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Modulation of enzyme–substrate selectivity using tetraethylene glycol functionalized gold nanoparticles

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Abstract

Tetraethylene glycol (TEG) functionalized gold nanoparticles with 2 nm core diameters (AuTEG) enhance α-chymotrypsin (ChT) enzyme activity in a substrate-selective fashion. We explored the hydrolysis of four different substrates and observed a marked increase in activity with the most hydrophobic substrate *N*-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (TP), while the other substrates remain virtually unaffected by the AuTEG 'crowding effect' in solution. The enhancement in catalysis is indicated by an increase in K_{cat}/K_m as obtained from Lineweaver–Burk analysis and we hypothesize it to arise from a macromolecular crowding effect analogous to that observed with high molecular weight poly(ethylene glycol) (PEG) polymers.

S Supplementary data are available from stacks.iop.org/Nano/20/434004

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Knowledge about various biochemical processes has conventionally been acquired through *in vitro* studies performed under dilute conditions [1]. However, biomolecules often exhibit higher activities in the intracellular environment than are observed *in vitro* due to macromolecular crowding [2]. Typical cellular environments feature substantial concentrations of biomacromolecules that occupy 20–40% of the total cell volume [3]. To mimic these cellular environments, poly(ethylene glycol) (PEG) is commonly used as a co-solute in aqueous solutions because of the biocompatibility of PEG coupled with its large volume [4]. This excluded volume creates macromolecular crowding reminiscent of cellular environments, conveying multiple attributes found in biological systems [5]. For these reasons, PEG has recently been utilized in biolabeling [6], biocompatibilization [7], protein stabilization [8] and drug delivery methods [9].

In previous research [10] we have used tetraethylene glycol (TEG) functionalized nanoparticles to probe the binding, enzymatic activity and stability of α-chymotrypsin (ChT). TEG functionalized gold nanoparticles provide us with the ability to control ligand orientation, ligand functionality, binding interactions, particle shape and particle size [11]. This versatility offers the distinct opportunity to mimic cellular macromolecular crowding with controlled TEG monolayer functionalization.

In this study we report that 2 nm core gold nanoparticles functionalized with a TEG linker (AuTEG; figure 1), selectively alters the enzymatic activity of ChT. As with PEG polymers, the TEG units order solvent molecules into large solvent networks [5b] that create macromolecular crowding resembling cellular environments. In contrast to previous studies with PEG polymers [12] and nanoparticles [13], this crowding is substrate-dependent. In our studies, we found that only the most bulky and hydrophobic substrate displays an enhanced ChT binding affinity and stability in the presence of AuTEG. All other substrates remain unaffected by the AuTEG macromolecular crowding. To our knowledge, this is the first demonstration of the modulation of substrate selectivity by molecular crowding. Moreover, the introduction of stability [14], magnetic [15] and semi-conducting [16]

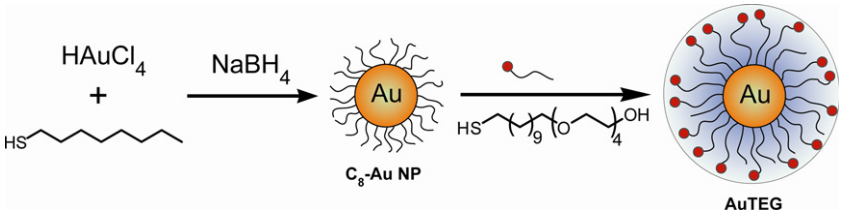

Figure 1. The AuTEG nanoparticle contains a 2 nm core and is functionalized with a mercaptoundecane ligand capped by TEG units using a place exchange reaction with octane thiol functionalized gold nanoparticles (C_8-Au NPs) by the Brust–Schiffrin method [17].

properties with carefully designed nanoparticles opens up ample opportunities for various biomedical applications.

2. Experimental section

2.1. General

α-Chymotrypsin (type II from bovine pancreas), *N*-succinyl-L-phenylalanine-*p*-nitroanilide (SPNA), *N*-glutaryl-phenylalanine-*p*-nitroanilide (GPNA) and *N*-benzyoyl-tyrosine-*p*-nitroanilide (BTNA) were purchased from Sigma. *N*-Succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanilide (TP) was purchased from Bachem. Synthesis of AuTEG was accomplished using our previous published procedure [18]. All experiments used 5 mM sodium phosphate buffer (pH 7.4) and were conducted at 30 °C.

2.2. Activity assays

Enzymatic activity assays were performed using a microplate reader (EL808IU, Bio-Tek Instruments). The enzymatic hydrolysis reaction was initiated by adding a substrate stock solution to a preincubated ChT–AuTEG solution to reach final substrate concentrations of [SPNA] = 2.08 mM, [GPNA] = 1.0 mM, [BTNA] = 100 μM and [TP] = 1.0 mM. The AuTEG concentration was 3.2 μM. The concentration of ChT varied with substrate. The hydrolysis of the substrate was measured using absorbance at 405 nm initially and then every 24 h for 3 days. The assays were performed in duplicates and triplicates. The standard deviation was usually less than 10%. A similar assay was performed using PEG (MW 1000) and PEG (MW 35 000). The concentrations of the PEG were 2 mM.

2.3. Lineweaver–Burk analysis

Stock solutions of TP substrate were made at various concentrations (6.4, 9.6, 16.0, and 22.4 mM) in ethanol. Concentrations of ChT and AuTEG remained constant at 0.1 μM and 1.6 μM, respectively. The enzymatic hydrolysis reaction was initiated by adding the substrate stock solution (16 μl) to a preincubated ChT–nanoparticle solution (184 μl) to reach a final substrate concentration. The assays were performed in duplicates and triplicates. The standard deviations were usually less than 10%.

2.4. Isothermal titration calorimetry

Experiments were performed using a VP-ITC Microcalorimeter, from Microcal, LLC. ChT (100 μM) was titrated into AuTEG (5 μM) solution and 50 injections of 5 μl were performed under constant stirring conditions (300 rpm). The change in heat was measured as a function of molar ratio.

2.5. Circular dichroism (CD)

Experiments were performed on a Jasco 720 spectropolarimeter using a quartz cuvette with a 1 cm path length. ChT (0.8 μM) was incubated with AuTEG (3.2 μM) for 30 min. Scans were recorded from 190 to 250 nm at a rate of 20 nm min^{-1}, sample interval at 0.2 nm and an 8 s response.

3. Results and discussion

Isothermal titration calorimetry (ITC) and gel electrophoresis were conducted to determine the nature of the interaction of AuTEG with ChT. ITC revealed no apparent complex formation between ChT and AuTEG (figure 2(a)). Gel electrophoresis supported the ITC data, as both ChT and AuTEG demonstrated distinct separate bands in the gel (supporting information available at stacks.iop.org/Nano/20/434004). Circular dichroism experiments showed the conserved secondary structure of ChT upon the addition of AuTEG (figure 2(b)), revealing its biocompatibility.

With the lack of particle–protein interaction verified, we next determined the consequences of addition of AuTEG upon enzymatic catalysis. ChT enzymatic activity was probed using four different substrates: SPNA, GPNA, BTNA and TP (figure 3(a)). The catalyzed hydrolysis reactions were followed spectroscopically by monitoring the formation of *p*-nitroanilide (figure 3(b)). Surprisingly, a two-fold increase in activity was observed for the TP substrate while the other three substrates, namely SPNA, GPNA and BTNA exhibited no change in enzymatic activity.

The selectivity of ChT for TP prompted further investigation. Previous research indicated that the TP substrate specifically exhibited a higher binding affinity to ChT than the other three substrates. Case *et al* [19] reported that ChT binding affinity showed a dramatic dependence upon peptide length. The proposed mechanism for this increase in binding is an extended active site with discrete subsites that bind hydrophobic amino acid residues [20]. Substrates with longer hydrophobic peptide chains such as TP bind more readily to these exterior sites, resulting in an increase in binding affinity.

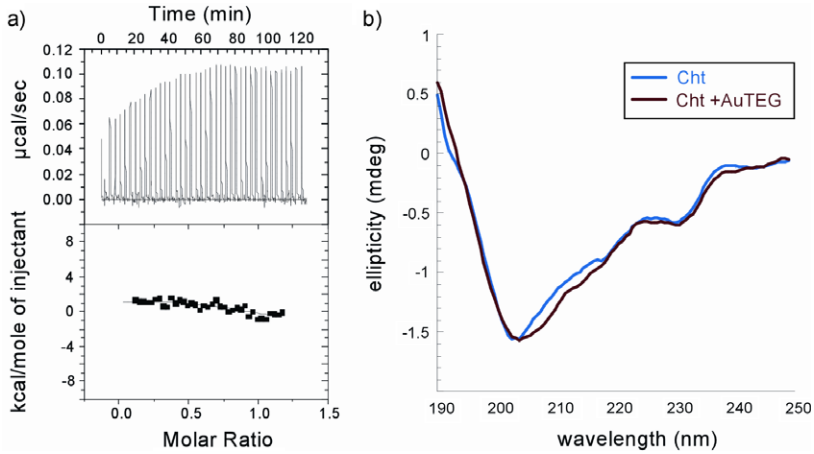

Figure 2. (a) Titration of the ChT (100 μM) into AuTEG (5 μM) shows no binding of AuTEG to ChT. (b) In the presence of AuTEG, ChT secondary structure is unaltered as seen via the conservation of minima at 232 and 202 nm.

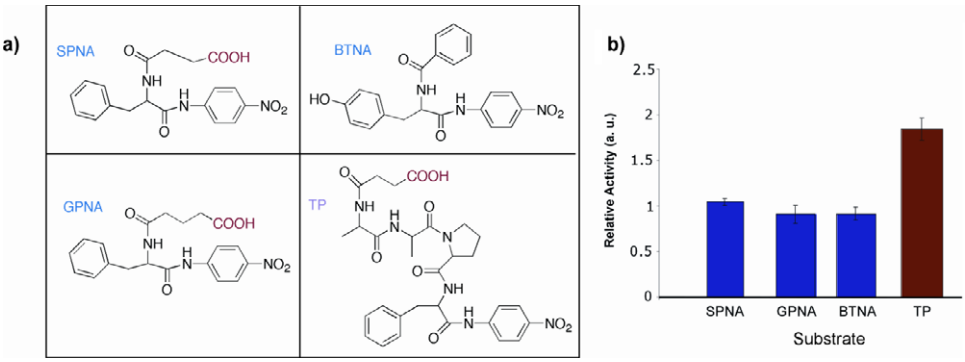

Figure 3. (a) Structures of SPNA, GPNA, BTNA and TP substrates. (b) Relative ChT activity in presence of AuTEG normalized to ChT activity without AuTEG in 5 mM sodium phosphate buffer solution (pH 7.4).

We investigated the Michaelis–Menten complex of ChT and TP in the presence of AuTEG to gain further insight into the selective enhancement of TP hydrolysis. Lineweaver–Burk analysis revealed critical information about TP binding affinity to ChT, specificity of ChT for TP and the rate of the hydrolysis reaction. The inverse velocity of the *p*-nitroanilide product formation was plotted versus the inverse TP concentration. Through Cornish-Bowden and Wharton statistics [21] K_m, K_{cat} and K_{cat}/K_m values were calculated (figure 4) to visualize the nature of the ChT–TP complex.

Changes in both K_m and K_{cat}/K_m indicated that AuTEG affects the TP binding affinity to ChT and the overall ChT selectivity. The decrease in K_m for ChT (4.3 to 2.1 mM) when AuTEG was added to the ChT solution (figure 4) indicates that there is an increase in affinity of ChT for the TP substrate. This increase in affinity is reflected in K_{cat}/K_m, which increased from 2300 to 5600 M^{-1} s^{-1}, a 2-fold increase in ChT–TP activity.

Our hypothesis for the increase in activity of ChT with the TP substrate is that AuTEG modulates the ChT–TP binding affinity via an excluded volume mechanism [22] that is similar to macromolecular crowding in polyethylene glycol (PEG) solutions [23]. The TEG units on AuTEG organize solvent molecules into large solvent networks,

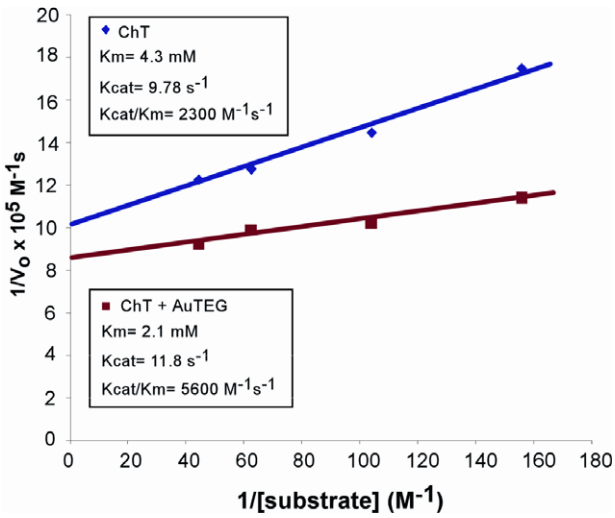

Figure 4. Lineweaver–Burk analysis of ChT in the absence and presence of AuTEG, demonstrating enhanced affinity of ChT for TP and an increase in specificity for TP (K_{cat}/K_m).

causing macromolecular crowding [24]. As a result of macromolecular crowding, the effective concentrations of TP and ChT should increase when AuTEG is added to

the ChT solution. This claim was supported by the apparent decrease in K_m for ChT: upon addition of AuTEG, less TP substrate was needed to provide the same ChT enzymatic activity, indicating a higher effective concentration of TP in the ChT + AuTEG solution. Dynamic light scattering (DLS) experiments revealed that the hydrodynamic diameter of the AuTEG is ∼16 nm (supporting information available at stacks.iop.org/Nano/20/434004), while the actual diameter of the AuTEG nanoparticle (including ligands) is ∼8 nm (supporting information available at stacks.iop.org/Nano/20/434004). The increase in particle size can only be explained as a large solvent network around the particles, since there is little or no aggregation of AuTEG, as evidenced by a very weak plasmon band at absorption at 520 nm [25]. In terms of the longer chain TP substrate, the AuTEG 'crowding effect' reduced the number of free water molecules surrounding the substrate. The TP hydrophobic amino acids are now selectively more exposed to ChT and can readily interact with the extended subsites of ChT as well as the active site, as observed via the increase in K_{cat}/K_m. The K_{cat} values indicated that the rate of the hydrolysis reaction was unaffected by the addition of AuTEG. The local active site environment of ChT retained its native structure (as seen previously with CD) and was unaltered by the presence of ordered solvent molecules. Due to the mere size of AuTEG, the nanoparticle is expected to be sterically excluded from any interaction with the active site of the ChT [26]. Therefore, it was reasonable to observe similar K_{cat} values upon addition of the AuTEG.

To test the molecular crowding hypothesis, two linear polymers, PEG 1000 (MW 1000 Da) and PEG 35 000 (MW 35 000 Da), were added as co-solutes for ChT. In these studies PEG 35 000 exhibited similar behavior as the AuTEG (figure 5), enhancing selectivity with the TP substrate. The smaller PEG polymer did not show any selectivity between substrates. Instead, it appeared to slightly inhibit the reaction of ChT and TP. This was reasonable because the smaller PEG 1000 may not be excluded from the active site as would be expected with the larger PEG and AuTEG molecules. The PEG 1000 may diffuse into the active site, blocking access for the TP substrate. This was supported by the DLS measurements that show an increase in the hydrodynamic diameter of ChT from 4.5 to 7.1 nm upon the addition of PEG 1000. Therefore, the organization of the solvent molecules may be directly correlated to the size and orientation of the polyethylene glycol units from the PEG polymer [27].

4. Conclusion

We demonstrated the ability to selectively control enzyme activity and stability through AuTEG macromolecular crowding interactions. Upon addition of AuTEG, enhanced selectivity of ChT is exhibited to the bulkier, more hydrophobic substrate TP due to the 'crowding effect' created by AuTEG in solution. This molecular crowding forces the TP substrate into the extended ChT subsites, causing a selective 2-fold increase in ChT activity, similar to high molecular weight PEG solutions. The shorter chain substrates, however, remain unaffected by this crowding effect because they do not interact with the extended ChT subsites. We believe that this method of introducing substrate selectivity along with enhanced activity could have utility in fundamental studies as well as biomedical applications.

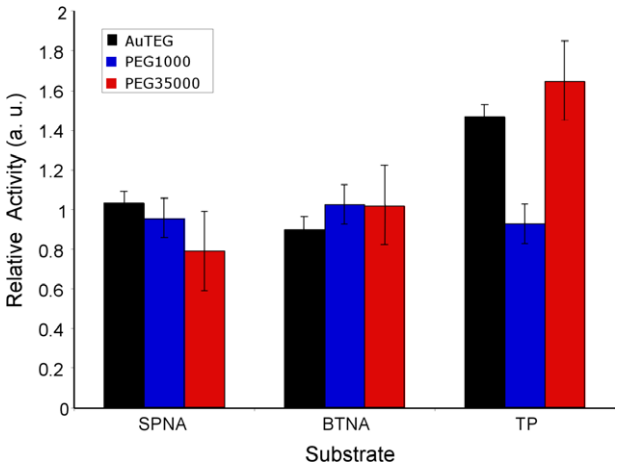

Figure 5. Relative activity of ChT to different substrates in the presence of PEG 1000, PEG 35 000 and AuTEG normalized to the ChT activity in the absence of the co-solutes. The long chain linear PEG polymer PEG 3500 displays similar selectivity to the hydrophobic TP substrate as AuTEG.

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References

[1] Zhou H-X, Rivas G and Minton A P 2008 *Annu. Rev. Biophys.* **37** 375
Minton A P 2001 *J. Biol. Chem.* **276** 10577
[2] Lightcap C M, Sun S, Lear J D, Rodeck U, Polenova T and Williams J C 2008 *J. Biol. Chem.* **283** 27314
Al-Haboori M 2001 *J. Biochem. Cell Biol.* **33** 844
Hu Z, Jiang J and Rajagopalan R 2007 *Biophys. J.* **93** 1464
[3] Ellis J R and Minton A P 2003 *Nature* **425** 27
Wenner J R and Bloomfield V A 1999 *Biophys. J.* **77** 3234
Davis-Searles P R, Saunders A J, Eric D A, Winzor D J and Pielak G J 2001 *Annu. Rev. Biophys. Biomol. Struct.* **30** 271
[4] Nakano S, Hisae K, Ohmichi T, Kawakami J and Sugimoto N 2004 *J. Am. Chem. Soc.* **126** 14330
[5a] Totani K, Ihara Y, Matsuo I and Ito Y 2008 *J. Am. Chem. Soc.* **130** 2101
[5b] Timasheff S N and Arakawa T 1985 *Biochemistry* **24** 6756
[5c] Vergara A, Paduano L and Sartorio R 2002 *Macromolecules* **35** 1389
[6] Schering C A, Zhong B, Woo J C G and Silverman R B 2004 *Bioconjug. Chem.* **15** 673
[7] Otsuka H, Nagasaki Y and Kataoka K 2004 *Langmuir* **20** 11285
Castellanos I J, Al-Azzam W and Griebenow K 2005 *J. Pharm. Sci.* **94** 327

Tao S L, Popat K C, Norman J J and Desai T A 2008 *Langmuir* **24** 2631
[8] Taluja A and Bae Y H 2007 *Pharm. Res.* **24** 1517
Arakawa T, Prestrelski S J, Kenney W C and Carpenter J F 1993 *Adv. Drug Deliv. Rev.* **10** 1
Otsuka H, Nagasaki Y and Kataoka K 2004 *Langmuir* **20** 11285
[9] Lin C-C and Anseth K S 2009 *Pharm. Res.* **26** 631
Filpula D and Zhao H 2008 *Adv. Drug Deliv. Rev.* **60** 29
Leuner C and Dressman J 2000 *Eur. J. Pharm. Biopharm.* **50** 47
Beronese F M 2001 *Biomaterials* **22** 405
Zahr A S, Villiers M and Pishko M V 2005 *Langmuir* **21** 403
[10] You C-C, Agasti S S, De M, Knapp M J and Rotello V M 2006 *J. Am. Chem. Soc.* **128** 14612
Fischer N O, Verma A, Goodman C M, Simard J M and Rotello V M 2003 *J. Am. Chem. Soc.* **125** 13387
Hong R, Fischer N O, Verma A, Goodman C M, Emerick T and Rotello V M 2004 *J. Am. Chem. Soc.* **126** 739
[11] Boal A and Rotello V M 2000 *J. Am. Chem. Soc.* **122** 734
Agasti S S, You C-C, Arumugam P and Rotello V M 2008 *J. Mater. Chem.* **18** 70
De M, You C-C, Srivastava S and Rotello V M 2007 *J. Am. Chem. Soc.* **129** 10747
[12] Stone M, Harvey S B, Kisiel W, Foster D and Nelsestuen G L 2002 *Biochemistry* **41** 15820
[13] Fischer N O, McIntosh C M, Simard J M and Rotello V M 2002 *Proc. Natl Acad. Sci. USA* **99** 5018
Hong R, Emerick T and Rotello V M 2004 *J. Am. Chem. Soc.* **126** 13572
[14] Stakenborg T, Peeters S, Reekmans G, Laureyn W, Jans H, Borghs G and Imberechts H 2008 *J. Nanopart. Res.* **10** 143
[15] Konwarh R, Karak N, Rai S K and Mukherjee A K 2009 *Nanotechnology* **20** 225107
[16] Gopee N V *et al* 2009 *Toxicol. Sci.* **111** 37
[17] Kanaras A G, Kamounah F S, Kjeld S, Kiely C J and Brust M 2002 *Chem. Commun.* 2294
[18] Verma A, Simard J M and Rotello V M 2004 *Langmuir* **20** 4178
Paulini R, Frankamp B L and Rotello V M 2002 *Langmuir* **18** 2368
[19] Case A and Stein R L 2003 *Biochemistry* **42** 3335
Hedstrom L 2002 *Chem. Rev.* **102** 4501
Peczuh M W and Hamilton A D 2000 *Chem. Rev.* **100** 2479
[20] Case A and Stein R L 2003 *Biochemistry* **42** 3335
Case A, Huskey P H and Stein R L 2003 *Biochemistry* **42** 4727
Kunugi S, Hirohara H and Ise N 1979 *J. Am. Chem. Soc.* **101** 3640
Blow D M 1976 *Acc. Chem. Res.* **9** 145
[21] Tinoco I, Sauer K, Wang J C and Puglisi J D 2002 *Physical Chemistry; Principles and Applications in Biological Sciences* (Englewood Cliffs, NJ: Prentice-Hall) p 314
[22] Ellis J R 2001 *Trends Biochem. Sci.* **26** 597
Kuntz I D and Kauzmann W 1974 *Adv. Protein Chem.* **28** 239
Hermans J 1982 *J. Chem. Phys.* **77** 2193
[23] Vergara A, Capuano F, Paduano L and Sartorio R 2006 *Macromolecules* **39** 4500
Lucarini M, Franchi P, Pedulli G F, Pengo P, Scrimin P and Pasquato L 2004 *J. Am. Chem. Soc.* **126** 9326
Dominak L M and Keating C D 2008 *Langmuir* **24** 13565
[24] Vergara A, Paduano L and Sartorio R 2002 *Macromolecules* **35** 1389
Lee L L Y and Lee J C 1987 *Biochemistry* **26** 7813
Simon L M, Kotorman M, Garab G and Laczko I 2002 *Biochem. Biophys. Res. Commun.* **293** 416
[25] Wei A 2004 Enhanced optical properties from metal nanoparticles and their ensembles *Nanoparticles, Building Blocks for Nanotechnology* (New York: Kluwer Academic/Plenum) p 173
[26] Timasheff S N and Arakawa T 1985 *Biochemistry* **24** 6756
[27] Nakano S, Hisae K, Ohmichi T, Kawakami J and Sugimoto N 2004 *J. Am. Chem. Soc.* **126** 14330