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Influence of “Flexible” versus “Rigid” Nanoparticles on the Stability of Matrix Metalloproteinase-7[†]

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Abstract

Matrix Metalloproteinase-7 (MMP-7) is invariably expressed in a variety of cancer cells, and exhibits the potentials to interact with differently charged macromolecular surfaces¹. To ascertain whether the nature of the charge carrying surfaces influences the stability as well as catalytic properties of the enzyme, we compared the effects of differently charged lipid (representative of “flexible”) and gold (“rigid”) nanoparticles. The experimental data revealed that the catalytic activity of MMP-7 is impaired only by the positively charged lipid nanoparticles, and it remains unaffected by their negatively charged or neutral counterparts. On the other hand, both positively and negatively charged gold nanoparticles impair the enzyme activity with nearly equal potency; no significant influence of neutral gold nanoparticles was noted on the enzyme activity. Unlike lipid nanoparticles, the charged gold nanoparticles mediated effects were found to be manifested partially via the inactivation of the enzyme. Arguments are presented that both the “rigidity” as well as the surface curvature of the lipid (“flexible”) vis a vis the gold (“rigid”) nanoparticles are responsible for eliciting differential influence on the catalytic activity as well as the stability of MMP-7.

Keywords

MMP-7; Matrilysin; Liposomes; Gold Nanoparticles; Electrostatic Interaction

1. INTRODUCTION

It has been well recognized that the transformation of normal cells to cancerous cells accompany a wide range of changes in the macro-molecular components of the physiological milieu². Some such changes involve expression of new class of enzymes and proteins which interact differently with their cognate partners, alter in lipid structures, metabolic processes, among others^{3–4}. Electrostatics serves as one of the important mediators of macromolecular interacting system, such as observed with proteins, protein-lipid and protein nucleic acid complexes. Protein surfaces interacting via electrostatic forces often yield highly selective macromolecular assembly, and they are often accomplished via bridging by small molecules^{5–7}. Hence, understanding of the electrostatic forces in aqueous solution, which control chemical processes and give rise to biological structure is essential for a variety of biotechnological applications, ranging from chemical synthesis to unraveling the physiological processes such as drug design, signal transduction via ionic fluxes, protein folding, localization, function related to health and disease^{8–10}.

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Over the past several years, sensitive, selective and convenient methods have been sought to detect proteins in the physiological system¹¹. However, protein detection is a challenging problem due to their structural diversity as well as the inability to selectively interact with their putative ligand molecules. It has been well recognized that several physiological processes are mediated via protein-lipid interactions, and the molecular mechanisms by which such interactions regulate the cellular function is of considerable interest from the point of view of developing a wide range of biotechnological applications¹².

The mechanistic studies on protein-lipid interactions have been customarily pursued using liposomes as the representative lipid nanoparticles (LNP). Aside from lipid nanoparticles, protein detection and desensitizations have recently been pursued using differently decorated gold nanoparticles (AuNPs) and quantum dots (QDs)^{13–15}. Due to size and shape controlled architecture of these metallic nanoparticles (NPs), detection and desensitization of protein biomarkers of many diseases have been facilitated⁹. Aside from growth and proliferation, cancer cells also possess the potentials to dislodge from their residual tissues and migrate to other organs and start proliferating there¹⁶. The latter “metastatic” process is accomplished via a series of biochemical reactions including disruption of extracellular matrix proteins¹⁷.

In physiological milieu, the level of extracellular matrix (ECM) assembly is dynamically controlled by synthesis of its individual components (such as collagens, elastin, etc.) and its degradation by proteolytic enzymes, Matrix Metalloproteinases (MMPs). Under normal condition, these processes are well “coordinated” but during metastasis, several MMPs are over-expressed, and their levels can be taken as the markers of a variety of cancers. MMPs are a family of more than 26 zinc and calcium dependent endopeptidases with diverse ECM proteins, such as collagen, gelatins, etc.¹⁸. Of various MMPs¹⁹, MMP-7 is structurally the simplest enzyme and it lacks fibronectin, hemopexin and other membrane anchoring domains. Despite being the least complex in architecture¹⁷ in the family, MMP-7 has been ascribed to be primarily involved in the inflammatory diseases and cancers¹⁶. Since MMP-7 preferentially cleaves some of the non-ECM substrates, which are confined to the cell membrane (e.g., E-cadherin, TNF- α , and other growth factors), its interaction with biological membranes has been sporadically considered²⁰. Recently we and other groups provided evidence that MMP-7 interacts with differently charged lipid membranes²¹. This has been unusual since most MMPs (except for the membrane anchored MMPs) are regarded as being soluble proteins, and they are believed to elicit their catalytic roles via randomly diffusing through the extracellular matrix.

A casual perusal of the electrostatic surface potentials of MMP-7 revealed a marked bipolar distribution of the positive and negative charges in the vicinity of the active site pocket and on the opposite end of the protein structure, respectively. Consistent with the above structural feature, we demonstrated that although the anionic LNPs interact more strongly with MMP-7 (Table 1), it does not inhibit the enzyme activity. On the other hand, cationic LNP inhibit the enzyme activity¹.

With our long term goal of desensitizing and alleviating the onset of carcinogenesis as well as metastasis, we became interested in the structural and functional consequences of interaction of MMP-7 to differently charged surface. In this endeavor, besides utilizing LNPs (representative of the “flexible” surface), we utilized differently charged AuNPs (representative of the “rigid” surface). The idea was to probe whether the catalytic and binding features of MMP-7 remained altered with the nature of the charged surfaces. To our surprise, we observed that beside charges, flexibility as well as surface curvatures of NPs differently modulates structural-functional features of MMP-7, and this paper elaborates on their molecular mechanism.

2. MATERIALS AND METHODS

2.1. Materials

The materials required for preparing the buffers and other chemicals were of reagent grade, and were used without further purification. The fluorogenic peptide substrate used was obtained from Calbiochem, CA. The differently charged lipids were purchased from Avanti® Polar Lipids, Alabaster, AL.

2.2. a). Cloning, Expression, and Purification of recombinant human MMP 7—

The enzyme was cloned, expressed in *E.coli* BL21 (DE3) cells and purified as described by Ganguly. *et. al* 1.

2.2. b). Preparation of differently charged Liposomes—Large Unilamellar Vesicles (LUVs) containing differently charged lipids were prepared by hydration, sonication and consequently extruding the thin films, formed by mixing appropriate amounts of chloroformic solutions of desired lipids in mol% as previously described ²².

2.2. c). Preparation of differently charged Gold Nanoparticles—The Gold colloid nanoparticle (2 nm size) suspension was purchased from Sigma ® Saint Louis, MO, USA. The gold-colloid nanoparticles (AuNPs) were functionalized by differently charged cationic, anionic and neutral AuNPs as described by Chen. *et. al.* ²³. A detailed protocol of the preparation of AuNPs will be published elsewhere.

2.3. Effect of differently charged liposomes on the enzymatic activity of MMP-7

The effects of differently charged lipid nanoparticles (LNPs) on enzymatic activity of MMP-7 were performed by measuring the initial rate of the MMP-7 catalyzed reaction using the fluorogenic substrate (obtained from Calbiochem, CA) of the following composition: MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ [Where MCA and Dpn stand for (7-Methoxycoumarin-4-yl)acetyl and N-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl, respectively], maintaining the excitation and emission wavelengths of 335 nm and 395 nm, respectively. The progress curve of the MMP-7 catalyzed reaction was monitored for about 300 seconds. The liposome concentrations (total lipid) varied between 0 and 250 µM while the concentration of MMP-7 was maintained at 0.3 µM in reaction mixture in 25 mM HEPES, 10 mM CaCl₂, pH 7.5.

2.4. Effect of differently charged Nanoparticles on the enzymatic activity of MMP-7

The effect of differently charged AuNPs on the MMP-7 activity was determined by using the same fluorogenic peptide as employed with LNPs. Due to difference in the inhibition effect of differently charged AuNPs on the enzyme activity of MMP-7, its concentration range was varied between 0 and 1 µM for cationic gold nanoparticle (Au-CNP) and between 0 and 3 µM for anionic gold nanoparticle (Au-ANP) and neutral gold nanoparticle (Au-NNP) respectively. The MMP-7 concentration during the above experiments was maintained at 0.3 µM.

2.5. Inhibition versus inactivation of MMP-7 by lipid and gold nanoparticles

This experiment was performed to ascertain the potencies of differently charged LNPs and AuNPs on inhibition versus inactivation of MMP-7. In this typical experiment, a relatively high concentration of MMP-7 (~ 8 µM) was pre-incubated with 2.1 µM CNP, and 6 µl was withdrawn from the stock sample and transferred to the reaction mixture for measuring the enzyme activity as already described in the above experiment. In this approach, the NPs were diluted in the reaction mixture, and their concentrations became similar to the ones that were

used during the inhibition experiment. The initial rates were compared to the rates when the NPs were mixed instantly without any pre-incubation period.

2.6. The CNP mediated secondary structural changes of MMP-7 via Circular Dichroic spectroscopic studies

To ascertain whether Au-CNP alters the secondary structural feature of MMP-7 upon binding, we performed the CD spectroscopic studies. The Au-CNP induced changes in the CD spectra of MMP-7 in the UV region (250 – 210 nm) were determined using 1 mm path length in quartz cuvette. During this experiment, the CD spectra of MMP-7 (50 μ M) was recorded as a function of increasing concentration (0 – 5 μ M) of Au-CNP. The increase in molar ellipticity as a function of Au-CNP concentration was determined in 5 mM phosphate buffer, pH 7.5. In order to reduce spectroscopic noise five scans were averaged, and the data were analyzed using the software provided by the manufacturer.

3. RESULT AND DISCUSSION

We purported to investigate the influence of differently charged LNPs and AuNPs on the catalytic activity of MMP-7. The idea has been to discern whether the “rigidity” of NPs carrying identical charges exhibit different influence on the structural and functional integrity of the enzyme. We previously reported that cationic LNPs selectively inhibit MMP-7 catalyzed reaction in 25 mM HEPES, 10 mM CaCl_2 , pH 8.5¹. To ascertain that the above feature was also valid at the physiological pH, the experiment was repeated in 25 mM HEPES, 10 mM CaCl_2 , pH 7.5.

Figure 1 shows the effects of increasing concentrations of differently charged LNPs on the enzyme activity of MMP-7 at pH 7.5 using the fluorogenic substrate (see Materials and Methods). The initial rate of the product formation was determined spectrofluorometrically by monitoring the progress curve of MMP-7 catalyzed reaction for 300 seconds. The excitation and emission wavelength of 335 and 395 nm, respectively, were maintained for monitoring the reaction progress in the above time regime. The data showed that unlike anionic and neutral LNPs, cationic LNP had inhibitory effect, as previously observed at pH 8.5. To quantitate the inhibitory potency of cationic LNP, we analyzed the experimental data according to the nonlinear regression analysis of Banerjee *et al.* 24, and found the K_i value as being equal to 18.5 μ M. Note that the latter value is about 4 fold higher than that obtained pH 8.5¹. Evidently the cationic LNP inhibits MMP-7 even at the physiological pH albeit with a somewhat lower potency.

To probe the effect of the nature of the NPs on the catalytic activity of MMP-7, we compared the above results with differently charged AuNPs. In contrast to LNPs, AuNPs were conceived to be non-flexible (i.e. “rigid”) in nature. Hence, if the surface charges were the only determinants for inhibiting MMP-7, both LNPs and AuNPs would be expected to yield an identical result. On the other hand, if the surface curvature and/or rigidity of NPs exhibited additive functions in inhibiting MMP-7, these NPs would behave differently even when they carried identically charges. To this end, we investigated the effect of differently charged AuNPs on the catalytic activity of MMP-7 under the same conditions used for the LNPs mediated inhibition. Figure 2 shows the inhibition of MMP-7 catalyzed reaction using differently charged NPs. Note that unlike LNPs, both Au-CNP and Au-ANP inhibit MMP-7 catalyzed reaction. On the other hand, Au-NNP exhibit marginal influence on the MMP-7 activity, similar to the neutral LNPs. The solid smooth line is the best fit of the data for the IC_{50} value is 0.178 ± 0.030 μ M. For Au-NNP the solid line represents the trend of the data points.

$$y = \frac{100\%}{\left(1 + \frac{(x)}{IC_{50}}\right)^s} \quad (\text{Eq. 1})$$

Where, Eq. 1 fits inhibition data to a two parameter equation, with the lower and upper limits of the background corrected as being equal to 0 and 100, respectively. The slope factor is taken to be 1.

We ascribed the experimentally determined IC_{50} values (from the data of Figure 2) to the inhibitory potency of Au-CNP and Au-ANP. On comparing the IC_{50} value of the above AuNPs with the K_i value of the cationic LNP, it is apparent that the former are more potent as well as less selective in inhibiting MMP-7 than the latter. The origin of the above difference is likely to partly lie in the “rigidity” as well as the surface curvature of AuNPs as compared to the cationic LNPs.

To ascertain whether the inhibitory feature exhibited by AuNPs was a true inhibition phenomenon and not due to partial inactivation of the enzyme, we performed the following experiment. The enzyme ($\sim 8 \mu\text{M}$) and Au-CNP ($2.1 \mu\text{M}$) were pre-incubated for different times, and 6 μl aliquots were withdrawn and transferred to the reaction mixture for measuring the enzyme activity. A control experiment was performed in the absence of Au-CNP. Figure 3 shows the enzyme catalyzed reaction progress curves under three different conditions: control (black line), incubation with Au-CNP for 0 time (red line), and 10 min (blue line). The rates of reactions (slopes) under the above conditions for the black, red, and blue lines were determined to be 0.38, 0.28, and $0.087 \Delta\text{F s}^{-1}$, respectively. On comparison of these reaction rates it is apparent that when the enzyme was pre-incubated with Au-CNP for 10 min, and an aliquot transferred to the reaction mixture (such that MMP-7 and Au-CNP concentrations were similar to those utilized in 0 time incubation experiment; red line), the enzyme's activity was impaired by 70%. Hence, besides Au-CNP dependent inhibition of MMP-7 (compare the slopes of the control and 0 time incubation experiments), the former inactivates the enzyme in a time dependent manner. It should be emphasized that such slow denaturation process was not observed in presence of inhibitory concentrations of cationic LNPs (data not shown). Hence, aside from the charges, rigidity/flexibility and/or surface curvature of the above NPs produce different influence on the catalytic activity of MMP-7.

We probed whether incubation of MMP-7 with Au-CNP resulted in changes in the secondary structure of the protein; we performed the Circular Dichroic spectroscopic studies. Figure 4 shows the changes in the molar ellipticity of MMP-7 as a function of increasing concentration of Au-CNP. Note that as the concentration of Au-CNP increases, the amplitude of the α -helical peak (at 222 nm) of MMP-7 decreases, suggesting that the enzyme is losing its secondary structural feature, and this feature can be easily ascribed to the Au-CNP mediated denaturation of the enzyme. No such pronounced effect was noteworthy when MMP-7 was titrated with increasing concentrations of LNPs (data not shown). These data are further supportive of the fact that aside from charges²⁵ the nature (particularly contributed by the rigidity/flexibility²⁶ and the surface curvature) of the NPs modulate the catalytic and/or structural features of MMP-7.

Figure 5 shows a model mechanism for the differential influence of cationic NPs on the structural-functional features of MMP-7. The negative electrostatic potentials in the vicinity of the active site pocket of the enzyme have been shown to form favorable contacts with both cationic LNP as well as Au-CNP. However, due to marked difference in the rigidity as well as the dimension (in the form of surface curvature) of LNP and Au-CNP, the latter induces the change in the enzyme conformation and thus leads to the denaturation of the protein. Hence,

Au-CNP causes both inhibition (due to complementary electrostatic interaction at the active site pocket) as well as inactivation of the enzyme. On the other hand, the lack of an extensive surface curvature coupled with the flexibility of the lipid bilayer, it is surmised that the cationic LNP only elicit the inhibitory influence on the MMP-7 catalyzed reaction.

Although this is the first demonstration of the influence rigid versus flexible NPs on the catalytic and structural features of MMP-7, there are literature precedents of our findings with other proteins^{27–29}. It has been argued that when proteins bind to the flexible surfaces (like LNPs), they adjust their conformations in response to the dynamic reorganization of the membrane. Depending on their intrinsic thermodynamic stabilities, both LNPs and their interacting protein partners can mutually cause structural deformities; such phenomena are often referred to as the “bending-dominated deformation” of flexible LNPs and “stretching-dominated response” of the intrinsically flexible protein³⁰. Besides, it has been known that proteins can attach to the Au-CNP surface via direct chemical cross linking or by “self assembly” via the electrostatic interaction^{25,31–34} such as that observed in the case of MMP-7. The proteins adsorbed on the “rigid” NPs surfaces often induce conformational changes, which are manifested in the changes in the local tertiary structures of protein followed by gradual unfolding to comparatively loose configuration, often leading to the denaturation of proteins. Hence, it is not surprising that unlike cationic LNP, Au-CNP causes inactivation of the enzyme presumably due to thermodynamic drive imposed by the preferential interaction of denatured (vis a vis native) form of MMP-7 to the AuNP surface.^{35–37} We are in the process of unraveling the molecular mechanism of interaction of MMP-7 to differently charged surfaces and we will report on these findings subsequently.

Acknowledgments

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ABBREVIATIONS

MMP-7	Matrix Metalloproteinase
AuNP	Gold Nanoparticles
LNPs	Lipid Nanoparticles
NPs	Nanoparticles
SUVs	Small Unilamellar Vesicles
Au-CNP	Cationic gold Nanoparticle
Au-ANP	Anionic gold Nanoparticle
Au-NNP	Neutral gold Nanoparticle

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Figure 1.

Effect of differently charged LNPs on MMP-7 catalyzed reaction. The time course of the MMP-7 catalyzed reaction was monitored (Ex = 335 nm, Em = 395 nm) using the fluorogenic peptide as a function of increasing concentrations of differently charged LNPs in 25 mM HEPES, 10 mM CaCl₂, pH 7.5. The symbol •-•, ○-○, and □-□ represent data obtained in presence of cationic, anionic and neutral LNPs respectively. [MMP-7] = 0.3 μM, [Substrate] = 10 μM at pH 7.5. Note unlike anionic and neutral LNPs, cationic LNP inhibits the MMP-7 catalyzed reaction. The solid smooth line is the best fit of the data according to Banerjee *et al* 20. The K_i value of the cationic LNP-MMP-7 complex was found to be 18.5 μM. For the anionic and neutral LNPs, the dashed line represents the trends of the data points.

Figure 2.

The effect of differently charged AuNPs on MMP-7 catalyzed reaction. Other experimental conditions are the same as in Figure 1. The symbol ●-●, ○-○, and □-□ are cationic, anionic and neutral AuNPs respectively. [MMP-7] = 0.3 μ M, [Substrate] = 10 μ M. Note that Au-CNP and Au-ANP (but not the Au-NNP) result in decrease in the MMP-7 catalyzed reaction. The solid smooth line is the best fit of the data for the anionic and cationic AuNPs. IC_{50} values for the Au-CNP and Au-ANP dependent inhibition data were obtained by fitting them by Eq 1 (see Results and Discussion), and its value was found to be 0.178 ± 0.030 μ M. For Au-NNP, the solid line represents the trends of the data points.

Figure 3.

Inhibition versus inactivation of MMP-7 by Au-CNP. The time course of the MMP-7 catalyzed reaction in the presence of Au-CNP is shown. Control experiment has been performed in the absence of Au-CNP. The time course of the reaction was monitored for about 1000 sec. Other conditions are same as in Figure 2. The black solid line, the red solid line and the blue solid line indicates the progress curves of MMP-7 alone, MMP-7 with 0.050 μM Au-CNP (0 time incubation) and MMP-7 (10 min pre-incubation time) respectively. The slopes ($\Delta F \text{ s}^{-1}$) of the progress curves were calculated for the above data were 0.38 (black line), 0.28 (red line), and 0.087 (blue line), respectively. Note that the incubation of MMP-7 with Au-CNP for 10 min resulted in an additional 70% loss in the enzyme activity.

Figure 4. Circular dichroic spectra of MMP-7 in the presence of increasing concentration of Au-CNP. The experiment was performed in 5 mM phosphate buffer, pH 7.5 between 210 – 270 nm region using Jasco J-815 spectropolarimeter. The concentration of Au-CNP varied increased from 0 to 5 μM . The solid green line indicates the CD spectra of 50 μM MMP-7 alone. The increasing concentration of Au-CNP results in the decrease in the magnitude of the negative ellipticity as shown by the vertical arrow.

Figure 5. Cartoon showing the influence of “flexible” versus “rigid” cationic surface on the structural feature of MMP-7.

Table 1

Types of Liposomes	K_d (μ M)
POPC	13.0 ± 1.4
POPS	0.73 ± 0.06
EOPC	5.0 ± 0.9

K_d values of differently charged LNPs - MMP-7 complexes¹