Video Article

In vitro Assembly of Semi-artificial Molecular Machine and its Use for Detection of DNA Damage

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Abstract

Naturally occurring bio-molecular machines work in every living cell and display a variety of designs [1-6]. Yet the development of artificial molecular machines centers on devices capable of directional motion, i.e. molecular motors, and on their scaled-down mechanical parts (wheels, axels, pendants etc) [7-9]. This imitates the macro-machines, even though the physical properties essential for these devices, such as inertia and momentum conservation, are not usable in the nanoworld environments [10]. Alternative designs, which do not follow the mechanical macromachines schemes and use mechanisms developed in the evolution of biological molecules, can take advantage of the specific conditions of the nanoworld. Besides, adapting actual biological molecules for the purposes of nano-design reduces potential dangers the nanotechnology products may pose. Here we demonstrate the assembly and application of one such bio-enabled construct, a semi-artificial molecular device which combines a naturally-occurring molecular machine with artificial components. From the enzymology point of view, our construct is a designer fluorescent enzyme-substrate complex put together to perform a specific useful function. This assembly is by definition a molecular machine, as it contains one [12]. Yet, its integration with the engineered part - fluorescent dual hairpin - re-directs it to a new task of labeling DNA damage[12].

Our construct assembles out of a 32-mer DNA and an enzyme vaccinia topoisomerase I (VACC TOPO). The machine then uses its own material to fabricate two fluorescently labeled detector units (Figure 1). One of the units (green fluorescence) carries VACC TOPO covalently attached to its 3'end and another unit (red fluorescence) is a free hairpin with a terminal 3'OH. The units are short-lived and quickly reassemble back into the original construct, which subsequently recleaves. In the absence of DNA breaks these two units continuously separate and religate in a cyclic manner. In tissue sections with DNA damage, the topoisomerase-carrying detector unit selectively attaches to blunt-ended DNA breaks with 5'OH (DNase II-type breaks)[11,12], fluorescently labeling them. The second, enzyme-free hairpin formed after oligonucleotide cleavage, will ligate to a 5'PO_4 blunt-ended break (DNase I-type breaks)[11,12], if T4 DNA ligase is present in the solution [13,14] . When T4 DNA ligase is added to a tissue section or a solution containing DNA with 5'PO_4 blunt-ended breaks, the ligase reacts with 5'PO_4 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'PO_4 blunt-ended DNA breaks.

This development exemplifies a new practical approach to the design of molecular machines and provides a useful sensor for detection of apoptosis and DNA damage in fixed cells and tissues.

Video Link

The video component of this article can be found at http://www.jove.com/video/3628/

Protocol

The sections for the molecular machine-based detection should be prepared first because their preparation takes more time than the assembly of the molecular device. The construct works well with 5-6µm-thick sections cut from paraformaldehyde-fixed, paraffin-embedded tissue blocks. Use slide brands which retain sections well, such as ProbeOn Plus charged and precleaned slides (Fisher Scientific) or similar. We recommend at first using a tissue with a well-known pattern of DNA damage which contains both DNase I- and DNase II-type breaks, such as dexamethasone-treated apoptotic rat thymus [13,14].

1. Preparation of sections

1. Place the sections in a slide rack and dewax in xylene for 15 min, transfer to a fresh xylene bath for an additional 5 minutes.
2. Rehydrate by passing through graded ethanol concentrations: 96% Ethanol -2x5min; 80% Ethanol - 5min; water - 2x5 min.

3. Digest section with Proteinase K. Use 100µL of a 50µg/mL solution per section. Incubate 15' at room temperature (23° C) in a humidified chamber. The time may need adjustment depending on the tissue type. Hard tissues might require longer digestion. Times of 15-25 min are usually used. Insufficient digestion may result in the weaker signal. Overdigestion on the other hand results in signal disappearance and section disruption.
4. Rinse in distilled water for 2x10 min.
5. Apply 100 µL per section of 2% BSA for preblocking. Incubate for 15 min at room temperature (23° C). During this time assemble the molecular machine.

2. Molecular machines assembly

All reagents are scaled for 25 µL total volume, which is sufficient for a single detection in an average size tissue section (10x10mm). The volume can be scaled up as needed.

1. Combine in a small plastic tube in this order:

1. Bidistilled water;
2. 15% polyethylene glycol-8000;
3. Solution of 66 mM-Tris HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM dithioerythritol, 1 mM ATP, (i.e. regular T4 DNA ligase buffer);
4. 70 pmoles of Oligonucleotide 1,
5. 215 pmoles (1.76 - 7.1 µg) VACC TOPO
6. 10 units T4 DNA ligase (500 U/mL)

2. Mix gently by pipetting. The molecular construct almost instantly self-assembles in this solution and can be used immediately at room temperature (23° C). Increasing the temperature to 37° C blocks the T4 DNA ligase-based component of the labeling.

3. Using molecular machines in tissue sections to dual label 5'OH and 5'PO4 DNA breaks

1. Aspirate the preblocking solution and apply 25µL of the full reaction mix containing 70 pmoles of Oligonucleotide 1, 53 - 215 pmoles (1.76 - 7.1 µg) VACC TOPO and 10 units T4 DNA ligase (500 U/mL) in solution of 66 mM-Tris HCl, pH 7.5, 5mM $MgCl_2$, 0.1 mM dithioerythritol, 1 mM ATP, and 15% polyethylene glycol-8000. The same sequence oligonucleotide carrying a single FITC label, Oligonucleotide 2, can be used instead for detection of a single type of DNA breaks. In such case omit ligase from the labeling reaction. Also in this situation a solution of 50mM Tris-HCL, pH 7.4, 15% PEG-8000 can be used instead of T4 DNA ligase buffer.
2. Incubate for 1 hr at room temperature (23° C) in a humidified chamber with a plastic coverslip. Protect from light. Lowering of the temperature to 16° C reduces the ligase-based signal; the temperature increase to 37° C completely eliminates the ligase-based signal. A partial inhibition of ligase sometimes occurs in the reaction mix, possibly due to contaminants introduced with topoisomerase preparations. Therefore, longer incubation (2-4 hours) might be required especially when significant numbers of ligase-labeled breaks are present. Although both ligase and topo signals can be observed at this stage, in many instances the ligase signal can be further enhanced by re-application of the reaction mix without VACC TOPO and dual-labeled Oligonucleotide, but containing a hairpin shaped oligonucleotide (Oligonucleotide 3) (35 mg/mL) and T4 DNA ligase (250 U/mL). To enhance signal proceed to Step 3, to see the reaction without enhancement go to Step 5.
3. Remove coverslips by gently immersing the slides vertically in a Coplin jar containing water at room temperature. Aspirate excess water.
4. Enhance ligase signal by applying 25 µL of reaction mix without VACC TOPO and Oligonucleotide 1, but containing Oligonucleotide 3: 66 mM-Tris HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM dithioerythritol, 1 mM ATP, and 15% polyethylene glycol-8000, 5 units T4 DNA ligase, 35 mg/mL Oligonucleotide 3 (blunt ended hairpin). The total volume of the labeling solution can be scaled up to accommodate the bigger sections. Incubate for 18 hr (overnight) at room temperature (23° C) in a humidified chamber with a plastic coverslip.
5. Remove coverslips by gently immersing the slides vertically in Coplin jar containing water at room temperature. Then wash section 3x10 min in distilled water.
6. Rinse with sodium bicarbonate buffer. Alkaline solution rinse enhances FITC fluorescence, which is pH sensitive and is significantly reduced below pH 7.
7. Cover section with an antifading solution (Vectashield with DAPI), coverslip and analyze the signal using a fluorescent microscope. Double-strand DNA breaks with 5'OH will fluoresce green, 5'PO_4 breaks - will fluoresce red (Figure 2).

4. Representative Results

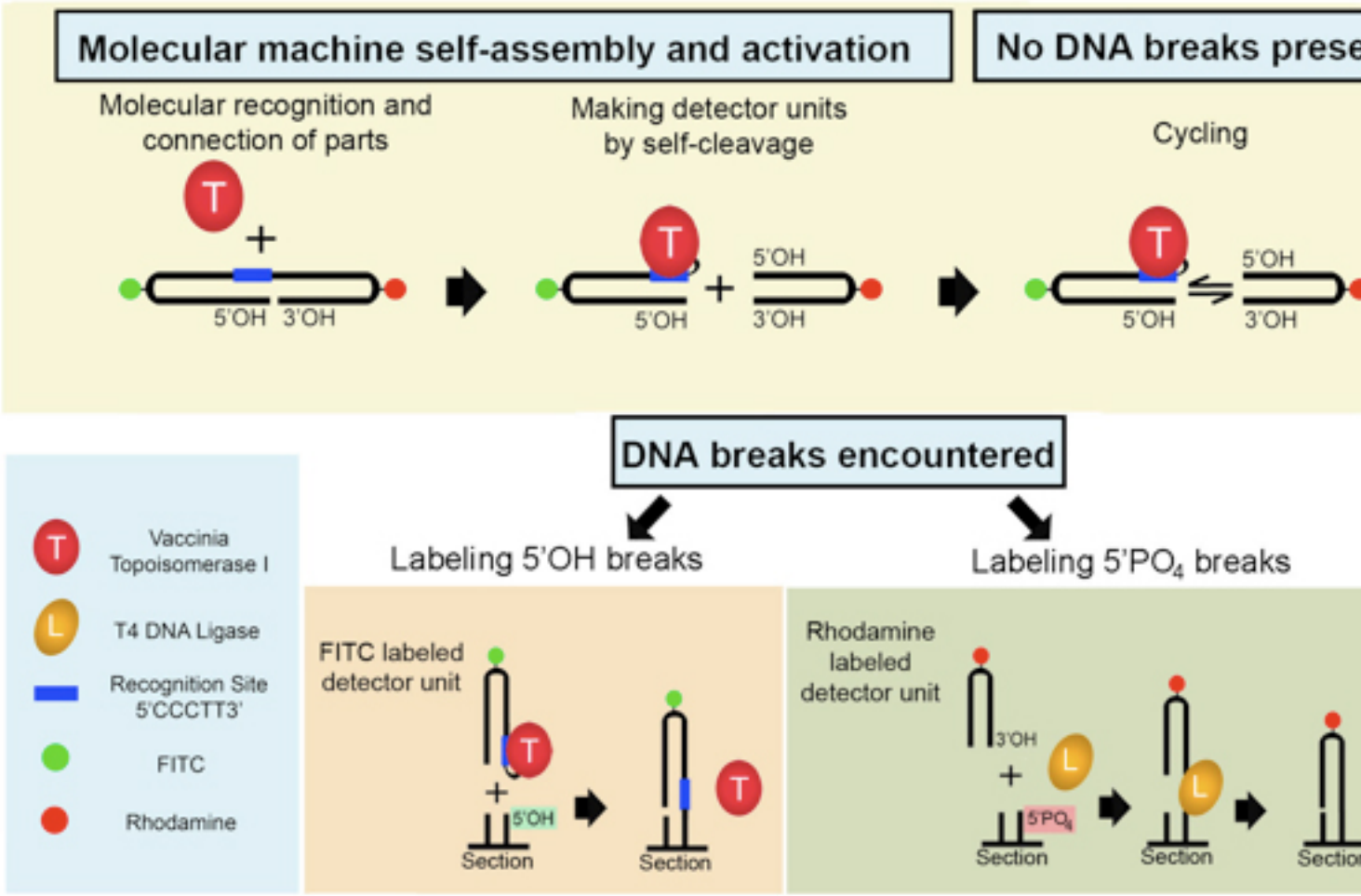

Figure 1. Semi-artificial molecular machine detects two types of DNA damage *in situ*. The fluorescent machine self-assembles when VACC TOPO binds to the double-hairpin 32-mer. The machine begins operation by splitting itself into two detector units via topoisomerase-made cut at the 3' end of the recognition sequence. This results in a cyclic process where the FITC-labeled unit continuously separates and religates back to the rhodamine-labeled unit. This persists until a detectable DNA break is encountered. When such an alternative acceptor (5'OH blunt end DNA break) is present in the tissue section, the FITC part will ligate to it. The remaining rhodamine part will attach to 5' PO_4 DNA blunt end breaks with the help of T4 DNA ligase. Consequently, both types of DNA breaks are simultaneously detected.

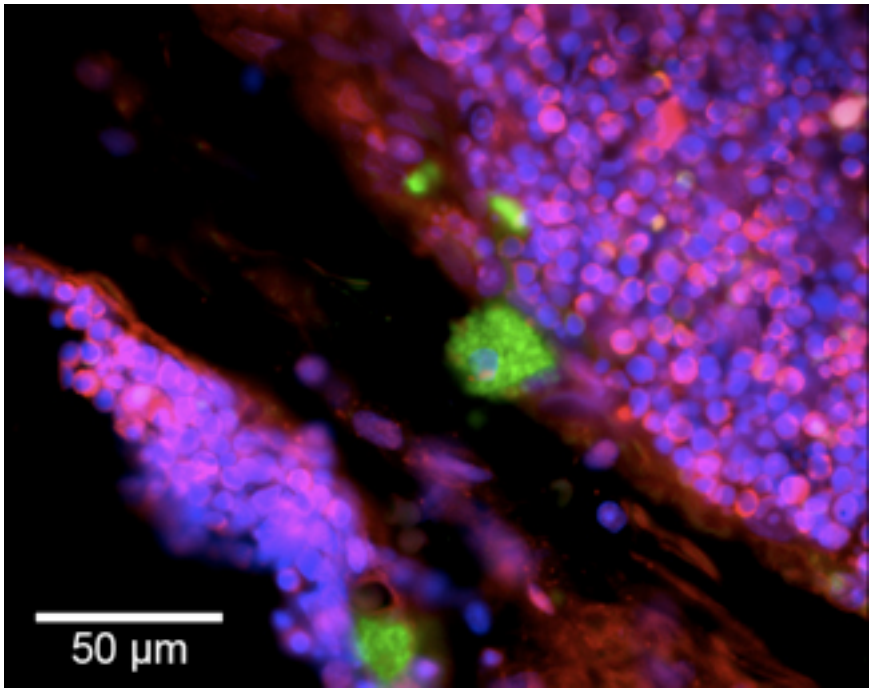

Figure 2. Molecular machine dual labels two types of DNA breaks in a tissue section of dexamethasone-treated thymus. Blunt-ended DNA breaks of DNase I- and DNase II-type are detected in the thymic cortical areas undergoing apoptosis. Green cytoplasmic fluorescence (5'OH DNA breaks) marks cortical macrophages digesting nuclear material of apoptotic thymocytes. This signal is produced by VACC TOPO, and localizes to phagolysosomes with DNA containing 5'OH double-strand breaks [11,12]. Red fluorescence (5'PO_4 breaks) labels nuclei of apoptotic thymocytes not engulfed by macrophages. Massive numbers of thymocytes simultaneously undergo apoptosis accompanied by generation of 5'PO_4 double-strand breaks, visualized by ligase-based labeling. These breaks are located at the nuclear periphery, forming ring-shaped patterns. All cellular nuclei are visualized by counterstaining with DAPI (blue fluorescence).

Discussion

In this video, we demonstrate how to assemble and use a dual-labeling DNA damage sensor. The sensor is a molecular machine driven by bio-molecular engine, a virus-encoded protein VACC TOPO linked with artificial components. The presented development exemplifies a bio-enabled approach which advocates adapting biological structures, architectures and actual parts and components of cells to the design of non-toxic molecular scale devices [12,15]. This approach resolves two issues inherent to the field of *in vivo* nanosensors: 1. the difficulty of making truly uniform and reproducible nano-constructs by using traditional nanomaterials; and 2. the potential toxicity, and high biological reactivity of nanoprobes, particles and other highly-dispersed nanomaterials. The sensor integrates a naturally-occurring molecular machine with artificial components which re-direct it to a new function of DNA damage labeling. The product of such integration is a semi-artificial molecular machine targeted to a new task. While topoisomerases, polymerases and other enzymatic machines are frequently used in biochemical research, they are not integrated with engineered components into individual molecular assemblies. Consequently in their routine use, they are not employed as semi-artificial devices [12].

Here we show how to use the sensor in the tissue section format for simultaneous labeling of DNase I- and DNase II-type breaks. Presently there are no other methods available to perform such simultaneous dual detection.

DNase I- and DNase II-type breaks are important markers of cell death progression, specifically labeling the apoptotic self-autonomous and phagocytic phases [11,16]. The sensor is a useful addition to the biomedical research arsenal dealing with the detection and detailed characterization of apoptosis.

Disclosures

No conflicts of interest declared.

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