was finally deprotected by reaction with TFA affording compound **4** in good yield (40%). For the CuAAC coupling with oligonucleotides, porphyrin **2** was dissolved in a mixture of *N*-methylpyrrolidone–water and **ODN-1** was added. The reaction was performed in the presence of CuI, di-*iso*-propylethylamine (DIEA) and sodium ascorbate at room temperature. Note that the reaction was performed with an excess of copper and that the free-base porphyrin is metallated during the reaction. The reaction was controlled by reverse phase HPLC (RP-HPLC, TEAA (10 mM)/MeCN) and small portions of **ODN-1** were added if necessary. The crude mixture was pre-purified by size-exclusion chromatography using water as an eluent to remove partially the unreacted azidoporphyrin, the salts and the organic solvent. The eluted fraction contained a mixture of **ODN-1**, porphyrin/ODN hybrids $\mathbf{P_1}$–$\mathbf{P_4}$ and still some unreacted porphyrin. The mixture was buffered to pH 7 with a concentrated solution of 1 M aq. TEAA buffer, purified by RP-HPLC and then lyophilised.



Scheme 1 Synthesis of the porphyrin/DNA hybrids from **3**: (i) PyBOP, DIEA, NMP, rt, 36%; (ii) CuI, DIEA, NMP/water, rt.

CuAAC with DNA are commonly performed directly in water or in a mixture of water and a highly polar organic solvent like methanol[48,49] or DMSO.[23,50] Nevertheless, due to the hydrophobicity of some molecules, other solvents such as DMF[27] can be used in addition to water to solubilise all reagents and perform the reaction. In our case, it appeared rapidly that no reaction occurred with these solvents. We decided to try the CuAAC under similar conditions using *N*-methyl-2-pyrrolidone (NMP) which is more commonly used for peptidic coupling. In a mixture of NMP and water (*ca.* 3 : 1 in volume), we were able to solubilise the porphyrin and the oligonucleotide improving the yield of the reaction. For the CuAAC, we used CuI instead of $CuSO_4$ because of the higher solubility of CuI in organic solvents. In addition, the use of DIEA permitted us to complex the copper catalyst avoiding the oxidative degradation of the oligonucleotide during the reaction.[51]

The porphyrin/DNA hybrids were purified by RP-HPLC allowing the separation of mono- to tetra-adducts; the hybrids were characterised by absorption spectroscopy, mass spectrometry and gel electrophoresis.

The chromatogram of the purification recorded at 260 nm with a diode array detector is presented in Fig. 1a. It shows six peaks eluted at 10.8%, 15.9%, 17.7%, 21.4%, 29.6% and 100% of acetonitrile; Fig. 1b shows the same purification recorded at 420 nm (where the absorption of the porphyrin is maximum). The absorption spectra of the different compounds were recorded during their elution (Fig. 1c): the first and last peaks correspond to unreacted ODN and porphyrin, respectively. The compounds eluted between 15.9% and 29.6% contain both DNA (absorption at 260 nm) and porphyrin (absorption at 420 nm). They correspond to $\mathbf{P_4}$, $\mathbf{P_3}$, $\mathbf{P_2}$ and $\mathbf{P_1}$, respectively. The absorption spectra show that the relative amount of

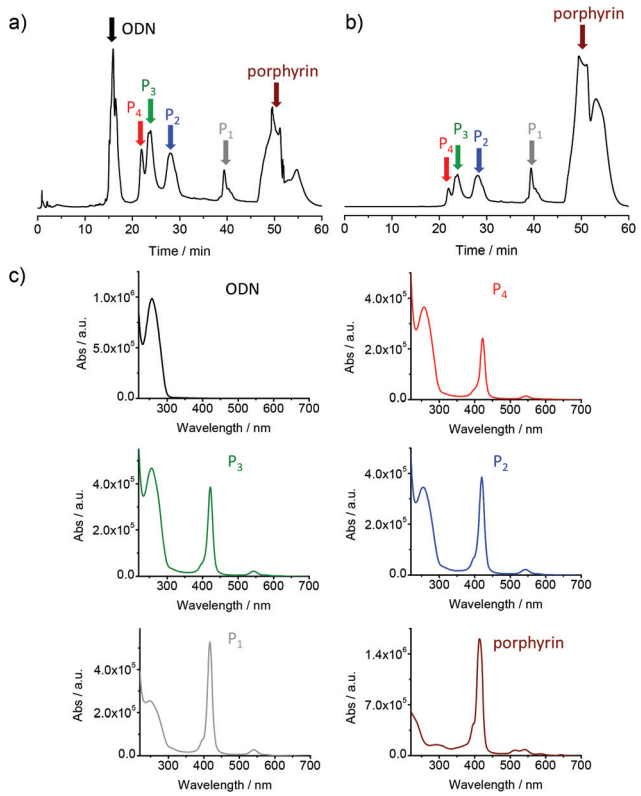


Fig. 1 RP-HPLC profile recorded at 260 nm (a) and 420 nm (b) of the crude reaction mixture obtained from the coupling of the 15-mer DNA fragment to the porphyrin residue. (c) UV-Vis absorption spectra of the samples of 15-mer DNA, $\mathbf{P_4}$ to $\mathbf{P_1}$ and metallated porphyrin **2**.

DNA decrease in the hybrids eluted at higher acetonitrile concentration. This is in perfect agreement with the structures of the hybrids: as the number of DNA chains decreases in the molecules, the apolar character induced by the central porphyrin molecule is more important thus increasing the interactions with the apolar stationary phase and then elution time.

In order to confirm the HPLC observations, different purified functionalised DNA fragments were characterised by polyacrylamide gel electrophoresis (denaturing PAGE). Then, 10 pmoles of DNA fragments (starting 15-mer sequence, $\mathbf{P_1}$, $\mathbf{P_2}$, $\mathbf{P_3}$ and $\mathbf{P_4}$) were labelled by ^{32}P at the 5′-end and then analysed by PAGE. This analysis univocally confirms the formation and the isolation of the porphyrin/DNA hybrids $\mathbf{P_n}$ with n = 1–4 (Fig. 2). Additionally the multiconjugation of the 15-mer DNA fragment onto the porphyrin scaffold was confirmed by MALDI-ToF mass spectrometry measurements (Table S1 and Fig. S1†). It is worth mentioning that the coupling reaction was also performed on the complementary strand: **ODN-2** (5′ TGA-ACT-GCA-GCT-CCU-propargyl 3′). After purification, the $\mathbf{P_{nc}}$ (n = 1–4) hybrids were characterised by gel electrophoresis (Fig. S2†) and mass spectrometry (see ESI†).

The molecular hybrid containing four DNA strands was combined with gold nanoparticles and the resulting assembly was characterised by transmission electron microscopy (TEM).

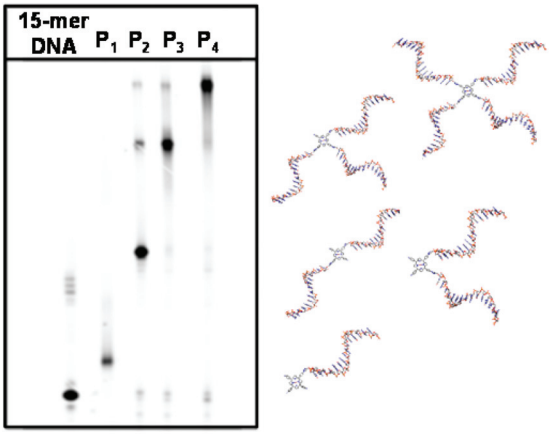


Fig. 2 Denaturing PAGE analysis (15%, 7 M urea) of the RP-HLPC purified porphyrin–DNA hybrids. First lane: starting 15-mer DNA (**ODN-1**); second lane: one porphyrin–one DNA fragment ($\mathbf{P_1}$); third lane: one porphyrin–two DNA fragments ($\mathbf{P_2}$); fourth lane: one porphyrin–three DNA fragments ($\mathbf{P_3}$) and fifth lane: one porphyrin–four DNA fragments ($\mathbf{P_4}$).

To this end, porphyrin/DNA hybrid $\mathbf{P_4}$ was sequentially hybridised with a complementary 14-mer DNA strand (5′ TGA-ACT-GCA-GCT-CC 3′) containing a thiol group at the 3′ end, and then incubated with gold nanoparticles (AuNPs) (see the Experimental section in ESI†). Note that the modified uridine on the DNA strands of $\mathbf{P_4}$ hybrid was not used for hybridisation in order to avoid potential steric interactions between the substituents in 2′ position of the ribose and an adenine base on the complementary strand. Fig. 3a shows a representation of the $\mathbf{P_4}$/AuNPs assemblies. TEM analysis of the gold nanoparticles alone does not show the presence of any aggregates (Fig. 3b). Conversely, in the $\mathbf{P_4}$/AuNPs hybrid sample (Fig. 3c and d), one can observe nanostructures formed with three and four AuNPs. It should be noted that most nanostructures formed possess three AuNPs instead of four, which can be attributed to a problem of steric hindrance (the 6 nm diameter of the AuNPs may be too large compared to DNA length).

The hybrid sample still contains a large amount of free AuNPs; it is most probably due to the procedure used to incorporate the nanoparticles in the assemblies. Indeed, the salt concentrations had to be low for this kind of analysis decreasing hybridisation capacities of complementary strands. Additionally, Balaz and co-workers[39] demonstrated that the covalently linked porphyrin on ssDNA slightly decreased the melting temperature of the dsDNA compared to the unmodified one. Nevertheless, TEM analysis permitted to confirm the formation of the nanostructures. In order to improve the yield in $\mathbf{P_4}$/AuNPs, a possibility would be to incorporate the complementary strand first on the gold nanoparticles and then, after purification, to perform the hybridisation with $\mathbf{P_4}$; a second possibility would be to increase the size of the oligonucleotide. Indeed, we are currently working on the latter solution, which is the increase from 15-base pairs to 21-base pairs

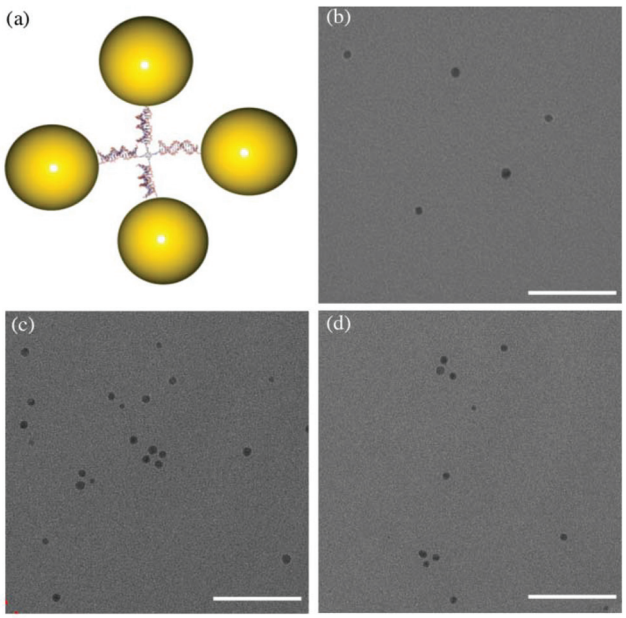


Fig. 3 (a) Schematic representation of the P_4/AuNPs assembly. (b) TEM images of the AuNPs particles alone. (c) and (d) TEM images of the self-assembly nanostructures with three (L-shaped) and four AuNPs. Scale bars represent 50 nm.

the length of the oligonucleotides incorporated in the porphyrin molecules.

Conclusions

In this paper, we described the synthesis of porphyrin–oligonucleotide hybrids based on the copper-catalysed *Huisgen* cycloaddition reaction. The hybrid containing four DNA strands ($\mathbf{P_4}$) was incubated with the 14-mer complementary strand bearing a thiol group, and then exposed to gold nanoparticles and imaged by TEM. The coupling reaction was also performed on the 15-mer complementary strand: **ODN-2** (5′ TGA-ACT-GCA-GCT-CCU 3′) to form $\mathbf{P}_{nc}$ (n = 1–4). The synthesis of these porphyrin–oligonucleotide building blocks constitutes the first step toward the formation of nanostructures and we are currently working on the construction of a 2-dimensional network based on the $\mathbf{P_4}$ and $\mathbf{P_{4c}}$ hybrids bearing the two complementary strands.

Experimental

Techniques

^{1}H NMR spectra were recorded with a NMR 300 MHz Bruker Avance Spectrometer with a solvent used as an internal reference. Purification of the porphyrin–oligonucleotide hybrids was performed with a Thermo-Fisher HPLC equipped with a diode array detector and on a column Hypersil GOLD 100 × 4.6 mm (Thermo-Fisher) with a gradient of acetonitrile (HPLC grade) in 10 mM aq. TEAA (triethylammonium acetate solution) buffer at room temperature. For the organic materials MALDI mass spectra were obtained from a Perseptive Biosystems Voyager DE-STR instrument. For the porphyrin–oligonucleotide hybrids, the MALDI mass spectra were obtained in the negative mode on a time-of-flight Biflex mass spectrometer (Bruker, Wissembourg, France) or in the positive mode on a time-of-flight Axima Performance (Shimadzu, Manchester, UK), both equipped with a 337 nm nitrogen laser and pulsed delay source extraction. The matrix was prepared by dissolving 3-hydroxypicolinic acid in 10 mM ammonium citrate buffer and a small amount of Dowex-50W 50 × 8–200 cation exchange resin (Sigma). The DNA sample (10 pmol; 1 μL) was added to the matrix (1 μL) on the target plate and allowed to dry. The resulting sample was placed on the target plate and allowed to dry. Spectra were calibrated using reference oligonucleotides of known masses. For gel analysis, oligonucleotides (10 pmoles) were labeled at the 5′-terminus with 10 μCi [γ-^{32}P]-ATP (2 pmol, 10 mCi mL^{-1}) Perkin-Elmer (Courtabœuf, France) upon incubation with T4 polynucleotide kinase (5 units, New England Biolabs) in 10 μL of the supplied buffer at 37 °C for 30 min. Unincorporated [γ-^{32}P]-ATP was removed by purification of the oligonucleotide on a MicroSpin column (GE Healthcare, UK). Then, the labeled DNA fragments were subjected to electrophoresis on a 15% denaturing polyacrylamide gel containing 7 M urea and finally revealed using a phosphor-Imager (Bio-Rad). For TEM analysis: 5 μL of AuNPs or 5 μL of 10 nM $\mathbf{P_4}$/AuNPs solution were deposited for 1 min on a 600-mesh copper grid covered with a thin carbon film, activated by glow-discharge in the presence of pentylamine.[52] The grids were washed with aqueous 2% (w/v) uranyl acetate, dried with ashless filter paper and observed in the bright-field mode, using a Zeiss 912AB transmission electron microscope. Images were captured at a magnification of ×67 500 using a ProScan 1024 HSC digital camera and iTEM acquisition software (Olympus Soft Imaging Solution).

Materials

meso-Tetra(4-carboxyphenyl)porphyrin **3** was purchased from Porphyrin Products Inc. Chemicals were purchased from Aldrich and were used as received. All the reagents, monomers and supports used in synthesis of the alkynylated DNA sequences were obtained from Chemgenes. The 3′-thiol-modified 14-mer oligonucleotide was purchased from Eurogentec. Solvents were purchased from Aldrich, SDS Carlo Erba and were used as received. For synthesis, CH_2Cl_2 (CaH_2, N_2) and THF (K/benzophenone, N_2) were distilled before use. The trifluoroacetic salt of 2-azidoethylamine **4** was synthesised according to the literature procedure.[47]

Synthesis

Oligonucleotides. The synthesis of oligonucleotide 15-mer ODN (**ODN-1:** 5′ GGA-GCT-GCA-GTT-CAU-propargyl 3′ and **ODN-2:** 5′ TGA-ACT-GCA-GCT-CCU-propargyl 3′) was carried out on an Applied Biosystems 392 DNA/RNA synthesiser using the phosphoramidite chemistry on a scale of 1 μmol. Upon completion, the alkyne function-containing oligonucleotides have been deprotected in concentrated aqueous ammonia for

16 h at 55 °C. After speed-vac evaporation of ammonia, the crude 5′-DMTr oligonucleotides were detritylated and purified on-line by RP-HPLC using a polymeric support.[53] After desalting by size exclusion chromatography, the oligonucleotides were then quantified by UV measurements at 260 nm. The purity and the integrity of the synthetic DNA oligomers were checked by RP-HPLC analyses together with MALDI-TOF mass measurements. Finally, the alkyne-modified DNA fragments were lyophilised and kept at −20 °C until their use in the CuAAC reaction.

***N*-(*tert*-Butyloxycarbonyl)-2-bromoethylamine 5.** To a cooled solution (0 °C) of 2-bromoazidoethylamine hydrobromide (1 g, 4.88 mmol) and di-*tert*-butyl dicarbonate (1.17 g, 5.37 mmol) in dichloromethane, triethylamine (748 μL, 5.37) was added dropwise. The reaction mixture was stirred for 1 h at 0 °C and overnight at RT. Then the reaction was diluted with dichloromethane (7 mL) and this solution was subsequently washed with 1 N $KHSO_4$ (3 × 7 mL) and brine (3 × 7 mL), dried over Na_2SO_4, filtered off and concentrated *in vacuo*. This afforded 1042 mg (4.650 mmol) of **5** as a yellow oil with a yield of 95%. The solid was used for the next step with no other purification. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.44 (s, 9H, $(CH_3)_3$), 3.45 (m, 2H, CH_2-Br), 3.51 (m, 2H, CH_2-NH), 4.96 (br s, 1H, NH).

***tert*-Butyl-2-azidoethylcarbamate 6.** A mixture of **5** (1.042 g, 4.65 mmol) with NaN_3 (604 mg, 9.3 mmol) was dissolved in DMF (21 mL) and stirred at RT overnight. Then, the reaction mixture was diluted in dichloromethane (21 mL) and this solution was washed with water (3 × 20 mL), dried over Na_2SO_4, filtered off and concentrated *in vacuo*. This afforded **6** as a yellow oil. The residue was used in the next step with no other purification. $R_{f\,4a}$ = 0.56; $R_{f\,4b}$ = 0.36 (dichloromethane).

TFA salt of 2-azidoethylamine 4. **6** was dissolved in dichloromethane (6 mL) and TFA (3 mL) was added dropwise. The solution was stirred for 2 h and then concentrated *in vacuo* resulting in a yellow oil. Dichloromethane was added to the oil leading to a precipitation. The solid was filtered off and washed several times with dichloromethane. The solid was dried overnight under vacuum affording 388 mg (1.939 mmol, yield = 42% from **5**) of **4** as an orange solid. ^{1}H NMR (MeOH-d_4, 400 MHz): δ 3.09 (t, 2H, J = 5.6 Hz, (CH_2)–N_3), 3.68 (t, 2H, J = 5.6 Hz, (CH_2)–NH_2).

Azido-porphyrin 2. *meso*-Tetra(4-carboxyphenyl)porphyrin **3** (50 mg, 63 μmol) was dissolved in a mixture of dry THF–DMF (6 mL, 5 : 1, v/v). TFA salt of 2-azidoethylamine **4** (100 mg, 504 μmol), PyBOP reagent (262 mg, 504 μmol) and dry DIEA (174 μL, 1 mmol) were sequentially added and the resulting reaction mixture was stirred at room temperature for 4 h. The reaction was checked for completion by TLC (CH_2Cl_2–MeOH, 9 : 1, v/v). Thereafter, the crude mixture was evaporated to dryness and the resulting residue was purified by chromatography on a silica gel column with a step gradient of MeOH (0–3%) in CH_2Cl_2 as the mobile phase, to give **3** as a purple solid (24 mg, 23 μmol, yield = 36%). ^{1}H NMR (400 MHz, DMF-d_7): δ ppm 9.17 (t, 4H, J = 5.2 Hz, NH), 8.96 (s, 8H, H-porphyrin), 8.45 (s, 16H, H-phenyl), 3.74–3.81 (m, 16H, CH_2–CH_2); ^{13}C NMR (75.5 MHz, DMF-d_7): 167.1, 134.4, 126.3, 120.0, 42.7, 39.8; IR ν_{max} (cm^{-1}) 715, 792, 863, 965, 1105, 1153, 1186, 1213, 1297, 1347, 1389, 1438, 1606, 2095, 2530, 2706, 2871, 2926, 3065, 3296; MS (MALDI-TOF, negative mode) m/z = 1061.4 $[M]^-$, calcd for $C_{56}H_{45}N_{20}O_4$: 1061.3938 g mol^{-1}.

General procedure for the CuAAC. A 10 mM solution of CuI in NMP (1 μmol, 100 μL) was mixed to a 50 mM solution of di-iso-propylethylamine (DIEA) in NMP (1 μmol, 20 μL). A 1 mM solution of ODN (**ODN-1** or **ODN-2**) in deionised water was added (50 nmol, 50 μL) followed by a 5 mM solution of **2** in NMP (20 μL, 100 nmol). The mixture was stirred at room temperature overnight. The reaction was followed by RP-HPLC with a gradient of acetonitrile from 0 to 100% in TEAA 10 mM. Possibly a 100 mM sodium ascorbate solution (1 μmol, 10 μL) is added several times to ensure the starting material conversion. After 24 h, NMP, salts and unreacted **3** were removed from the crude mixture by size exclusion chromatography (NAP-25 column Sephadex G-25 DNA grade, GE Healthcare) using deionised water as an eluent. The fractions containing the oligonucleotide derivatives were pooled (~1 mL) and dried under reduced pressure. The reaction was started again with the same conditions but without addition of **3** to maximise the amount of tris- and tetra-adducts. The crude mixture was purified against a Sephadex column and the oligonucleotide derivatives were then purified by RP-HPLC with a gradient of acetonitrile from 0 to 100% in TEAA 10 mM. The compounds are obtained according the following order of elution: unreacted ODN, **P_4**/**P_{4c}**, **P_3**/**P_{3c}**, **P_2**/**P_{2c}**, **P_1**/**P_{1c}**, and then unreacted porphyrin. The different fractions were lyophilised and kept at −20 °C.

Gold nanoparticles. The nanoparticles were synthesised according to the procedure described by Brust and Schiffrin.[54] Tetraoctylammonium bromide (410 mg, 750 μmol) dissolved in toluene (30 mL) and $HAuCl_4$ (100 mg, 250 μmol) in deionised water (10 mL) were added under vigorous stirring. After a few minutes, the aqueous phase was separated and a solution of $NaBH_4$ (110 mg, 2.9 mmol) dissolved in water (8 mL) was rapidly added (5 s) with vigorous stirring. The mixture was then stirred for 2 h at RT. The 2 phases were separated and the organic layer was subsequently washed with HCl 0.1 N (2 × 5 mL), $NaHCO_3$ sat. (2 × 10 mL) and NaCl sat. (10 mL). The organic layer was finally dried over Na_2SO_4 and filtered off. The gold nanoparticles solution in toluene was kept at 4 °C.

Assembly of porphyrin/DNA hybrids. A mixture of 10 μM **P_4** and 40 μM complementary 14-mer DNA strand (5′ TGA-ACT-GCA-GCT-CC 3′), containing a thiol group in 3′ position, is annealed at 90 °C for 5 minutes and cooled slowly to room temperature (RT) in Tris 10 mM, pH 7.5, NaCl 50 mM and $MgCl_2$ 10 mM buffer. 5 μL of the mixture is incubated overnight at RT with 5 μL of AuNPs. The **P_4**-AUNPs solution is diluted 20 times in annealing buffer for TEM imaging.

Acknowledgements

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