Rational Design and Experimental Analysis of Short-Oligonucleotide Substrate Specificity for Targeting Bacterial Nucleases

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ABSTRACT: An undecamer oligonucleotide probe based on a pair of deoxythymidines flanked by several modified nucleotides is a specific and highly efficient biosensor for micrococcal nuclease (MNase), an endonuclease produced by Staphylococcus aureus. Herein, the interaction mode and cleavage process on such oligonucleotide probes are identified and described for the first time. Also, we designed truncated pentamer probes as the minimum-length substrates required for specific and efficient biosensing. By means of computational (virtual docking) and experimental (ultra-performance liquid chromatography—mass spectrometry and matrix-assisted laser desorption ionization time-of-flight) techniques, we perform a sequence/structure—activity relationship analysis, propose a catalytically active substrate–enzyme complex, and elucidate a novel two-step phosphodiester bond hydrolysis mechanism, identifying the cleavage sites and detecting and quantifying the resulting probe fragments. Our results unravel a picture of both the enzyme–biosensor complex and a two-step cleavage/biosensing mechanism, key to the rational oligonucleotide design process.

1. INTRODUCTION

Nucleic acids have been proven useful recognition molecules for the development of several diagnostic strategies, taking advantage of their flexibility to be adapted to various transduction mechanisms, such as fluorescence, electrochemistry, piezoelectric, and colorimetric mechanisms.1 In particular, the use of nuclease-activatable oligonucleotide probes (NAOPs) as biosensors has proven its potential as a groundbreaking solution in early diagnosis of serious infectious diseases.2,3 The detection of bacterial infection,4–6 malignant cells in biopsies of breast cancer,7–9 or food contamination by pathogenic bacteria10 has been previously reported to be feasible on the basis of their nuclease activity profile.10–12 A key aspect in the development of any diagnostic tool based on oligonucleotide probes is the necessity of designing probes which are both efficient and specific.13 Efficiency is directly related to the extent to which the target nucleic acid cleaves the synthetic substrate. In this respect, the oligonucleotide probe needs to be readily cleaved by the target nuclease even when the latter is in very low concentrations, such as in the early stages of infection. In addition, in order to avoid false positives in the diagnosis, a reliable biosensor needs to be resistant to cleavage by other nucleases that may be present in the sample under analysis.

In this line, in a previous work,4 Hernandez et al. described the efficiency and specificity of a library of oligonucleotide probes in the detection of bacterial infection caused by Staphylococcus aureus (S. aureus) based on the activity of their secreted endonuclease, the micrococcal nuclease (MNase). In that study, the MNase activity was detected through a fluorescence resonance energy transfer (FRET) method, using specific oligonucleotide probes modified at the 5′-end with a fluorophore (fluorescein amidite, FAM) and at the 3′-end with a fluorophore quencher (tide quencher 2, TQ2), as depicted in Figure S1. Once the oligonucleotide is cleaved by the enzyme, the fluorophore is released, and the fluorescence intensity is quantified in order to estimate the nuclease activity. Among all the NAOPs tested,4 the one consisted of a pair of deoxythymidines (dTts) flanked by several 2′-O-methyl-modified nucleotides, 5′-mCmUmCmGdTdTmCmGmUmC-3′ [named the TTprobe in previous reports5 and hereafter the FRET-dTdTprobe when used in FRET experiments or the nude-dTdTprobe when used in ultra-performance liquid chromatography mass spectrometry (UPLC–MS) and matrix-assisted laser desorption ionization time-of-flight.
(MALDI-TOF) experiments; see Figure 1], noticeably exhibited the greatest sensitivity and specificity to be digested by MNase. The FRET-dTdTprobe has also been used to test S. aureus antibacterial susceptibility with high accuracy in comparison with the gold-standard broth microdilution method and very recently was also used as a 6-mer oligonucleotide linker to covalently functionalize a hydrogel coating with vancomycin, aiming to enable a rapid release of vancomycin within the periprosthetic implant infected with S. aureus.

The great advances in the nucleic detection technology reported so far lack a rational and atomic-level description of the interaction mode and catalytic reaction of the oligo–enzyme complex. An unprecedented description of the catalytic interactions between the said nucleo- or FRET-dTdTprobe and MNase is proposed here for the first time based on the coherent results obtained with the full-length oligomer and shorter pentameric sequences designed both for docking purposes and for the identification of the minimum-length required in the probe candidate. Computational and experimental techniques were used, which combined with the previous knowledge on the phosphodiester bond hydrolysis mechanism (Figure 2) have reported consistent results, providing a basic and essential understanding of the process. Importantly, we describe probe candidates with low molecular weights compared with that of previous reports that work efficiently as biosensors.

2. EXPERIMENTAL SECTION

2.1. Nuclease-Activatable Oligonucleotide Probes. All NAOPs were purchased at Biomers.net (Germany) with and without the FAM fluorophore and TQ2 quencher at the 5′- and 3′-ends, respectively, flank the sequence in the otherwise identical FRET-dTdT probe.

Figure 1. Chemical structure of the nude-dTdTprobe. FAM fluorophore and TQ2 quencher at the 5′- and 3′-ends, respectively.
Table 1. ARF Intensity of Fluorescence for the FRET-NAOPs, Obtained from Digestion Experiments Upon Incubation with Either Purified MNase or One of the Five Pathogen Strains Cultured

| Probe/Strain | M. Nase | S. aureus | P. mirabilis | K. pneumoniae | P. aeruginosa | S. pneumoniae |
|--------------|---------|-----------|--------------|---------------|--------------|--------------|
| DNA          | 22.4 (+0.8) | 18.9 (+0.8) | 7.2 (+0.3) | 1.2 (+0.1) | 1.0 (+0.0) | 1.2 (+0.1) |
| poly A       | 6.2 (+0.2)  | 6.0 (+0.2)  | 3.6 (+0.1) | 1.0 (+0.0) | 1.0 (+0.0) | 1.0 (+0.0) |
| poly T       | 17.6 (+0.3) | 14.3 (+0.4) | 12.2 (+0.3) | 12.2 (+0.0) | 12.2 (+0.0) | 12.2 (+0.0) |
| poly AT      | 38.1 (+0.5) | 32.2 (+0.7) | 17.8 (+0.2) | 1.3 (+0.0) | 1.3 (+0.0) | 1.5 (+0.0) |
| 2'Ome        | 1.6 (+0.16) | 1.1 (+0.1)  | 1.0 (+0.1) | 1.0 (+0.1) | 1.6 (+0.2) |
| 2'F          | 3.5 (+0.2)  | 6.6 (+0.3)  | 4.9 (+0.3) | 0.9 (+0.1) | 0.9 (+0.1) | 1.5 (+0.1) |
| FRET-dTdT    | 29.7 (+0.17) | 22.6 (+0.13) | 2.5 (+0.2) | 1.1 (+0.1) | 1.1 (+0.1) | 1.3 (+0.1) |

with 1 μL of the dTdT probe NAOP (with or without FAM and TQ2) at a concentration of 50 pmol in 8 μL of PBS +/+ and incubated at 37 °C for 60 min. Thereafter, the reaction was stopped thermically by freezing it in dry ice and was kept frozen in an ultrafreezer at −80 °C until the MALDI assays were performed. The sample preparation was carried out by the deposition of 0.5 μL of the sample (the NAA reaction) directly onto a polished stainless-steel plate (Bruker Daltonics, Bremen, Germany) and mixed with 0.5 μL of the matrix solution, composed of a dissolution of 10 mg of 2,4,6-trihydroxynicotinamide and 5 mg of dionammonium citrate in 1 mL of deionized water. MALDI-TOF-MS analysis was performed using an Ultraflextreme III time-of-flight mass spectrometer equipped with a Nd:YAG laser (Smartbeam II, 355 nm, 1 kHz) and controlled by Flex Control 3.3 software (Bruker Daltonics, Bremen, Germany). The acquisitions were carried out in the positive-ion linear mode at a laser frequency of 1 kHz. The spectrum was acquired at 30% laser fluency and was recorded in the m/z range from 1500 to 5000. The detector cutoff was set at m/z 1300, and the spectrum resulted from the accumulation of 3000 laser shots. All the spectra were analyzed using FlexAnalysis software 3.0 (Bruker Daltonics, Bremen, Germany).

2.5. LC–MS Analysis. After some optimization procedures to set the most favorable conditions to perform the UPLC–MS experiments, the standard conditions defined for the NAAs were as follows: 1 μL of MNase (20 U/μL) reacted with 1 μL of pentadecamer (1 nmol nuclease substrate) in a 1.5 mL hplc vial, and 198 μL of the sample (the NA probe) was injected into the UPLC system, using an Acquity BEH C18 column (100 mm × 2.1 mm, 1.7 μm) from Waters (Milford, MA, USA). The samples were eluted using a flow rate of 300 μL min⁻¹ and using as a mobile phase 100 mM triethanolamine in water (A) and 100 mM triethanolamine in acetonitrile/water (9:1) (B). The gradient method was as follows: 0–5 min at 95% A, 5–3.5 min to 50% A, 3.5–3.7 min to 1% A, 3.7–4.2 min at 1% A, 4.2–4.3 min to 95% A, and 4.3–5 min at 95% A. The UV detector wavelengths were set at 262 and 280 nm, and the injection volume was 10 μL. The MS detection was carried out using the electrospray ionization TOF mass spectrometer LCT Premier XE from Waters (Milford, MA, USA) with an electrospray ionization source, working in the negative/W mode. The MS range acquired was between m/z 100 and 2000. The capillary and cone voltages were set at 1000 and 100 V, respectively. For other parameters, the desolvation gas temperature was 350 °C, and the source temperature was 120 °C. The desolvation and cone gas rates were set at 600 and 30 L h⁻¹, respectively. MassLynx v4.1 software was used to analyze the chromatograms and spectra (Waters, Milford, MA, USA).

2.6. Computational Studies. 2.6.1. Virtual Docking. Due to the limitations (maximum number of atoms) of the technique, virtual docking experiments were carried out with suitable molecular models of the nucleotide dTdT probe. Virtual docking of several short-oligonucleotide probes to the MNase receptor structure was made with the Glide software package of Schrödinger Inc. A careful analysis (beyond the scope of this report) of this entire set of structures deposited in the PDB for this nuclease pointed to PDB ID = 1SNC (deposited by Loll and Lattman) as the most appropriate to perform the virtual docking study. The latter crystal structure consists of the ternary complex of residues 7–141 of staphylococcal nuclease, Ca²⁺, and the inhibitor thymidine-5',5'-diphosphate (THP) refined at 1.65 Å.

2.6.2. Receptor Preparation and Grid Generation. The structure of residues 7–141 of staphylococcal nuclease was downloaded from the protein data bank (PDB ID: 1SNC) and prepared with Schrödinger’s Protein Preparation Wizard suite in order to add water molecules, assign bond orders, create zero-order bonds to the Ca²⁺ metal, and optimize the H-bond network. The preprocessing was carried out with default methods, except that water molecules beyond 3 Å of any heavy atoms were removed. Minimization and refinement were performed to remove local clashes prior to further standard equilibration protocols. The resulting structure contained seven different water molecules, five of which establish key interactions with the crystallized THP ligand but are located far from the active site on the enzyme. In view of the latter, two alternative Glide grid files were generated for the MN receptor, differing in the amount of water molecules, using an enclosing box of ca. 46 Å centered on the Ca²⁺ atom, which properly covered the active site of the enzyme (see the Supporting Information for further details).

2.6.3. Ligand Preparation and Docking. Pentameric oligonucleotides (ligands) to be screened were manually generated using the Build facility in Maestro 12.5 and prepared for docking using LigPrep with the OPLS_2005 force field. To set the ionization and tautomerization state of compounds at a pH range of 6–8, Epik v16207 was used, with a maximum number of 10 generated structures. The binding mode and affinity of the ligands were estimated using an extra precision Glide docking protocol, with and without positional NOE and metal coordination constraints.

2.7. Identifying Catalytically Relevant Poses. The hydrolysis of oligonucleotides promoted by MNase was proposed to proceed via the cleavage of a 5'-P-O bond to yield a free 5'-hydroxyl group and a terminal 3'-phosphate monoester group (see Figure 2). The hydrolysis of one single phosphate bond may consist of four consecutive steps involving a catalytic water molecule, three amino acids, and a Ca²⁺ ion. The efficiency and specificity of such hydrolysis depend on the nature of the substrate sequence for each nuclease.

According to the authors, hydrolysis may follow an SN2-type mechanism (see Figures 2 and S4), starting with the nucleophilic attack of the oxygen atom of a catalytic water molecule (HOH225) on the P atom of a Ca²⁺-coordinated phosphate group. The attack is
believed to be favored upon the polarization of the coordinated phosphate group via an H-bond network governed by several coordination water molecules as well as residues ARG35 and ARG87. The latter residue was also proposed by the authors as being the acidic residue that transfers a proton to the 5′-oxygen atom of the phosphate group, yielding the final cleavage products composed of a 5′′-OH fragment and a 3′′-phosphate. Actually, the Ca2+ metal center cooperating with guanidinium residues showed a nearly 35 000-fold rate enhancement of DNA hydrolysis compared to that of the mutant without the guanidinium groups.10 Similar mechanisms have been reported for other endonucleases, even for the cleavage of double-stranded DNA. In the present work, catalytically relevant pentamer/MNase poses were identified among those which were characterized by (i) phosphate group coordination to the Ca2+ cation, accompanied by the establishment of key H-bonds with neighboring residues or water molecules that would polarize the phosphate group; (ii) coordination of a catalytic water molecule (HOH225) to a basic residue (such as GLU43) as well as to the 3′-O atom of the coordinated phosphate group; and (iii) H-bonding of an acidic residue (such as ARG 87) to the 5′-O atom of the Ca2+-coordinated phosphate group.

3. RESULTS AND DISCUSSION

3.1. Digestion of NAOPs by MNase: Fluorescence Assay. Pathogen specificity of seven NAOPs (DNA, poly A, poly T, poly AT, 2′OMe, 2′F, and FRET-dTdT; see Table 1 for sequence details) was tested by an NAA carrying out the cleavage of a catalytic water molecule (HOH225) to a basic residue (such as GLU43) as well as to the 3′-O atom of the coordinated phosphate group; and (iii) H-bonding of an acidic residue (such as ARG 87) to the 5′-O atom of the Ca2+-coordinated phosphate group.

Figure 3. Screening of the nuclease activity profile of five pathogen strains and MNase (0.1 U/μL). Results reported as an increase in the mean fluorescence intensity as a result of the degradation of each NAOP. The bars represent the average of triplicate fluorescence measurements (±s.d.).

respectively, with respect to the control buffer for the three mentioned bacterial strains. On the other hand, NAOPs poly A, poly T, and poly AT showed similar digestion profiles, with the poly AT probe showing the largest activation ratio among all. Due to the observed lack of specificity in the digestion of those NAOPs by S. aureus, they were discarded as molecular candidates for bacterial detection. On the contrary, two chemically modified NAOPs, 2′OMe and 2′F, were largely resistant to be cleaved by all strains, emitting barely any fluorescence in any of the FRET experiments. In that respect, we conclude that the FRET-dTdT probe was the only NAOP specifically digested by the S. aureus bacterial supernatant and pure MNase, with ARFs in comparison with the control buffer of 22.6 (±1.3) and 29.7 (±1.6), respectively. These results are in good agreement with our previous findings, confirming a highly efficient and specific degradation of the FRET-dTdT probe (reported previously as the TT probe).13 As required for the specific detection criterion, the FRET-dTdT probe withstood digestion by all other bacterial strains, showing insignificant ARF fluorescence values of 2.5 (±0.2), 1.1 (±0.1), 1.1 (±0.1), and 1.3 (±0.1) when incubated with bacterial supernatants of P. mirabilis, K. pneumoniae, P. aeruginosa, and S. pneumoniae cultures, respectively.

3.2. Identification of the Cleavage Site(s) in the dTdTProbe by MS. The cleavage site(s) and origin of the great selectivity and efficiency of the hydrolysis of the described FRET-dTdT probe by S. aureus MNase are summarized in the following lines, contributing therefore key knowledge for the improvement of the design of NAOPs that will specifically and efficiently target the detection of this or other specific nuclease activity. With that purpose, the degradation products (oligonucleotide fragments) generated upon the incubation of MNase with the nude-dTdT probe were identified by MALDI-TOF spectrometry and virtual docking experiments.

Two alternative constructs of the said FRET-dTdT probe were included in the study described below, that is, with (the FRET-dTdT probe) and without (the nude-dTdT probe) the
FAM fluorophore at the 5′-end and the TQ2 quencher at the 3′-end, respectively. The FAM and TQ2 labels, necessary to detect and quantify the digestion of the probe in the FRET experiments, were first proven not to alter the cleavage site(s) or reaction efficiency. In this regard, upon incubation with MNase, digestion products of both the nude-dTdT probe (MW: 3500.15 Da) and the FRET-dTdT probe (MW: 4673.33 Da) were analyzed by means of MALDI-TOF experiments (Figures 4 and S2), which revealed that both probes undergo the same two scissions and generate equivalent oligonucleotide fragments. In the case of the nude-dTdT probe, the starting construct of 3500.15 Da (subpanel in Figure 4) is cleaved into two detectable fragments (Figure 4), with masses m/z: 1356 and 1878 Da, compatible with fragments S′-mCmUmCmG and dTmCmGmUmUmC-3′, respectively. Also, a signal consistent with dTdTmCmGmUmUmC-3′ was found as a minor product (2202 Da). As the first attempt, these fragments obtained imply that the cleavage occurs at the 5′ thymine position. Sequence S′-mCmUmCmGdT was not observed under these reaction conditions. Thus, a third, low-mass (322.21 Da), and nondetectable fragment corresponding to single dT−OH is expected to be a product of the nuclease activity on the nude-dTdT probe as well (note that the S′-3′-nuclease activity of MNase promotes the cleavage of phosphodiester groups, generating the 3′-hydrolyzed phosphate ends (−PO₄H₂) and the protonated S′-oxygen atoms in the leaving fragment). Analogous results were obtained in experiments run with the FRET-dTdT probe (see Figure S2), confirming the existence of two cleavage sites, and evidenced that the fluorophore and quencher used in the FRET experiments located at the 5′- and 3′-ends of the oligonucleotide sequences, respectively, do not alter the cleavage pattern and presumably the cleavage efficiency.

As proposed in Scheme 1, these results are compatible with two alternative mechanisms for double digestion of the probes by MNase, that is, either an initial hydrolysis of the mGdT phosphate bond followed by the cleavage of the key dTdT nucleotide pair in the dTdTmCmGmUmUmC-3′ fragment (route I) or, alternatively, an initial hydrolysis of the dTdT pair followed by the hydrolysis of the phosphate group linking together the mGdT pair (route II).

The actual route for the double cleavage mechanism was unequivocally identified to be both, in parallel, starting by route II and followed by route I and II (see Scheme 1).

3.3. Unraveling the Double Cleavage Mechanism. The proposed double cleavage mechanism of the FRET-dTdT probe was further investigated by means of virtual docking as well as UPLC experiments. Virtual docking experiments were performed with shortened molecular models of the nude-dTdT probe, consisting of four pentameric oligonucleotide probes, pent#1 to pent#4, (see Figure S5, Table 2, and Supporting Information Figures S6 and S7 for additional details). The latter were tailored to address several important questions in the field of the design and development of oligonucleotide-based biosensors: (a) find the minimum substrate necessary to carry out the biosensing action with both specificity and efficiency; (b) improve the physicochemical properties that may favor a larger biodistribution in potential in vivo applications; and (c) decrease the entropy/degrees of freedom of the probe, which would simultaneously favor the receptor binding process/energetics in both experimental and virtual docking studies. Also, the performance of experiments with shortened sequences of the nude-
dTdT probe will benefit from the docking of ligands having less degrees of freedom.

The design of the pentamers consists of four truncated versions of the nude-dTdT parental probe, having dTdT at different positions, as follows: pent#1 with dTdT at positions 5–9; pent#2 at positions 4–8; pent#3 at positions 3–7; and pent#4 at positions 2–6. As discussed below, pent#3 and pent#4 were experimentally proven to serve as appropriate molecular models of the parental nude-dTdT probe in terms of cleavage site identification, whereas pent#1 and pent#2 were not, being later characterized as oligonucleotides lacking the MNase recognition site.

The selection/identification of the most appropriate model of the nude- or FRET-dTdT probe to be used with docking purposes was carried out by incubating the pentamers with S. aureus MNase and analyzing their digestion products by means of UPLC–MS (Figure 6). The latter experiments revealed, for instance, that pent#1 undergoes one single and yet poor phosphate bond hydrolysis under the reaction conditions used (Figure 6a). Briefly, pent#1 yielded small quantities of fragments 5′-dTdT and mC-mG-mU-3′, yet the native nondigested pentamer was largely found on the analyzed digestion mixture, suggesting that the cleavage efficiency was relatively low. Interestingly, pent#2 was found to be completely resistant to digestion (Figure 6b), while pent#3 and #4 were largely digested by MNase (Figure 6c,d), and they both underwent two equivalent scissions, as did the original nude-dTdT probe: cleavage at the 5′-side deoxythymidine position, yielding fragments 5′-mC-mG and dT-dT-mC-3′ in the case of

Table 2. Sequences of the Pentameric Probes Tested

| probe | sequence |
|-------|----------|
| pent#1 | 5′-dT-dT-mC-mG-mU-3′ |
| pent#2 | 5′-mG-dT-dT-mC-mG-3′ |
| pent#3 | 5′-mC-mG-dT-dT-mC-3′ |
| pent#4 | 5′-mU-mC-mG-dT-dT-3′ |

Figure 5. Chemical structure and molecular weight (MW, Da) of the four pentamers derived from the nude-dTdT probe: (a) pent#1, (b) pent#2, (c) pent#3, and (d) pent#4.

Figure 6. UPLC chromatogram of the four pentamers and digestion products after 1 h incubation time with MNase: (a) pent#1; (b) pent#2; (c) pent#3; and (d) pent#4 (see Figure S6). The dT nucleotide is colored orange in each probe’s digestion fragment.
pent#3, and subsequent hydrolysis of the dTdT phosphate bond, returning fragments dT-mC-3' and dT [phosphate-bearing dT(PO4H2)]. Likewise, in the case of pent#4, the first cleavage yielded fragments 5'-mU-mC-mG and dT-dT-3', while the latter fragment returned two deoxythymidine bearing dT(PO4H2). Likewise, in the case of pent#4, the first cleavage yielded fragments 5'-mU-mC-mG and dT-dT-3', while the latter fragment returned two deoxythymidine monomers after the second hydrolysis [dT and dT(PO4H2)].

Since these results were equivalent to those obtained with the native nude-dTdT probe, pent#3 and pent#4 were identified as appropriate molecular models and therefore used in virtual docking experiments as substrates to be docked to the enzyme, which shed light into the binding mode and conformational properties of the catalytic complex. Pent#2 was also included in the virtual study as a negative control of the cleavage process.

Thus, flexible docking of pent#2, pent#3, and pent#4 to the enzyme structure (see the Experimental Section and Supporting Information) was carried out, which generated a large variety of poses (snapshots of alternative binding modes of the ligand—receptor pair), derived from the large number of degrees of freedom still present in the pentameric probes. Poses were analyzed and classified as catalytically relevant or irrelevant depending on the presence or absence in the generated complex of certain key interactions between the probe and the enzyme, which were identified by Cotton et al. in previous reports on the phosphodiester bond hydrolysis mechanism (see the Experimental Section and Supporting Information, Figure S5). The application of these criteria allowed the identification of catalytically meaningful poses in the case of pent#3 and pent#4, but no meaningful poses were found for pent#2. Thus, Figure 7 shows the ligand interaction diagrams of the selected poses of pent#2, pent#3, and pent#4, where the phosphate group preceding the dTdT pair in pent#3 and pent#4 coordinates to the central cation, and simultaneously, all other relevant interactions with the key reported residues in the active site of the enzyme were also established. Interestingly, no catalytically relevant pose was identified for the case of pent#2; multiple poses of this pentamer showed phosphate group coordination to the central calcium cation, but they could not be considered as relevant due to the absence of other catalytically required interactions.

A closer look at the binding modes of pent#2, pent#3, and pent#4 to MNase revealed that the recognition of mG by means of its insertion in a pocket flanked by residues GLU43 and ASP19 may facilitate the coordination to the Ca2+ cation (see Figures S4—S8 in the Supporting Information). The large affinity of the said pocket toward mG is also observed in pent#2, which is the only pentamer with two mG nucleotides and actually accommodates 5'-mG in the identified pocket instead of mG-3. The latter mG insertion promotes an alternative binding mode and conformation of the pentamer (as compared to pent#3 and pent#4), which translates into noncatalytically relevant poses. Therefore, the presence of the mCmGdT tandem in the pentamer (and the original

Figure 7. Surface representation (top) and ligand interaction diagram and ΔG binding energies (bottom), respectively, of catalytically nonrelevant poses of pent#2 (a,d) and catalytically relevant poses for pent#3 (b,e) and pent#4 (c,f). Red circles highlight the phosphate group coordinated to the calcium cation. The colors indicate the residue (or species) type: red—acidic (Asp and Glu); green—hydrophobic (Ala, Val, Ile, Leu, Tyr, Phe, Trp, Met, Cys, and Pro); purple—basic (Hip, Lys, and Arg); blue—polar (Ser, Thr, Gln, Asn, His, Hie, and Hid); light gray—other (Gly and water); and darker gray—metal atoms. Interactions with the protein are marked with lines between ligand atoms and protein residues: solid pink—H-bonds to the protein backbone; dotted pink—H-bonds to protein side chains; green—π–π stacking interactions; and orange—π–cation interactions.
dodecamer probe) seems to be crucial for the proper recognition and posterior hydrolysis of the dTdT phosphate bond; that is, the mCmG nucleotide pair sitting by the dTdT dimer is responsible for improving the cleavage performance and specificity of MNase, which implies that at least two chemically modified nucleotides are needed at the 5′-thymine cleavage site. Alternatively, poses obtained with pent#2, where the phosphate group between both dT nucleotides was coordinated to Ca2+, lacked some or all of the catalytically relevant interactions, and this pentamer was therefore not considered further as an appropriate molecular model of the nude-dTdT probe. Even so, pent#2 was used as a negative control in the virtual docking experiments described below, that is, as a ligand that should not yield positive docking results.

To find out more information about the catalytic hydrolysis and elucidate important details of its catalytic order of action, UPLC kinetic experiments of the incubation of MNase (0.1 U/μL) with pent#4 (5 pmol/μL) were carried out in an 80 min reaction (Figure 8 and Supporting Information Table S2). A total of 16 UPLC chromatograms were acquired, derived from an injection volume of 10 μL every 6.66 min. UPLC chromatograms at four representive incubation times (t = 0, t = 7, t = 28, and t = 80 min) shown in Figure 8a−d revealed a double cleavage process started by pent#4, as concluded by the transient detection of fragment mUmCmGdT at t = 5 min (Figure 8e).

This intermediate is present during the first few minutes, which was completely digested by t = 20 min into fragments mUmCmG (Figure 8f) and 3′-phosphate deoxythymine dT (see a complete chromatogram in Figures S20−23). Simultaneously, the native pentamer (Figure 8g) was digested into fragments mUmCmG (Figure 8f) and the dTdT pair (Figure 8h).

The time evolution of four of the five fragments generated during the reaction (Figure 8e−h) allowed us to elucidate the catalytic cycle (see Figure 8i) of the reaction of S. aureus MNase on pent#4. This reaction implies a double hydrolysis mechanism, starting with dTdT pair hydrolysis in the first instance and followed by mGdT pair hydrolysis in the second (see Table S2).
Moreover, we have confirmed experimentally that FRET-pent#4 (fluorophore-labeled pent#4) maintains the detection capabilities of the parental FRET-dTdT probe (Figure S25). Importantly, we have also observed a significant improvement in the ARF of FRET-pent#4 [125.7 (±2.73)] over that of the parental FRET-dTdT probe [27.5 (±1.16)], indicating that more efficient fluorescence quenching was obtained by this truncated version (Figure S26).

Our findings are summarized in Figure 9, where general pictures of the nude-dTdT probe in complex with MNase before and after hydrolysis are represented.

4. CONCLUSIONS
In summary, we confirm the specificity and selectivity of the dTdT nuclease activatable oligonucleotide probe to specifically detect S. aureus based on its MNase activity; also, we describe for the first time the full hydrolysis process undergone by the full-length probe, which implies double subsequent cleavages at the 5′-side position of both dT nucleotides, starting between the dTdT dimer and followed by the 5′-mG-dT cleavage. Appropriate molecular pentameric models of the nude-dTdT probe and a fluorophore-labeled pentamer (FRET-pent#4) were designed and also validated experimentally, and their virtual binding mode was used to identify key catalytic interactions with the calcium cation, ARG35, ARG87, and a catalytic water molecule H-bonded to residue GLU43. Docking experiments pointed to a particular pair of modified nucleotides (mCmG) at the 5′-side of the nuclease’s cleavage site as necessary for probe recognition and binding. Specifically, the insertion of mG in a pocket flanked by residues GLU43 and ASP19 was found to be critical to trigger the subsequent hydrolysis of the probe at the active site pocket of MNase. These results represent an important step forward in the understanding of the mode of action, specificity, and efficiency of NAPOs in the context of nuclease activity detection and diagnosis, as well as the first description of the plausible truncation and reduction in size to a minimum of five nucleotides of an efficient oligonucleotide substrate.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00884.

Chemical structure of the FRET-dTdT probe, details on FRET assays, MALDI-TOF experiments, phosphate hydrolysis mechanism, virtual docking experiments, UPLC experiment results, mass spectra, and details related to the catalytic cycle of the cleavage process proposed, pent#4’s FRET assays and activation ratio (PDF)

SMILES formula of the nude-dTdT probe and pentamers pent#1−pent#4 (CSV)

Docking models of the binary complexes between 1SNC and pent#1−pent#4 (PDB)

Docking models of the binary complexes between 1SNC and pent#2 (PDB)

Docking models of the binary complexes between 1SNC and pent#3 (PDB)

Docking models of the binary complexes between 1SNC and pent#4 (PDB)

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Author Contributions
T.J. and J.B. contributed equally. All authors have given approval to the final version of the manuscript.

Notes
The authors declare the following competing financial interest(s): The authors T.J. and F.J.H. are inventors in a patent that describes the use of oligonucleotides for targeting nucleases. SOMAprobes is a company that develops specific oligonucleotide probes for diagnostics. No other competing interests are declared.

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ACRONYMS AND ABBREVIATIONS
A, Agar; ATCC, American Type Culture Collection; a.u., arbitrary units; dT, deoxythymidines; FAM, fluorescein amide; FRET, fluorescence resonance energy transfer; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; UPLC-MS, ultra-performance liquid chromatography mass spectrometry; ESI-TOF, electrospray ionization time-of-flight; MALDI-TOF-MS, matrix-assisted laser desorp-
tion/ionization-time-of-flight mass spectrometry; MNarr, micrococcal nuclease; NA, nutrient agar; NAA, nuclease activity assay; NAOPs, nuclease-activatable oligonucleotide probes; NB, nutrient broth; SAR, sequence/structure–activity relationship; S. aureus, Staphylococcus aureus; THB, Todd Hewitt broth; TEP, thymidine-3′,5′-diphosphate; TQ2, tide quencher 2; UPLC-MS, ultra-performance liquid chromatography mass spectrometry; TSB, Trypticase soy broth; YE, yeast extract; XP, extra precision

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