Published in final edited form as:

Chem Commun (Camb). 2018 September 11; 54(73): 10248–10251. doi:10.1039/c8cc04601h.

Real-time tracking of single-molecule collagenase on the native collagen and partially-structured collagen-mimic substrates

James Froberg^a, Woo-Sik Choi^a, Abbas Sedigh^b, Tayebah Anajafi^c, Jasmin Farmakes^b, Zhongyu Yang^b, Sanku Mallik^c, D. K. Srivastava^b, and Yongki Choi^{*,a}

^aDepartment of Physics, North Dakota State University, Fargo, North Dakota, 58108, United States, yongki.choi@ndsu.edu

^bDepartment of Chemistry and Biochemistry, North Dakota State University, Fargo, North Dakota 58108, United States

^cDepartment of Pharmaceutical Sciences, North Dakota State University, Fargo, North Dakota 58108, United States

Abstract

The dynamic interactions of an individual matrix metalloproteinase-1 were imaged and monitored in the presence of either triple-helical or non-triple-helical, partially-structured collagen-mimic substrates. The enzyme exhibited ten-fold increased catalytic turnover rates with the structurally modified substrate by skipping the triple-helix unwinding step during the catalytic pathway.

Graphical abstract

Real-time imaging and tracking proteolytic activities of individual enzymes with their native and structurally modified substrates

About 90% of deaths due to cancer involve tumors that metastasize to spread around the body.^{1, 2} During proliferation, cancer cells overexpress and up-regulate matrix metalloproteinases (MMPs), which play a pivotal role in the degradation of almost all proteins of the extracellular matrix (ECM).³ Although MMPs are closely involved in

*Corresponding author.

Electronic Supplementary Information (ESI) available: [Materials and methods, MMP1 expression and purification, MMP1 assaying, CD measurements, and nano-circuit device fabrication, electronic measurements, and signal transduction mechanism]. See DOI: 10.1039/x0xx00000x

multiple human cancers, the MMPs' proteolytic activities with the collagen, a major element of ECM and the most abundant in human tissue, remain incompletely understood.^{4, 5}

The triggered release of drugs from liposomes has been recognized as a promising cancer therapeutic approach, in which the liposomes release their contents when the membranes are destabilized by an external trigger such as enzymes or pH.^{6, 7} For example, MMP-targeting liposomes synthesized by a triple-helical collagen-mimic lipopeptide have demonstrated the release of the anti-cancer contents triggered by MMPs at the target tumor site and the reduction of tumor size by the contents.^{6, 8, 9}

The enzymatic degradation of such liposomes depends on the triple-helical structure and cleavage site of the lipopeptides.^{8, 10} The increased helical conformation of the lipopeptides enhances thermal and structural stability, while the helicity decreases the catalytic efficiency of MMPs to cleave and process the substrates, with a much more pronounced effect for MMP1.^{8, 11} Thus, it is critical to develop novel lipopeptides that can provide structural and thermal stability to form three-dimensional liposomes with high susceptibility to MMP1 for the effective degradation.

Here, we synthesized the non-triple-helical, partially-structured collagen-mimic lipopeptides that include stearic acid and two GPO repeating units ([CH₃(CH₂)₁₆CONH-GPQGIAGQR(GPO)₂GG; O represents 4-(*R*)-hydroxyproline], Fig. S1), in which the conjugation of stearic acid gives rise to the structural stability and the reduced helicity provides high susceptibility for degradation by MMP1. Neither GPO-free lipopeptides nor lipopeptides with multiple GPO repeats (*n* > 3) produce stable, non-triple-helically cross-linked structures.^{6, 8} Thus, the lipopeptides serves as an ideal analog substrate to examine the relationship between the enzyme kinetics and minor variation of substrate's structure.

However, investigating dynamic interactions between individual MMPs and substrates is difficult using conventional fluorescence-based techniques for two main reasons. First, temporal, spatial, and population averaging effects prevent probing of individual biological events¹² with substrates which could be heterogeneous in size and feature a cross-linked structure involving bindings of GPO repeats and fatty acids.^{6, 10} Second, signals from the optical techniques are limited by the complete dissociation of fluorophore-quencher units in the substrates regardless of their helicity, which not only preclude the observation of single cleavage events occurring each single-strand, but also interfere with enzymes' specificity and processivity.^{13–15}

To overcome these issues, two complementary single-molecule techniques are applied here. We performed atomic force microscopy (AFM) measurements and electronic-based single-molecule nanocircuit measurements.^{16–18} Our measurements compare MMP1's catalytic ability, efficiency, processivity, and rate-limiting factors for the native collagen and lipopeptides substrates at a single-molecule level. The results provide detailed information about enzyme-ligand interaction at single-molecule resolution, providing new insights into designing effective drug carriers for enzyme-triggered contents-release.

Initially, the catalytic activities of MMP1 to both collagen and lipopeptides were investigated through AFM measurements (Fig. 1). Figures 1a-1d demonstrate typical shape

and dimension of the native, triple-helical collagen monomers before and after incubating with MMP1 for an hour in an assaying buffer (100 mM Tris-HCl, pH 7.5, 10 mM CaCl₂, 100 mM NaCl₂). The AFM images clearly show individual collagen monomers with an average length of 301 nm and height of 1 nm. After incubating with MMP1, collagen fragments in length, compared to the intact collagen monomers, appeared in the AFM image. Figure 1d compares the overall length distributions of the intact collagen and cleaved collagen fragments, splitting from a single peak at 301 nm to two peaks at 77 nm and 219 nm with no changes of their heights. These observations reflect the hydrolysis of the specific amide bond between G and I in the collagen molecule by MMP1, creating two collagen fragments, one $\frac{1}{4}$ and the other $\frac{3}{4}$, which are consistent with previous AFM studies.^{19–21}

Similar to the native collagen, the lipopeptides were prepared and imaged by the AFM under the same conditions (Figs. 1e–1g). The AFM images show the lipopeptides in rod-like shapes with various lengths, heights (2–10 nm) and cross-links (Fig. 1e). After incubating the lipopeptides with MMP1 for an hour, the AFM images revealed many short fragments in both lengths and heights, indicating almost complete digestion of the lipopeptides by MMP1 (Fig. 1f).

The increased height and various lengths of the lipopeptides suggest that the lipopeptides form bundles of single lipopeptides. However, the lipopeptides do not have the minimum number of GPO repeats, which twist to form a triple-helical conformation and a collagen fibril with the native collagen. Thus, both the hydrophobic interactions between fatty acids at one end and the weak interaction between two GPO repeats at the other end could contribute to the formation of bundles in non-helical, linear conformation, producing the rod-like lipopeptides. Also, non-specific interaction between the lipopeptides and bundles provide additional freedom to branch out, cross-link, and stack each other randomly, yielding various heights in AFM images.

The non-triple-helical conformation of the lipopeptides was further supported by our circular dichroism spectroscopy measurements, showing no positive peak at $\lambda = 220\text{--}225$ nm in a wide range of temperatures from 5 to 60 °C (Fig. S2).

Although the lipopeptides are bundled up and partially-structured with many single lipopeptides, almost all bundles and cross-links were cleaved by the MMP1, indicating the high susceptibility to MMP1. These observations in AFM measurements proved the preserved proteolytic activity of MMP1 with both substrates regardless of triple-helicity and crosslinked structures.

Next, the real-time interactions between individual MMP1 and both substrates were monitored using liquid-AFM imaging in the buffer solution (Fig. 2). Figures 2a–2d depict time-series AFM measurements of the binding and cleavage of the individual collagen molecule by MMP1. Initially, the AFM probe scanned over the collagen and identifies single collagen monomers (Fig. 2a). Following the addition of MMP1 (6 μM), the AFM probe re-scanned the same collagen monomer, capturing a MMP1 attachment to the monomer and formation of a MMP1-collagen complex (Fig. 2b). Next, subtle conformational changes of the complex were observed while continuously monitoring the complex, suggesting either

unwinding of the triple-helical collagen or the hydrolysis of the amide bond (Fig. 2c). During these processes, we observed the increase in height after MMP1 binding to the collagen (Figs. 2a-2b, 2i), as well as the slight decrease in height due to subtle conformational changes of the complex (Figs. 2b-2c, 2i). After cleavage, the MMP1 diffused away from the complex, leaving two short collagen fragments (Fig. 2d). A similar interaction between an individual MMP1 and lipopeptide was monitored and recorded (Figs. 2e-2h). Figures 2i-2j present the height profiles of the substrates and complex, which were consistent before and after cleavage and with the AFM measurements performed in dry conditions (Fig. 1).

The AFM images in Fig. 2 visualize real-time intermolecular interaction between MMP1 and collagen substrates at single-molecule resolution. MMP1 formed the MMP-substrate complex at an off-center position of the substrates and then dissociated from the same position, leaving two short fragments. Previous work has demonstrated that MMP8 is able to nonspecifically bind non-cleavage sites of collagen monomers, but unable to cleave¹⁹ and that MMP1 randomly binds and diffuses along the collagen fibrils to find the cleavage sites.²² When MMP1 recognizes and bind the cleavage sites, cleavage takes place as demonstrated in our real-time AFM measurements. We note that the slow catalytic activity observed here could result from the surface effect, which limits diffusion of both MMP1 and collagen and interferes with conformational motions of MMP1 while binding, unwinding, and catalyzing the surface-bound collagen.

To further investigate the kinetics during the catalysis and complement the real-time AFM observations, we performed single-molecule nanocircuit measurements, which provide excellent temporal resolution ($< 20 \mu\text{s}$) and long period time ($> 20 \text{ min}$) measurements. For MMP1-nanocircuits, individual MMP1 molecules were attached to the sidewalls of single-walled carbon nanotube (SWNT) field-effect transistors (FETs) through the pyrene-iminodiacetate- Cu^{2+} linker molecules (Fig. S1), in which the Cu^{2+} ion binds to N-terminal His-tag of MMP1 and the pyrene moiety binds to the sidewall of the SWNT through π - π interaction.^{16, 18} Thus, MMP1 can freely interact with substrates and form the MMP1-substrate complex. When MMP1 or the complex undergoes conformational changes to bind, unwind, hydrolyze, and release the products, motions of charge residues associated with the conformational transition induce current fluctuations ($I(t)$) underlying the SWNT-FET through a charge gating effect that has been proven by our previous work.^{17, 18}

Therefore, the MMP1-nanocircuits directly record MMP1's dynamic conformational changes as it degrades its substrates. Figure 3 depicts typical $I(t)$ signals measured with the nanocircuit under different conditions. In the absence of the substrates as control measurements, $I(t)$ signals exhibited no meaningful features which serve as a baseline current (Fig. 3a). Following the addition of collagen monomers ($1 \mu\text{g/ml}$) to the MMP1-nanocircuit, several current spikes above the baseline current were captured (Fig. 3b). Such current spikes maintained for a short period time ($\sim$ few ms) with a magnitude of $\sim 6 \text{ nA}$ (Fig. 3b inset). Figure 3c illustrates the similar $I(t)$ fluctuations between two current states, but substantially increased number of spikes when the lipopeptides were added to the same MMP1-nanocircuit.

Crystallographic and computational studies have identified that the active site pocket of MMP1 is initially narrow (closed conformation, diameter of ~ 5 Å), which allows triple-helical collagen molecules to bind to MMP1's pocket surface, but hinders triple-helical conformation (diameter of ~ 15 Å) from entering into the catalytic zinc atom located inside of the pocket ~ 7 Å away from the entrance surface.^{23, 24} Following unwinding of the triple-helix, the single strand could enter into the catalytic site to be cleaved by two fragments.^{24, 25} Next, the pocket opens (open conformation) to remove the cleaved fragments and closes it for adoption and hydrolysis of following strands.²⁴ During such conformational rearrangement, motions of charged side chains of Arg108 and Glu110 in proximity to SWNT binding sites (the N-terminus) electrostatically gate the SWNT channel current, resulting in two distinguishable current states.^{26, 27} Thus, we assign the low and high current states of the signals to correspond to closed and open conformation of the MMP1 (Fig. S5).

Therefore, the individual spikes in Figs. 3b-3c represent conformational transitions of MMP1 associated with the catalytic processing of the substrates. The hydrolysis of the triple-helical collagen is known to be a very slow process due to the unwinding of the triple-helix before cleavage events,^{14, 28} resulting in only a few spikes during the measurements. However, the catalytic activity of MMP1 with the lipopeptides has substantially increased, proving that MMP1 does not require triple-helical conformations, nor is it hindered by the cross-links of the fatty acid or GPO repeats for its processivity.

Figures 3d-3e show the probability distribution of the duration in the low (τ_{lo}) and high (τ_{hi}) current states accumulated from more than 600 s of recordings. The distributions fit an exponential, suggesting a single mean value of τ . In the presence of the native collagen substrate, the mean τ_{lo} and τ_{hi} were measured to be 26 s and 0.78 ms, respectively (Figs. 3d-3e, blue color). With the lipopeptides, the mean τ_{lo} (2.9 s) was decreased by a factor of 10, but no significant changes in the mean τ_{hi} (0.74 ms) (Figs. 3d-3e, red color). The overall turnover rate, $1/(\tau_{lo} + \tau_{hi})$, also increased almost ten-fold due to the major contribution of τ_{lo} to the rate. While unusually long durations for each state at the distribution tail are slightly off from the fit, occurrence of such events are extremely rare ($< 5\%$), and thus no significant contribution to the mean duration τ . The mean τ values, turnover rates, variance analysis, and relative energy differences of each substrate are summarized in Table 1.

The rate $k=0.038\text{ s}^{-1}$ of MMP1 with the collagen monomers are in agreement with the k_{cat} from previous bulk measurements.^{28, 29} Thus, the current fluctuations correspond to enzymatic turnover events. The increased rate of $k=0.345\text{ s}^{-1}$ with the lipopeptides suggest more catalytic events occurring by MMP1. Since the lipopeptides do not have triple-helical structures, MMP1 could cleave them without the intermediate step of unwinding the helical conformation. This effective catalytic pathway resulted in the significant reduction of duration in the closed conformation (88 %), implying that the unwinding step could be the major rate-limiting step during the entire catalytic cycle.

To further assess the number of rate-limiting steps in the reaction pathway, the mean normalized variance $r=\sigma^2/\tau^2$ of the τ_{lo} and τ_{hi} is used.³⁰ For the collagen, the variance $r_{lo}=0.71$ ($= 1/n$, n is the number of the rate limiting steps) suggests that the conformational transition from closed to open involves at least two steps, but non-identical, rates. In

contrast, the variance $r_{lo} \approx 1$ for the lipopeptides indicates the duration of the conformational transition is rate-limited by a single-step Poisson process. Taken together, we conclude that one of the major rate-limiting steps in the closed conformation is the unwinding of the triple-helical conformation before cleavage, which disappeared during substantially reduced τ_{lo} with the non-triple helical lipopeptides.

Despite the major differences in τ_{lo} and r_{lo} , the mean $\tau_{hl} = 0.74\text{--}0.78$ ms and the variance $r_{lo} = 0.98\text{--}1.00$ were almost identical for both substrates. These results indicate that the open conformation lasts briefly to release the cleaved fragments from the active site pocket, which is the only rate-limiting step for the conformational transition from open to close to process a following non-helical collagen strand. In other words, the kinetics and rates from the hydrolysis to products release remain remarkably identical for both substrates. Although the structural stability of the lipopeptides and its contribution to the catalytic efficiency have not been identified in the present report, we could conclude that MMP1 can efficiently catalyze the lipopeptides compared to the structurally-stable, triple-helical collagen substrates.

Finally, the thermodynamic energy differences E between the two current states, corresponding to the ratio of τ_{lo} and τ_{hl} , were investigated. In MMP1, the open conformation is much shorter than the closed conformation, indicating that this conformation requires higher energy to access. Using Boltzmann statistics, this energy difference can be calculated as $E = k_B T \ln(\tau_{hl}/\tau_{lo})$.³¹ E were measured to be 6.25 kcal/mol and 4.96 kcal/mol for the collagen and lipopeptides, respectively. The reduction of 1.29 kcal/mol between triple-helical and single-strand conformation can probably be interpreted as the extra energy required for the unwinding of the triple-helix. Although the energy barrier was reduced by ~20 %, the overall catalytic turnover of MMP1 resulted in a drastic increase by a factor of 10. Furthermore, E values were obtained when either substrate is bound to the MMP1. In the absence of either substrate, no two-state $I(t)$ signal was observed, indicating that the open conformation is inaccessibly higher in energy associated with dissociation of the products.

In conclusion, our AFM measurements demonstrated the real-time cleavage of the triple-helical collagen and non-triple-helical, partially-structured lipopeptide substrates by MMP1 at single-molecule resolution. From nanocircuit measurements, we observed that the kinetic rates and rate-limiting steps were highly sensitive to the substrate's structure, resulting in ten-fold increased catalysis with lipopeptides. These results provide a detailed look of kinetics during the overall MMP1-ligand reaction, helping design new lipopeptides for MMP-targeting, drug-containing liposomes.

We thank Prof. Philip Collins at UC Irvine for supplying SWNT-FET nano-devices. This research was supported financially by NIGMS NIH R15GM122063 and P30GM103332 to Y.C. and NIGMS NIH R15GM110637 to D. S.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Notes and references

1. Mehlen P and Puisieux A, Nat. Rev. Cancer, 2006, 6, 449. [PubMed: 16723991]
2. Taketo MM, Cancer Prevention Research, 2011, 4, 324–328. [PubMed: 21372032]
3. Page-McCaw A, Ewald AJ and Werb Z, Nat. Rev. Mol. Cell Biol, 2007, 8, 221–233. [PubMed: 17318226]
4. Lu PF, Weaver VM and Werb Z, J. Cell Biol, 2012, 196, 395–406. [PubMed: 22351925]
5. Egeblad M and Werb Z, Nat. Rev. Cancer, 2002, 2, 161–174. [PubMed: 11990853]
6. Elegbede AI, Banerjee J, Hanson AJ, Tobwala S, Ganguli B, Wang R, Lu X, Srivastava DK and Mallik S, J. Am. Chem. Soc, 2008, 130, 10633–10642. [PubMed: 18642903]
7. Kale AA and Torchilin VP, Journal of Liposome Research, 2007, 17, 197–203. [PubMed: 18027240]
8. Banerjee J, Hanson AJ, Gadam B, Elegbede AI, Tobwala S, Ganguly B, Wagh AV, Muhonen WW, Law B, Shabb JB, Srivastava DK and Mallik S, Bioconjugate Chemistry, 2009, 20, 1332–1339. [PubMed: 19601658]
9. Banerjee J, Hanson AJ, Nyren-Erickson EK, Ganguli B, Wagh A, Muhonen WW, Law B, Shabb JB, Srivastava DK and Mallik S, Chemical Communications, 2010, 46, 3209–3211. [PubMed: 20424776]
10. Shoulders MD and Raines RT, Ann. Rev. Biochem, 2009, 78, 929–958. [PubMed: 19344236]
11. Minond D, Lauer-Fields JL, Nagase H and Fields GB, Biochemistry, 2004, 43, 11474–11481. [PubMed: 15350133]
12. Lu HP, Curr. Pharm. Biotechno, 2009, 10, 522–531.
13. Lauer-Fields JL, Tuzinski KA, Shimokawa K.-i., Nagase H and Fields GB, J. Biol. Chem, 2000, 275, 13282–13290. [PubMed: 10788434]
14. Lauer-Fields JL, Broder T, Sritharan T, Chung L, Nagase H and Fields GB, Biochemistry, 2001, 40, 5795–5803. [PubMed: 11341845]
15. Robichaud TK, Steffensen B and Fields GB, J. Biol. Chem, 2011, 286, 37535–37542. [PubMed: 21896477]
16. You S, Froberg J, Yu J, Haldar M, Sedigh A, Mallik S, Srivastava DK and Choi Y, Chemical Communications, 2017, 53, 3307–3310. [PubMed: 28261707]
17. Choi Y, Olsen TJ, Sims PC, Moody IS, Corso BL, Dang MN, Weiss GA and Collins PG, Nano Lett, 2013, 13, 625–631. [PubMed: 23323846]
18. Choi Y, Moody IS, Sims PC, Hunt SR, Corso BL, Weiss GA and Collins PG, Science, 2012, 335, 319–324 [PubMed: 22267809]
19. Sun HB, Smith GN, Hasty KA and Yokota H, Analytical Biochemistry, 2000, 283, 153–158. [PubMed: 10906235]
20. Bozec L and Horton M, Biophys. J, 2005, 88, 4223–4231. [PubMed: 15778444]
21. Rosenblum G, Van den Steen PE, Cohen SR, Bitler A, Brand DD, Opendakker G and Sagi I, PLOS ONE, 2010, 5, e11043. [PubMed: 20585385]
22. Sarkar Susanta K., Marmer B, Goldberg G and Neuman Keir C., Current Biology, 2012, 22, 1047–1056. [PubMed: 22578418]
23. Chung L, Shimokawa K.-i., Dinakarbandian D, Grams F, Fields GB and Nagase H, J. Biol. Chem, 2000, 275, 29610–29617. [PubMed: 10871619]
24. Ivano B, Vito C, Marco F, Claudio L, Massimiliano M and Joo YK, Angew. Chem. Int. Edit, 2006, 45, 7952–7955.
25. Bella J, Eaton M, Brodsky B and Berman H, Science, 1994, 266, 75–81. [PubMed: 7695699]
26. Li J, Brick P, O'Hare MC, Skarzynski T, Lloyd LF, Curry VA, Clark IM, Bigg HF, Hazleman BL, Cawston TE and Blow DM, Structure, 1995, 3, 541–549. [PubMed: 8590015]
27. Ivano B, Vito C, Linda C, Marco F, F.G.C. GC, Petr H, Claudio L, Giacomo Pand M.C. TJ, FEBS Lett, 2012, 586, 557–567. [PubMed: 21945315]
28. Welgus HG, Jeffrey JJ and Eisen AZ, J. Biol. Chem, 1981, 256, 9511–9515. [PubMed: 6270089]

29. Ohuchi E, Imai K, Fujii Y, Sato H, Seiki M and Okada Y, J. Biol. Chem, 1997, 272, 2446–2451. [PubMed: 8999957]
30. Min W, English BP, Luo G, Cherayil BJ, Kou SC and Xie XS, Accounts Chem. Res, 2005, 38, 923–931.
31. Xie SN, Single Molecules, 2001, 2, 229–236.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1.

AFM topography of collagen substrates before and after incubating with MMP1. (a) Single, triple-helical collagen molecules. (b) Cleaved collagen fragments after incubating MMP1. (c) Height profiles of collagen molecules extracted from the cross-sectional distance perpendicular to the molecules in (a) and (b). (d) Length distribution of collagen molecules before (top) and after (bottom) incubating with MMP1. (e) Non-triple-helical, partially-structured lipopeptides. (f) Cleaved lipopeptides after incubating with MMP1. (g) Height profiles of the lipopeptides in (e) and (f). All scale bars are 200 nm

Fig. 2. Real-time interactions between an individual MMP1 and a substrate. Time-series AFM images of (a)-(d) the collagen molecule and (e)-(h) the lipopeptide after perfusion of MMP1. The images were recorded at 8 min. and 6 min. intervals for the collagen and lipopeptide, respectively. All scale bars are 100 nm. Height profiles of (i) the collagen and (j) lipopeptide as indicated in number in the AFM images.

Fig. 3.

Electronic current $I(t)$ fluctuations of the MMP1-nanocircuit. (a) In the absence of substrates (buffer only), no current fluctuations were observed. The addition of (b) the collagen and (c) lipopeptides resulted in the current spikes, fluctuating between the low and high current states, which corresponds to the enzymatic turnover events. Comparison of probability distributions of (d) the low state (τ_{lo}) and (e) high state (τ_{hi}) for both substrates. Single exponential fits are shown as solid lines, determining the mean value of τ .

Table 1

Single molecule kinetic parameters

Substrates	τ_{lo} (s)	r_{lo}	τ_{hi} (ms)	r_{hi}	Rate (s^{-1})	E (kcal/mol)
Collagen	26 ± 1.8	0.49 ± 0.34	0.78 ± 0.04	0.98 ± 0.44	0.038	6.25
Lipopeptide	2.9 ± 0.26	0.71 ± 0.32	0.74 ± 0.05	1.00 ± 0.26	0.345	4.96