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Semi-artificial Fluorescent Molecular Machine for DNA Damage Detection

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Abstract

The design of artificial molecular machines is complicated because the mechanics used in macromachines is not readily adaptable for nano environments. We constructed a semi-artificial molecular device, which contains a naturally occurring molecular machine—a vaccinia virus encoded protein—linked with an artificial part. The self-assembled construct makes two fluorescently labeled detector units. It is the first sensor capable of selectively detecting different types of DNA breaks, exemplifying a practical approach to the design of molecular devices.

Introduction

Multiple naturally occurring molecular machines operate in every living cell performing critical tasks.^{1–6} A molecular machine can be defined as a structural fabrication, commonly an assembly of polymeric molecules that can convert energy or information from one form or location to another.³ Generally it is a macromolecule or macromolecular complex, which performs a specific and useful function acting locally as an individual; energy for a machine is taken from various physical sources (photons, thermal motions, etc.) and can be used or conserved by forming and disrupting chemical bonds.^{4,5}

The naturally occurring molecular machines include a number of enzymes, such as polymerases and topoisomerases,^{7,8} as well as nonenzymatic natural cellular components. They range from very simple, such as a denaturing–reassembling DNA duplex or a restriction enzyme–DNA complex,⁴ to the most intricate, such as a ribosome.⁶

Research in the area of artificial molecular machines has resulted in creation of a variety of imaginative artificial molecular devices;^{9–13} however, no practical molecular machines have been introduced. Although the naturally occurring molecular machines display a variety of designs,^{1–6} the major effort in the development of artificial molecular machines has concentrated on the design of molecular devices capable of converting absorbed energy into directional motion, i.e., molecular motors, as well as on their scaled-down mechanical parts (wheels, axels, pendants, etc).^{13–15} These developments emulate approaches used in the design of macromachines, even though such physical properties of macrosystems as inertia and momentum conservation are not readily usable in the nanoworld environments because of Brownian motion interference.⁶ The difficulties in adapting the macromachine designs to the very different conditions of the nanoscale environment can explain why no practical molecular machines have been introduced so far. Alternative designs, which do not rely on unidirectional

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molecular motors for their performance and do not emulate mechanical macromachines, could be simpler and easily adaptable to the specific conditions of the nanoworld. The most practical approach is to use naturally occurring molecular machines as parts of the new designs by combining them with artificial components. Such combinations would result in semi-artificial molecular devices. Although cellular enzymes, such as polymerases, restrictases, and topoisomerases, which function as molecular machines, are widely used in biochemistry and molecular biology, they are not linked with artificial components into the stable molecular-size contraptions. Therefore, in their conventional applications they cannot be viewed as semi-artificial molecular machines. Here we present the first useful semi-artificial molecular machine. Our molecular device is designed and assembled as an autonomous nanoscale sensor, which is made of a fluorescently tagged oligonucleotide and a protein (topoisomerase) molecule. It selectively labels two major types of DNA breaks and can be used as a DNA damage sensor working in solution and in fixed tissue sections.

Methods

Oligonucleotides

The oligonucleotides were prepared by Synthetic Genetics, San Diego, CA.

Oligo 1 (double-hairpin vaccinia topoisomerase I cleavable oligonucleotide, single fluorescein labeled (VACC – RC):

5'-AAGGGACCTGCGCAGGTCCCTTAA-CGCATATGCGTT-3'

Oligo 2 (double-hairpin vaccinia topoisomerase I cleavable oligonucleotide, dual labeled with fluorescein and rhodamine (VACC – RC DUAL):

5'-AAGGGACCTGCGCAGGTCCCTTAA-CGCATRATGCGTT-3';

F: FITC-dT, **R:** Tetramethylrhodamine-dT;

Test Oligo 3 (hairpin for reaction testing in solution as the source of blunt ends):

5'-GCGCTAGACCTGGTCTAGCGC-3';

Oligo 4 (blunt-ended rhodamine-labeled hairpin probe for in situ ligation with T4 DNA ligase)¹⁶:

5'-GCG CTA GAC **CRG** GTC TAG CGC-3';

R – Tetramethylrhodamine-dT.

Apoptotic Thymus

Sprague–Dawley rats (150 g) were injected subcutaneously with 6 mg/kg dexamethazone (Sigma) dissolved in 30% dimethyl sulfoxide in water. Animals were sacrificed after 24 h and the thymus was fixed in 4% paraformaldehyde. After incubating 18 h in paraformaldehyde, the tissue fragments were placed in 70% ethanol and then taken through graded alcohols to 100% ethanol. They were placed overnight in chloroform and embedded in paraffin.

Vaccinia Topoisomerase I

Vaccinia DNA topoisomerase I was expressed in *E. coli* and purified as described.¹⁷ The commercial enzyme preparation used in our initial studies was purchased from Epicenter (Madison, WI) but had lower and inconsistent activity.

Vaccinia Topoisomerase I-Based Labeling of DNA Ends in Solution

Reaction mixture (10 μ L) containing 50 mM Tris-HCl (pH 7.4), 100 pmol of oligo 1 (VACC-RC) or oligo 2 (VACC-RC DUAL), 100 pmol of test oligo 3, 150 pmol (1.2 μ g/ μ L) vaccinia topoisomerase I was incubated for 1 h at 37°. The samples were electrophoresed through a 20% polyacrylamide gel for 90 min at 180 V and stained with SYBR Green (Molecular Probes).

Vaccinia Topoisomerase I-Based Labeling of DNA Ends in Tissue Sections

6 μ m thick sections of the normal bovine adrenal gland were deparaffinized with xylene, rehydrated in graded alcohol concentrations, washed in water, and treated with proteinase K (50 μ g/mL) for 15 min at room temperature (23°C). Sections were washed in water (2 $\times$ 10 min), and treated with 100 ng/ μ L of DNase I (Roche, Indianapolis, IN) in 50 mM Tris-HCL, pH 7.4, 10 mM MgCl₂ overnight at 37 °C, or with 500 ng/ μ L DNase II in the buffer supplied with the enzyme (Sigma Chemicals, St. Louis, MO) for 30 min at 37 °C. Sections were washed in water (3 $\times$ 10 min), and preblocked with 2% BSA (15 min, 23 °C). The blocking solution was aspirated, and the reaction mixture (25 μ L) containing 50 mM Tris-HCL, pH 7.4, 100 pmol oligo 1 (VACC-RC), 15% PEG-8000, 215 pmol vaccinia topoisomerase I was applied to sections. The sections were incubated 1 h at 37 °C in a humidified chamber, washed in water (3 $\times$ 10 min), then T4 DNA ligase-based labeling was performed in the same tissue sections, as previously described.¹⁶ The sections were rinsed with sodium bicarbonate, covered with Vectashield with DAPI (Vector Laboratories, Burlingame, CA), and cover-slipped.

Combined Vaccinia Topoisomerase I–T4 DNA Ligase-based Labeling of DNA Ends in Tissue Sections

6 μ m thick sections of dexamethazone-treated rat thymus were deparaffinized with xylene, rehydrated in graded alcohol concentrations, washed in water, and treated with proteinase K (50 μ g/mL) for 15 min at room temperature (23 °C). After two 10 min washes in water, the sections were blocked with 2% BSA for 15 min at 23°C. The solution was aspirated and the reaction mix (25 μ L) containing 66 mM-Tris HCl, pH 7.5, 5 mM MgCl₂, 0.1 mM dithioerythritol, 1 mM ATP, and 15% poly(ethylene glycol)-8000, 70 pmol (900ng) oligo 2 (VACC-RC DUAL), 215 pmol vaccinia topoisomerase I, and 10 units T4 DNA ligase (500 U/mL) was applied to the sections. The sections were incubated in a humidified box (1 h, 23 °C). They were then briefly washed in water. Although both ligase and topo signals were clearly observed at this stage, the ligase signal was further enhanced by applying the reaction mix, without vaccinia topoisomerase and VACC-RC DUAL, but containing oligo 4 (blunt-ended hairpin) (35 mg/mL) and T4 DNA ligase (250 U/mL). Sections were incubated in a humidified box (16 h, 23 °C). They were then briefly washed in water. Sections were then counterstained with 4,6-diamidino-2-phenylindole (DAPI).

In a mock reaction, no signal was observed when an equal volume of 50% glycerol in water was substituted for T4 DNA ligase, and vaccinia topoisomerase I was deactivated by preincubation with 4 μ g of proteinase K for 10 min at 23 °C. Proteinase K was subsequently inactivated by boiling. Control sections of normal bovine adrenal and normal rat thymus were unstained after ligase-mediated labeling (beyond a couple of apoptotic cells in the thymus), as we have discussed elsewhere.¹⁸ No signal was detected in the control series, after the pretreatment with 215 pmoles of vaccinia topoisomerase I for 2 h at 37 °C.

Images were captured using an Olympus IX-70 fluorescent microscope and a MicroMax digital videocamera (Princeton Instruments, Inc.). Composite images were created in MetaMorph 4.1 (Advanced Scientific, Inc.). (A Chroma Technology band-pass filter set was used: FITC excitation D490/40, emission 520/10; rhodamine excitation D560/40, emission 620/30; DAPI excitation D360/40, emission 460/20.)

Results and Discussion

The Principle of the Design

Our molecular device is made by combining two molecules. (1) A self-complimentary dual-labeled 38-mer oligonucleotide. The oligonucleotide contains a CCCTT3' vaccinia topoisomerase I recognition sequence located adjacent to the nick formed by folded 3' and 5' ends (Figure 1). (2) A vaccinia DNA topoisomerase I molecule, which is a virus-encoded eukaryotic type IB type topoisomerase.¹⁹

The molecular device operates at room temperature and at neutral pH. To detect two types of breaks it requires the presence of ATP, Mg²⁺, and T4 DNA ligase; otherwise, it detects a single type of DNA break (bearing 5'OH).

In operation, our molecular-size construct uses its own material to make two detector units that undergo cycles of separation–rejoining until they encounter their target DNA breaks. The construct cannot actively propel itself or its working parts, therefore it is not a nanomechanical device of a type preferentially discussed in the literature. Its design exemplifies principles that we consider instrumental for the development of future useful molecular-level devices.

The machine goes through the following cycle of transitions (Figure 1):

1. Self-assembly. The topoisomerase molecule noncovalently binds to the CCCTT3' recognition sequence located in the central part of the oligonucleotide and occupies nucleotides from –9 to +13 (in relation to the nick position+1) and 13 nucleotides on each side of the nick on the opposite strand.²⁰ The specific binding of the enzyme to the CCCTT motif places it into a lower energy state and it remains in place until it has cut the DNA.
2. Fabrication of two fluorescent detector parts. The machine operation starts when the topoisomerase molecule, bound to the oligonucleotide, creates an additional nick at the 3' end of the CCCTT3' recognition sequence.²¹ This strand cleavage leads to creation of two separate blunt-ended hairpins, which will dissociate from each other. The cleaved phosphodiester bond energy is conserved by formation of a covalent link between the 3' phosphate of the cut DNA strand and a tyrosyl residue of the enzyme (Tyr-274).²²
3. Idling. The free energy gain for the breakage reaction is small, on the order of +1 kcal/mol, making the reaction freely reversible.²³ Therefore, vaccinia topoisomerase, which remains bound to the CCCTT motif located on the downstream hairpin, will religate it back to the end of the upstream hairpin. This leads to cycles of self-attachment–disattachment of detector units as thermal motion randomly separates and brings together enzyme-bearing and enzyme-free hairpins, which are repeatedly religated and then recut by topoisomerase.
4. Labeling of DNA breaks. In the presence of another acceptor, such as a blunt-ended DNA break with 5'OH, the topoisomerase-carrying hairpin will ligate to it instead and will fluorescently label it. Blunt-ended oligonucleotide duplexes, similarly activated by vaccinia topoisomerase, were shown to specifically ligate to the blunt-ended acceptor.²⁴ In principle, the hairpins with 3' or 5' overhangs can also be produced by the same approach for detection of staggered DNA ends shown specific for apoptotic but not necrotic DNA cleavage.¹⁸ However, blunt ends are the most advantageous targets for detection in cellular DNA, as they represent the most frequent type of ds DNA breaks in apoptosis.²⁵ Therefore, the machine design was modified to detect these DNA breaks.

5. The second enzyme-free hairpin formed after oligonucleotide cleavage will ligate to a 5'PO₄ blunt-ended break, if T4 DNA ligase is present in the solution.^{26,27} When T4 DNA ligase is added to a tissue section or a solution containing DNA with 5' PO₄ blunt-ended breaks, the ligase reacts with 5'PO₄ DNA ends, forming semi-stable enzyme–DNA complexes. The blunt ended hairpins will interact with these complexes releasing ligase and covalently linking hairpins to DNA, thus labeling 5' PO₄ blunt-ended DNA breaks.
6. Self-disassembly. Topoisomerase separates from the oligonucleotide or ligated hairpin.
7. Repeat self-assembly and repetition of all subsequent cycles.

The cleavage of a 37-mer oligonucleotide and religation of the enzyme-bearing detector unit to a blunt-ended acceptor with a 5'OH end was verified in solution (Figure 2a), and then the molecular device bearing a single fluorophore was tested in tissue section format detecting DNase II but not DNase I cuts (Figure 3a).

The dual-labeled construct that bears fluorophores on both hairpins was tested in solution and has successfully detected a blunt-ended 21-mer with 5'OH ends (Figure 2b). Gel electrophoresis of the solution containing the operating constructs permitted observation of the separate detector units seen as red or green fluorescent bands (Figure 2b). The dual-labeled machine performance was then examined in tissue section format in sections of dexamethazone-treated rat thymus. It simultaneously detected the primary DNase I-like cleavage in apoptotic thymocyte nuclei and DNase II-like breaks in the cytoplasm of cortical macrophages, ingesting apoptotic cells (Figure 3b).

The primary type of DNA cleavage in dexamethazone-treated thymus has been determined to be of DNase I type.²⁸ DNase II type breaks generated during lysosomal digestion of phagocytized apoptotic cells were known to exist but have not been detected before using biochemical bulk DNA purifications.^{28,29} Our molecular construct labels individual breaks in situ and can visualize both DNase I- and II-type breaks even in small numbers of cells and thus increases the sensitivity of DNA damage detection.

The new molecular device places a single FITC fluorophore at the end of each DNA break. Therefore, in a confocal microscope setting detection of DNA breaks on a level of a single break is possible.³⁰ The fluorescent signal intensity is directly proportional to the number of breaks because a single fluorophore corresponds to a single break, therefore the dynamic of DNA break generation can be analyzed in such a setting.

In preliminary experiments we found that the sensitivity of the system can be increased several orders of magnitude by substituting fluorophores with biotins and visualizing the results of the reaction using an avidin–peroxidase system (TSA system from PerkinElmer Life Sciences, Boston, MA) or by adding longer tails of fluorescent DNA (not shown). However, we consider such substitutions redundant and impractical for the most widely used task of apoptosis detection, which can be achieved using a regular fluorescent microscope. The number of breaks in apoptosis rises from about 50 000 per genome at the initial high molecular weight DNA degradation at the earliest stages of apoptosis to 3×10^6 during later internucleosomal DNA fragmentation stage.³¹ Using a dot spot test we showed that a nonconfocal fluorescent microscope optical system (Olympus IX-70 microscope with MicroMax digital videocamera) can visualize 1.25 fmole of FITC spotted as a 1.3 mm dot, which corresponds to ~45 000 FITC molecules per the surface area occupied by a nucleus 0.01 mm in diameter (Figure 2c). Therefore, using a single fluorophore-carrying molecular construct allows visualization of the full spectrum of breaks present in apoptosis, from the initial early stage to the massive DNA cleavage stage. The fact that both DNA breaks of DNase I type (bearing 5' PO₄) and DNase

II type (bearing 5' OH) are selectively labeled is advantageous over the currently used TUNEL assay (terminal transferase based labeling). TUNEL cannot detect DNase II-type breaks at all,³² although this type of DNA cut represents a major type of DNA cleavage in apoptosis in at least some cells.^{33–35}

Any molecular machine is fundamentally required to perform a useful function.⁴ Here we present the first practical design directly applicable for the detection of DNA damage in fixed cells and tissues. From the enzymology point of view, our molecular construct is in fact a designer fluorescent DNA–enzyme complex put together to perform a specific useful function. This assembly is by definition a molecular machine, as it contains one.^{36,37} However, its artificial part (a fluorescently labeled and self-hybridized oligonucleotide) directs its performance toward a new function of DNA break labeling.

Several features keep this design simple yet functional in the nanoscale environment: (1) The machine is a nonmotor driven molecular device, although it uses the same force that drives directional Brownian ratchet mechanisms of molecular motors and pumps.³⁸ Guided directional motion is not essential for its work because random movement is an efficient way of surveying the section or a solution for DNA breaks. Our presented data show that in many instances 1 h is sufficient to complete DNA damage detection (Figure 3). (2) The construct uses both “bottom-up” and “top-down”³⁹ approaches, as it first self-assembles from two molecules and then cleaves itself into smaller detector units. This “bottom-up-top-down” strategy mimics the pathways used by enzymatic systems in vivo⁴⁰ and ensures both simple design and durable performance, as no external building blocks or guided assemblies are required for the fabrication, which is accomplished by the simple act of cutting. (3) The device does not maintain permanent structure as its parts continuously disassociate and reassociate, therefore it does not require a “stiff” frame⁴¹ to be able to fabricate its working units and to withstand persistent molecular collisions.

Our molecular construct relies on mechanisms that are unique for the nanoenvironment; therefore, unlike “scaled-down” machines, it cannot be “scaled-up”. Although the “scaled-down” approach, which straightforwardly extends the macroscopic concepts of machine motors and parts such as wheels, axles, belts, etc. to the nanoscale constructs,¹⁴ has its place and might in the future result in the development of useful molecular machines, our molecular construct demonstrates that molecular devices that use mechanisms developed in the evolution of biological molecules, are simpler and uniquely suitable for nanoscale environments. The described construct can be used for apoptosis and DNA damage detection in solution and in fixed cells and tissues.

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Figure 1.

Light-emitting molecular machine and its usage for DNA damage detection in situ. The machine self-assembles when vaccinia topoisomerase I (TOPO) binds to the double-hairpin oligonucleotide. The machine operation starts when TOPO cleaves the oligonucleotide at the 3' end of the recognition sequence. As a result the TOPO-activated FITC-labeled portion of the oligonucleotide continuously separates and religates back to the rhodamine-labeled part. When other acceptors, such as blunt-ended DNA breaks with 5'OH are present in tissue section, the FITC labeled part will attach to them. If T4 DNA ligase is present in reaction solution, it will ligate the remaining rhodamine-labeled part to DNA blunt ends with 5' PO₄. Therefore, both types of DNA breaks are detected simultaneously and directly.

Figure 2.

Detection of blunt-ended 5'OH bearing DNA ends in solution using fluorescent molecular machines. (a) The double-hairpin FITC-labeled oligonucleotide 1 (lane 1) was treated with vaccinia topoisomerase (lane 2) for 1 h at 37 °C, resulting in cleavage into two parts: 14-mer and FITC-labeled 23-mer. The cleaved FITC-labeled part of the oligo with attached topoisomerase (23-mer+TOPO-I) did not enter the 20% acrylamide gel and is located in the well (bright green FITC fluorescence); the cleaved unlabeled part of the oligonucleotide 1 (14-mer) is stained by SYBR Green (dark green fluorescence); the unreacted oligonucleotide without attached topoisomerase (37-mer) is FITC-labeled. The addition of the 21-mer acceptor hairpin, with 5'OH blunt-ends (lane 3) results in appearance of the FITC-labeled 44-mer, due to ligation to the 23-mer TOPO-activated oligo. (b) The 38-mer double-hairpin dual-labeled with FITC and rhodamine (lane 1) reacted with vaccinia topoisomerase for 1 hr at 37 °C and was treated with proteinase K (4 μ g, 10 min at 37 °C) to remove the enzyme (lane 2), vaccinia topoisomerase cleaved the double-hairpin producing 23-mer FITC-labeled and 15-mer rhodamine-labeled hairpins; the addition of the 21-mer unlabeled acceptor with 5'OH blunt ends prior to proteinase K treatment results in ligation producing the FITC-labeled 44-mer (lane 3). (c) Graded dilutions of a 37-mer oligonucleotide, labeled with a single FITC fluorophore, were spotted onto nylon membrane in 0.3 μ L aliquots (spot diameter $\sim$ 1.3 mm). Images were taken using an Olympus IX-70 microscope and a MicroMax digital videocamera. 1.25 fmol (1.23×10^{-15} mol) of FITC in a spot corresponds to 45×10^3 FITC molecules/per the surface area occupied by a cell nucleus 0.01 mm in diameter (1 mm grid shown in red).

Figure 3.

DNA damage detection in tissue sections using molecular machines: 5'OH blunt-ended DNA breaks detection (a) dual detection of 5'OH and 5'PO₄ blunt-ended DNA breaks (b). (a) DNase I and DNase II treated sections. Sections of the normal bovine adrenal were treated with either DNase I, producing 3'OH/5'PO₄ blunt-ended breaks or DNase II, producing 3' PO₄/5' OH blunt-ended breaks. The vaccinia topoisomerase I-based molecular machines bearing single fluorophore and T4 DNA ligase selectively labeled only one type of DNA breaks each. Bar = 25 μ m. (b) Dexamethasone-treated apoptotic rat thymus. Simultaneous dual detection of two types of cuts in the thymic cortical areas undergoing apoptosis using molecular machines bearing two fluorophores. Cortical macrophages with engulfed nuclear material (green

fluorescence) and apoptotic thymocytes (red fluorescence) are labeled. Green cytoplasmic fluorescence reveals lysosomes containing DNA with 5'OH double-strand breaks. Surrounding thymocytes undergo apoptosis and have 5'PO₄ double-strand breaks, located at the periphery of their nuclei (red fluorescence). The lower image was additionally counterstained with DAPI to visualize cell nuclei (blue fluorescence). Red, ligase-based detection; green, topoisomerase-based detection; blue, DAPI staining of cellular DNA. Bar = 15 μ m.