Dynamic modulation of proximity-induced enzyme activity using supramolecular polymers

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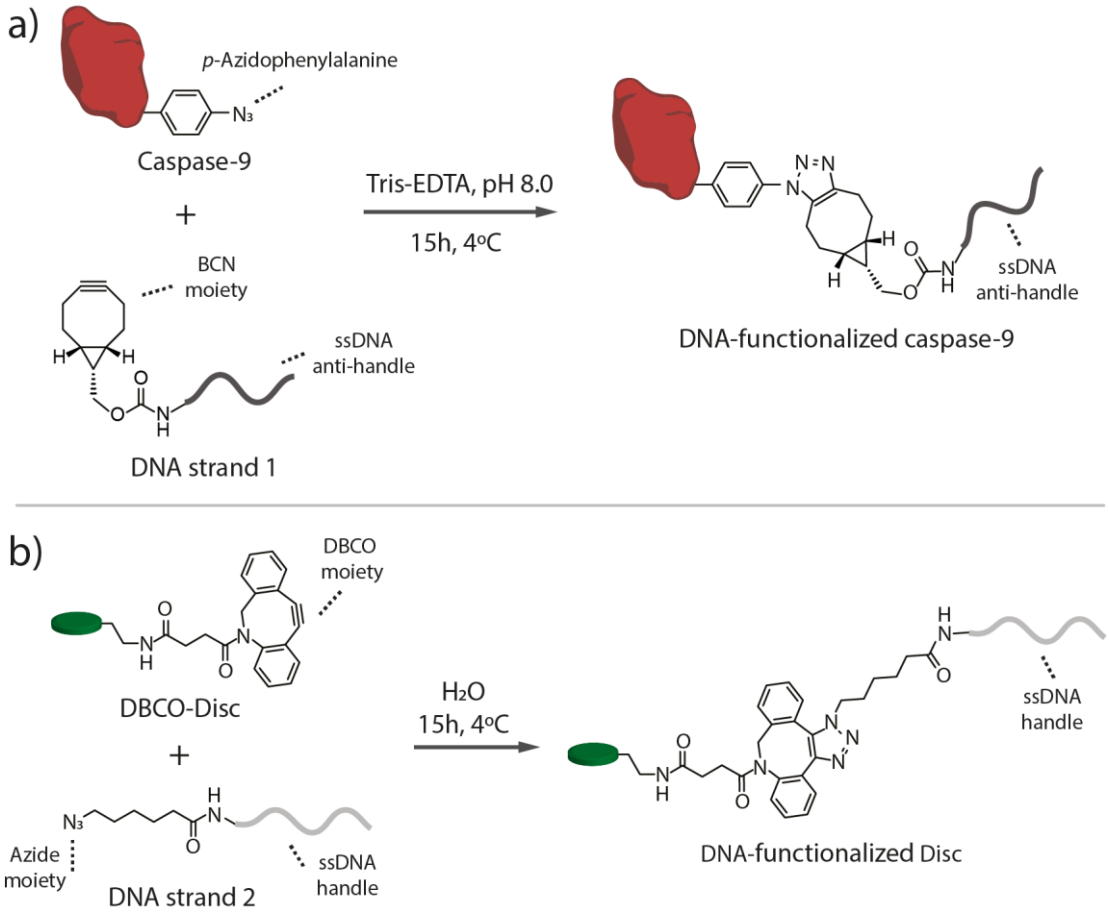
Chemicals and materials

All reagents and solvents were obtained from commercial sources and used without further purification. The non-natural amino acid *p*-azidophenylalanine was obtained from Bachem. Synthetic tetrapeptide caspase-9 substrate Ac-LEHD-AFC was purchased from Enzo Life Sciences. The amino-functionalized and azido-functionalized DNA strands were obtained HPLC-purified from Integrated DNA Technologies and dissolved in DNase/RNase-free water at 250 and 100 µM respectively. All the experiments were performed using Tris-EDTA buffer solution and CHAPS hydrate obtained from Sigma Aldrich and RNase-Free $MgCl_2$ (1M) and NaCl (5 M), RNase-free obtained from ThermoFisher Scientific. Corning® 384-well Black Round Bottom Polystyrene Not Treated Microplate was obtained from Fisher Scientific. Eppendorf® Safe-Lock tubes 1.5 mL and DNA LoBind Tubes, DNA LoBind, 0.5 mL were obtained from Eppendorf.

Expression and purification of caspase-9

Expression and purification of caspase-9 was performed as described previously[1]. Briefly, the catalytic domain of human caspase-9 (140-416) with an N-terminal amber stop codon was encoded on a pET28a plasmid and synthesized by GenScript. The construct contains an N-terminal His-SUMO tag (the SUMO tag was included to improve stability and solubility during expression[2]) and a C-terminal Strep-tag. The pEVOL-pAzF vector, encoding for the orthogonal aminoacyl-tRNA synthetase–tRNA pair, was a kind gift from Peter Schultz (Addgene plasmid #31186). The plasmid encoding for caspase-9 was co-transformed with the pEVOL plasmid into E. coli BL21(DE3) competent bacteria (Novagen) after which protein expression was performed overnight at 18 °C with 1 mM of *p*-azidophenylalanine in the culture medium. After mechanical cell lysis, purification was performed sequentially by both Ni^{2+}-affinity chromatography and Strep-Tactin affinity chromatography, using the N- and C-terminal affinity tags on caspase-9, respectively. Cleavage of the N-terminal His-SUMO tag was performed by adding SUMO protease dtUD1 (1:500, purified according to standard procedure[2]) during overnight dialysis. After purification, proteins fractions were combined and concentrated using Amicon 10 kDa MWCO centrifugal filters (Merck Millipore) to a final concentration of ~1.5 mg/mL (~47 µM), and then snap frozen in liquid nitrogen and stored in 100 µL aliquots at -80°C. The concentration of caspase-9 was determined by measuring the absorption at 280 nm (NanoDrop 1000, Thermo Scientific) assuming an extinction coefficient of 3.1×10^4 M^{-1} cm^{-1} (ref. 3). Total yield after purification typically was ~4 mg/L culture medium. Molecular weight was confirmed using liquid chromatography quadrupole time-of-flight mass spectrometry (Waters ACQUITY UPLC I-Class System coupled to a Xevo G2 Q-ToF) by injecting a 0.1 µL sample into an Agilent Polaris C18A RP column with a flow of 0.3 mL/min and a 15-60% acetonitrile gradient containing 0.1% formic acid[1].



Scheme S1. Schematic representation of the functionalization of caspase-9 and discotic monomers with single-stranded DNA. a) Functionalization of caspase-9 with single-stranded DNA **1** using strain promoted click chemistry between an azide moiety located at the caspase-9 and a BCN moiety located at the DNA strand. b) Synthesis of DNA-functionalized Disc (DNA-Disc) by reacting azido-functionalized strand **2** with DBCO-decorated Disc by Strain Promoted Click Chemistry.

Functionalization of DNA anti-handle strand

Functionalization of amino-modified DNA strand **1** (Table S1) with a bicyclononyne moiety was performed on a 100 µL scale by adding 2 mM (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl *N*-succinimidyl carbonate (BCN-NHS, 4 mM stock solution in dry DMSO) to 100 µM of DNA in 1×PBS (pH 7.4), and incubating for 2 h at 25°C under continuous shaking. Excess BCN-NHS was removed by two rounds of ethanol precipitation. Briefly, 900 µL ice-cold 100% ethanol and 20 µL 3 M potassium acetate (pH 5.5) was added directly to the reaction mixture and incubated for 30 min at -30°C. After centrifugation (14,000 g for 30 min at 4°C) the supernatant was removed and the pellet was reconstituted in 100 µL water. This procedure was repeated once and after centrifugation the pellet was washed with 95% ice-cold ethanol (v/v, in water). The mixture was centrifuged again (14,000 g for 10 min at 4°C) and the pellet was reconstituted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -30°C. Concentrations were determined using absorption at 260 nm (NanoDrop 1000, Thermo Scientific), assuming extinction coefficients reported by the manufacturer.

Caspase-9 enzyme-DNA conjugation and purification

The DNA-protein conjugation procedure has been described previously in detail[1]. Typically, conjugation reactions were carried out on a 500 µL scale using 10 µM protein and 30 µM BCN-DNA in reaction buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% CHAPS (w/v), pH 8.0) for ~16 hr at 4°C (Scheme S1a). Competing thiol-yne reactions of BCN-DNA with the 11 cysteines in caspase-9 were suppressed by pre-incubating the protein with 1 mM β-mercaptoethanol for 30 min at 4°C[4]. Small-scale Strep-Tactin affinity chromatography was performed to remove excess BCN-DNA, while ion-exchange chromatography was performed to remove unreacted protein. Elution fractions containing pure enzyme-DNA conjugates were pooled, supplemented with glycerol (5% (v/v) final concentration), snap frozen in liquid nitrogen, and stored at -80°C in 5 µL aliquots. The concentration of purified enzyme-DNA conjugates was determined with gel densitometry on SDS-PAGE, as described[1]. Enzymatic activity of DNA-functionalized caspase-9 after purification was analyzed by measuring the kinetic parameters, which were found to be in the same range as values reported in literature[1].

DNA-decorated monomers synthesis and purification

Azido-functionalized DNA strand **2** (Table S1, Scheme S1b) (50 µL, 100 µM) was reacted with an excess of compound DBCO-Disc by strain promoted click chemistry (as previously reported by us[5]) (50 µL, 250 µM) at 4 °C with shaking overnight. The crude was saturated with 5 µL of UltraPure™ DNase/RNase-Free NaCl (5 M) solution and dissolved in 300 µL of ice-cold isopropanol. The samples were incubated at -20°C overnight. The resulting suspension was centrifuged at 4°C, 14,000 rpm and the supernatant containing unreacted DBCO-Disc was discarded. The reaction was evaluated using UV-Vis. Concentrations were determined using absorption at 260 nm and 350 nm (NanoDrop 1000, Thermo Scientific), assuming extinction coefficients reported by the manufacturer for the DNA strand and assuming an extinction coefficient for the disc of 50,000 M^{-1} cm^{-1}.

Caspase-9 activity assays

Enzyme activity was measured using the synthetic tetrapeptide caspase-9 substrate LEHD (dissolved in dry DMSO at 10 mM), which is cleaved by caspase-9 after the aspartic acid residue releasing and unquenching the fluorescent dye 7-amino-4-(trifluoromethyl)coumarin (AFC). In a typical measurement, the substrate was added to a final concentration of 167 µM and proteolytic cleavage was monitored over time in 384-well plates (60 µL reaction volume) by measuring fluorescence (λ_{exc} = 400 nm, λ_{em} =505 nm) in a Tecan Spark 10M plate reader. Activity measurements were performed at 18°C similar to previous studies published by us. [1,5] Raw data of all activity assays were extracted, converted, and formatted using OriginLab.

Table S1 | Amino- and azido-functionalized DNA strands that results in the DNA-decorated protein and the DNA-decorated discotic. The DNA sequence **1** was based on sequences reported in the literature with minimal secondary structure and melting temperatures > 40 °C[6]. Both sequences were tested with NUPACK to detect any possible undesired interactions[7]. Bold thymine nucleotides were added as a spacer. For functionalization, an amine was introduced in the 5´-end of the caspase-9 sequence and an azide (Az) moiety was introduced in the 5´-end of the discotic DNA sequence.

Strand	Use	Sequence (5’ to 3’)
1	Caspase-9	H_2N - **TTTTT**GAGTGAGTCGTATGA
2	Supramolecular nanoscaffold	Az - **TTT**TCATACGACTCACTC

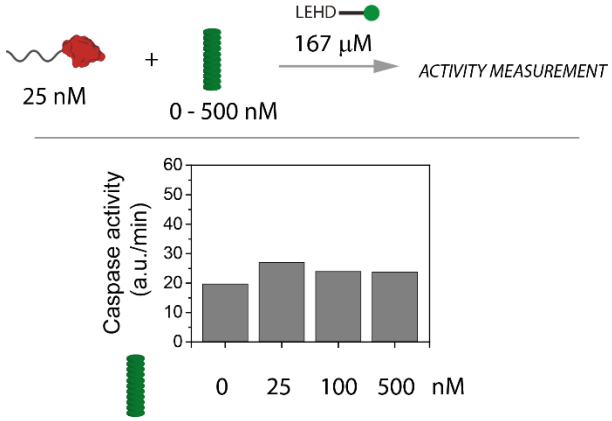


Figure S1. Control experiments ruling out possible unspecific interactions between the components in the system. A constant concentration of caspase-9 at 25 nM was incubated with varying concentrations of Inert-Disc from 0 to 500 nM. The initial slopes from the first 20 to 60 minutes after the addition of 167 µM of enzyme-substrate, extracted from the intensity traces (not shown) are plotted in the graph, showing similar activities for all samples.

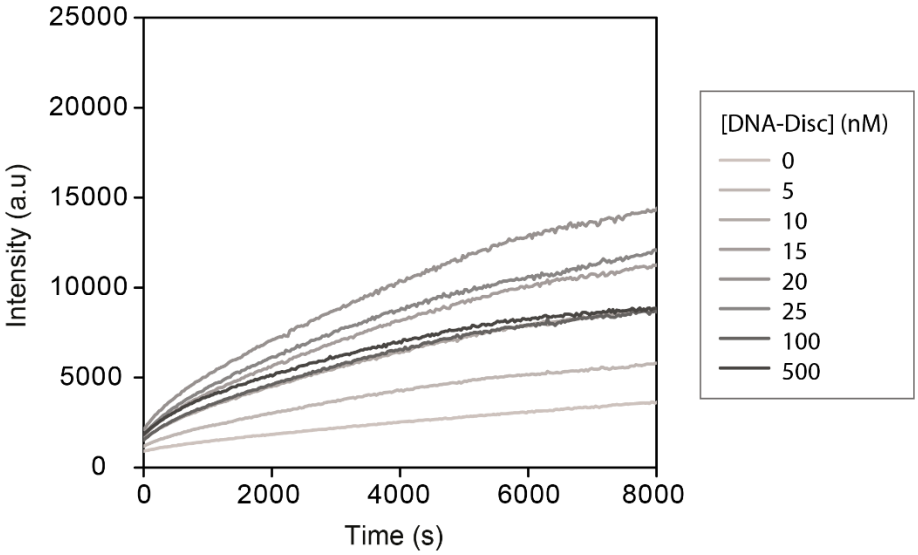


Figure S2. Caspase-9 activity probed by the cleavage of the fluorogenic substrate Ac-LEHD-AFC. The graph shows the fluorescence traces over time at different DNA-Disc concentrations. All activity assays were performed using a constant concentration of caspase-9 at 25 nM and a constant concentration of Ac-LEHD-AFC at 167 µM. First, the caspase-9 was incubated with the pertinent amount of DNA-Disc (0-500 nM), for 2h at 4°C in order to ensure complete hybridization between the complementary DNA strands. Protease activity was monitored after the addition of the Ac-LEHD-AFC substrate. The increasing fluorescence upon substrate cleavage was measured every 30 seconds when exciting the sample at λex= 400 nm, and recording the fluorescence at λ_{em} = 505 nm.

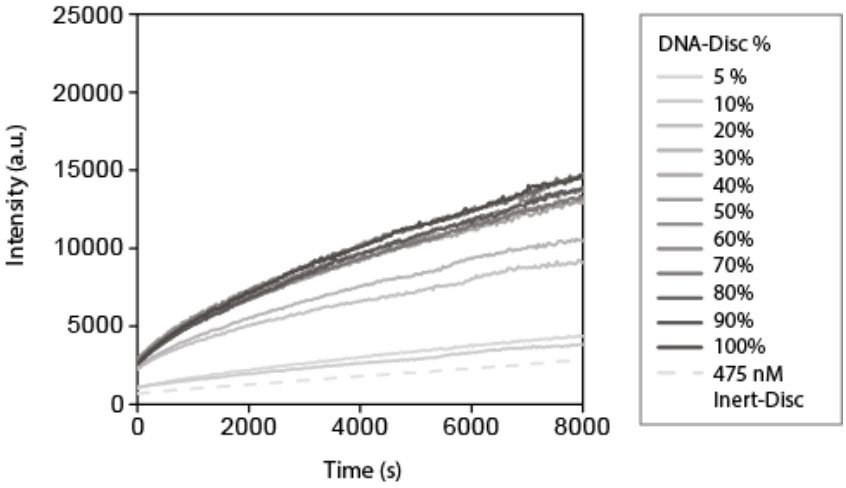


Figure S3. Caspase-9 activity probed by the cleavage of the fluorogenic substrate Ac-LEHD-AFC. The graph shows the fluorescence traces over time at different DNA-Disc densities displayed by the nanoscaffold. All activity assays were performed using a constant concentration of caspase-9 at 25 nM, a constant concentration of Ac-LEHD-AFC at 167 μM, and a constant concentration of DNA-Disc at 25 nM. First, the DNA-Disc was incubated with varying amounts of Inert-Disc in order to obtain the different DNA handle densities on the nanoscaffold. Previous co-assembly studies of the DNA-Disc and Inert-Disc showed complete intermixing after one hour of incubation.[5] Therefore, the supramolecular nanoscaffolds at the different handle densities were first incubated for one hour to ensure monomer intermixing, followed by their incubation with caspase-9 for an additional two hours at 4°C in order to ensure full hybridization between the complementary DNA strands. Protease activity was monitored after the addition of the Ac-LEHD-AFC substrate. The increasing fluorescence upon substrate cleavage was measured every 30 seconds when exciting the sample at λ_{ex}= 400 nm, and recording the fluorescence at λ_{em} = 505 nm.

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