Supramolecular Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713649759

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To cite this Article Villalonga, Reynaldo , Fragoso, Alex , Cao, Roberto , Ortiz, Pedro D. , Villalonga, Maria L. and Damiao, Angelo E.(2005) 'Supramolecular-mediated Immobilization of Trypsin on Cyclodextrin-modified Gold Nanospheres', Supramolecular Chemistry, 17: 5, 387 — 391
To link to this Article: DOI: 10.1080/10610270500126743
URL: http://dx.doi.org/10.1080/10610270500126743

Supramolecular Chemistry, July–August 2005 Vol. 17 (5), pp. 387–391

Supramolecular-mediated Immobilization of Trypsin on Cyclodextrin-modified Gold Nanospheres

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Received (in Austin, USA) 14 December 2004; Accepted 28 February 2005

Bovine pancreatic trypsin was immobilized on β- and γ-cyclodextrin coated gold nanospheres via supramolecular associations. The enzyme retained 100%–120% of its catalytic activity and its thermal stability at 50°C was 2–2.5 fold increased in the presence of the β- and γ-cyclodextrin modified metal nanoparticles, respectively. The influence of these immobilization processes on the conformational properties of the enzyme was studied by fluorescence spectroscopy. These results open a new perspective to the possible application of cyclodextrin-modified gold nanospheres as water-soluble carriers for enzyme immobilization.

Keywords: Cyclodextrin; Enzyme stability; Gold nanospheres; Trypsin

INTRODUCTION

Special interest has been devoted to use nanoparticles as three-dimensional matrix for enzyme immobilization in aqueous solution [1,2]. Such enzyme-colloid systems exhibit potential applications as catalysts in fine organic chemistry [3] and for preparing novel biosensor devices [4], a prospective area in nanotechnology.

Supramolecular associations with cyclodextrin (CD) derivatives have been recently described by our groups as a useful strategy for increasing the stability of enzymes against heat treatment and proteolytic degradation, without affecting their catalytic behaviors. Chemical [5,6] and enzymatic [7] covalent attachment of CD derivatives and polymers [8] at the enzyme surface, as well as addition of water-soluble CD-containing polysaccharides [9] have been the strategies developed. Additionally, we reported a new procedure for immobilizing protein structures on the CD-coated surface of silver electrodes [10] based on the ability of CDs to form inclusion complexes with hydrophobic guests [11]. In the present work, we describe the supramolecular-mediated adsorption of bovine pancreatic trypsin (EC 3.4.21.4) on the surface of CD-modified gold nanospheres (Scheme 1), and the influence of this interaction on the thermal stability and catalytic activity of the enzyme.

EXPERIMENTAL SECTION

Materials

N-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) and bovine pancreatic trypsin (36 U/mg *vs* BAEE [12]) were obtained from Merck. CDs were purchased from Amaizo (analytical grade) and used as received after checking purity by ^{1}H NMR. All other chemicals were of analytical grade.

Preparation of CD-modified Gold Nanospheres

The nanospheres were prepared by the method developed by Kaifer and coworkers using perthiolated-βCD and γCD derivatives as capping molecules [13], and [CD]/[$AuCl_4^-$] ratios of 0.20 and 0.077, respectively. The synthesis of perthiolated derivatives of βCD and γCD was also performed as previously described [13]. The amount of oligosaccharides attached to the nanoparticles was estimated by the redissolution method [14] to be

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ISSN 1061-0278 print/ISSN 1029-0478 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/10610270500126743

SCHEME 1

10 and 12 molecules of βCD and γCD respectively, assuming that the nanoparticles are perfect spheres. High-resolution electron microscopy (HREM) images of the nanospheres were obtained using JEOL 4000-EX instrument, operating at 400 kV. The particle size distribution was obtained from digitalized amplified micrographs by averaging the larger and smaller axis diameters measured in each particle.

Analytical Determinations

Esterolytic activity of native and modified trypsin was determined at 25°C in 67 mM Tris/HCl buffer, pH 8.0, using BAEE as substrate [12]. One unit of esterolytic activity was defined as the amount of enzyme that hydrolyses 1.0 μmol BAEE per minute at 25°C. Protein concentration was estimated by the Lowry method using bovine serum albumin as standard.

The molecular weight of the preparations was determined by analytical GPC on TSK G3000SW column (7.5 × 60 cm), calibrated with protein standards from the Oriental Yeast Co., Ltd. (Japan).

The fluorescence emission spectra of native and immobilized trypsin, before and after 1 h incubation at 55°C, were measured with 1.03 nM enzyme solutions in 50 mM sodium acetate buffer, pH 5.0 using a RF-1501 Shimadzu spectrofluorophotometer. The protein solution was excited at 280 nm and emission scanned between 300–400 nm.

RESULTS AND DISCUSSION

Dark red and water soluble gold nanospheres were prepared by *in situ* modification of growing colloids with perthiolated-βCD and γCDs. These particles showed average diameters of 2.9 and 4 nm, respectively, as determined by HREM (Fig. 1). The nanospheres showed the characteristic surface plasmon resonance due to gold with a maximum absorption around 510 nm (Fig. 2).

The interaction of trypsin with the CD-capped nanoparticles was studied by electronic spectroscopy. Upon addition of trypsin, the intensity of the surface plasmon resonance increases for both types of nanospheres, indicating interaction of the enzyme protein molecules with the metal surface of the colloids. It has been shown that adsorbate-induced aggregation of metal colloids leads to increased absorption of the plasmon resonance [4]. Similar increased absorptions combined with a red shift of the maximum have been explained by an increase of the local refractive index in the vicinity of the colloid surface [15] and has been observed for other biomolecules [16]. Interestingly, in our case the increase on absorbance is significant although the red-shift observed is negligible.

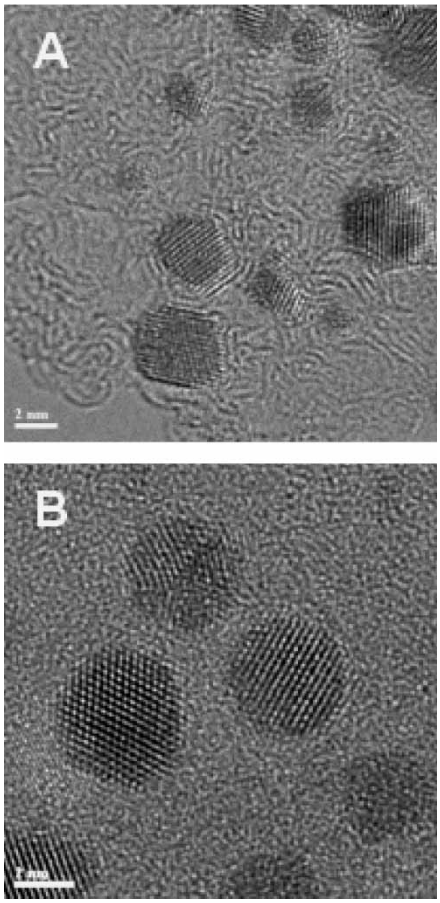

FIGURE 1 HREM image of βCD- (A) and γCD-gold nanospheres (B).

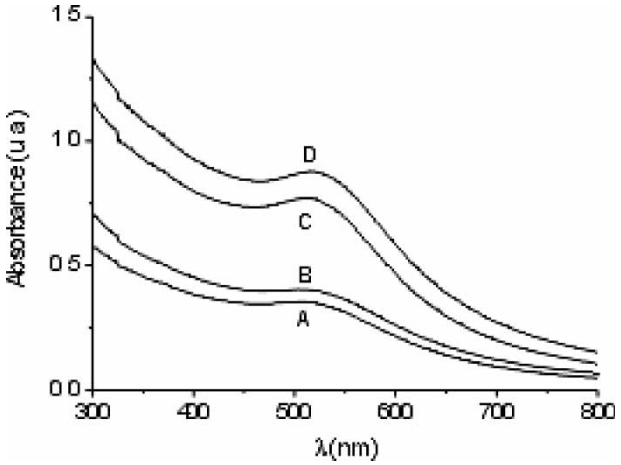

FIGURE 2 UV–vis spectra of the β and, γCD-modified gold nanospheres (0.5 mg nanosupport/ml) at 25°C in 50 mM sodium acetate buffer, pH 5.0 in the absence and in the presence of 20μg/ml trypsin. A: βCD-gold nanospheres, B: βCD-gold nanospheres + trypsin, C: γCD-gold nanospheres, D: γCD-gold nanospheres + trypsin.

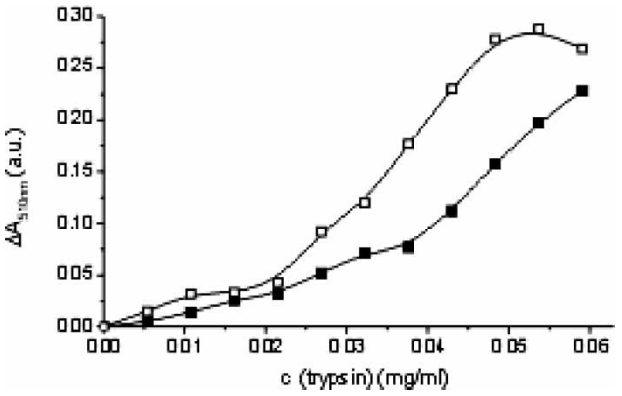

FIGURE 3 Effects of trypsin concentration on increase in surface plasmon resonance of β (■) and, and γCD-modified gold nanospheres (□) (0.5 mg nanosupport/ml) at 25°C in 50 mM sodium acetate buffer, pH 5.0.

A possible mechanism for trypsin-induced aggregation of the gold colloid could be the supramolecular interaction between the aromatic residues located at the surface of trypsin and the CD moieties coating the metal core, leading to the formation of intercolloid supramolecular associations. This fact is not surprising considering that it has been previously demonstrated that CDs retained their host properties after immobilization on gold nanospheres [13].

Figure 3 reports the influence of protein concentration on the increase in the surface plasmon resonance of both CD-modified gold nanoparticles. ΔA_{510nm} increased for the γCD-gold colloid with increasing trypsin concentration up to 0.048 mg/ml and then slowly reached a saturation value. A detailed study of this solution reveals that this colloidal system becomes unstable at trypsin concentrations higher than 0.048 mg/ml, appearing a fine precipitate with time. On the contrary, the trypsin-βCD-gold system was stable in all range of protein concentration studied, increasing $\Delta A_{510\,nm}$ as the enzyme is added. These differences in colloid stability suggest that the interaction of trypsin with γCD particles is stronger, leading to a faster precipitation and could be associated with higher content of CD hosts on the particle surface with respect to the βCD particles. As γCD hosts have a greater diameter, the possibility of forming inclusion complexes with a broader variety of aromatic amino acid residues or lateral chains of the protein is favored thus increasing the enzyme-particle interaction. However, the possible inconvenience of precipitation during the activity measurements (vide infra) is avoided working at trypsin concentrations lower than 0.048 mg/ml.

The formation of high molecular weight species in both systems trypsin-CD Au nanospheres was confirmed by HPLC experiments, obtaining broads with maximum at 107 kDa and 123 kDa, respectively. It is expected that multimeric enzyme aggregates were formed in trypsin solution by supramolecular cross-linking with the CD-capped Au nanoparticles.

The esterolytic activity of trypsin (20 μg/ml) toward BAEE remained the same after addition of the βCD-coated particles at 0.5 mg/ml final concentration. Interestingly, in the presence of the γCD-modified colloid at same concentration the esterolytic activity of the enzyme was increased in about 20%. In previous reports, we have demonstrated that CDs covalently attached to the surface of trypsin actually increase its catalytic activity [5,6,8], presumably by increasing the local substrate concentration by interaction with neighboring CD hosts. This mechanism is also possible in this case, as CDs are expected to be located in close proximity to the enzyme surface. It is also possible that a most active enzyme conformation is induced for trypsin by interaction with the γCD-coated nanospheres, although this hypothesis is not easy to verify.

Enzymes are quickly inactivated at high temperatures due to irreversible unfolding and protein aggregation [17]. The later appears as the predominant mechanism for trypsin thermal inactivation, according to previous studies [18]. Figure 4 shows the time course of inactivation of trypsin preparations at 50°C in pH 5.0 buffer. The addition of CD-modified gold nanospheres conferred resistance to the enzyme against heat treatment: the half-life times of trypsin at this temperature were 2- and 2.5-fold increased in the presence of the βCD- and γCD-gold colloids, respectively. This result suggests that the aggregation processes occurring in trypsin at high temperatures are avoided by its supramolecular association with the nanospheres. As the thermoestability also depends on protein rigidity, is

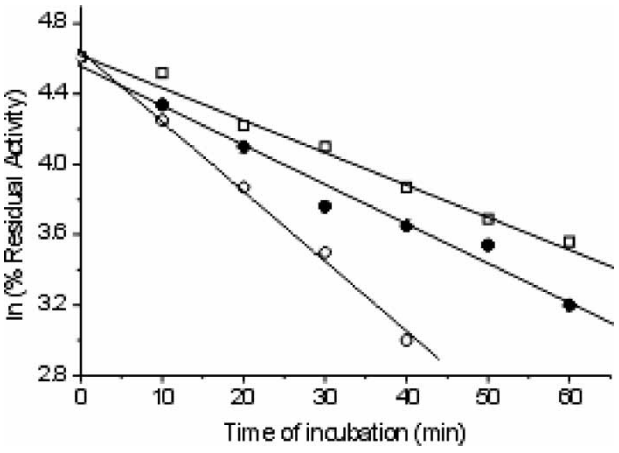

FIGURE 4 Kinetics of thermal inactivation of trypsin (20 μg/ml) at 50°C in the absence (○) and in the presence of β (■) and, and γCD-modified gold nanospheres (□) (0.5 mg nanosupport/ml). Enzyme preparations were incubated at 50°C in 50 mM sodium acetate buffer, pH 5.0 during 1 h. Samples were removed at scheduled times, chilled quickly, and assayed for enzymatic activity.

it clear that the CD-gold colloids confer the protein a less favorable environment to unfolding as they surround and entrap the enzyme preventing inter-protein hydrophobic interactions. Similar thermal stabilization was reported for trypsin in the presence of CD-containing polysaccharides in aqueous media [9].

On the other hand, exposed secondary hydroxyl groups of CDs at the surface of the nanoparticles confer high hydrophilic characteristics to these colloids. This hydrophilic microenvironment could also stabilize trypsin molecules after supramolecular adsorption, preventing protein denaturation at high temperatures.

Figure 5 shows the fluorescence spectra of native and immobilized enzyme forms before and after thermal treatment at 55°C. The fluorescence spectra of native and immobilized trypsin on βCD-modified gold nanoparticles upon excitation at 280 nm showed an identical emission maximum at 340 nm, which is characteristic of tryptophan residues partially shielded from the surrounding medium [19]. This fact suggests that the conformational structure of the enzyme was retained after immobilization on the βCD-coated nanoparticles. However, the fluorescence spectra of the catalyst prepared with the γCD-gold colloids showed a slight shift to lower wavelengths, indicating that a more compact protein structure is formed.

Thermal treatment at 55°C resulted in the unfolding of non-immobilized enzyme, leading to a distinct red shift in the wavelength maximum, accompanied by a decrease in the fluorescence intensity. On the contrary, both immobilized trypsin forms showed, after identical heat treatment, smaller decrease in the fluorescence intensity and a slight shift to lower wavelengths. These results indicate that a more compact protein structure is formed after thermal incubation of the trypsin-CD gold nanoparticles adducts. This phenomenon could be explained by the hydrophobic nature of the proposed supramolecular interaction between the protein and the CD-caped metal nanospheres. In fact, it has been previously demonstrated that the stabilizing energy of hydrophobic interactions increases with the increase of temperature [20]. This kind of "chaperone-like activity" over protein structures has been previously reported for CDs [21], but not for CD-coated nanosized supports.

It should be highlighted that this change in the fluorescence emission spectrum was noticeably

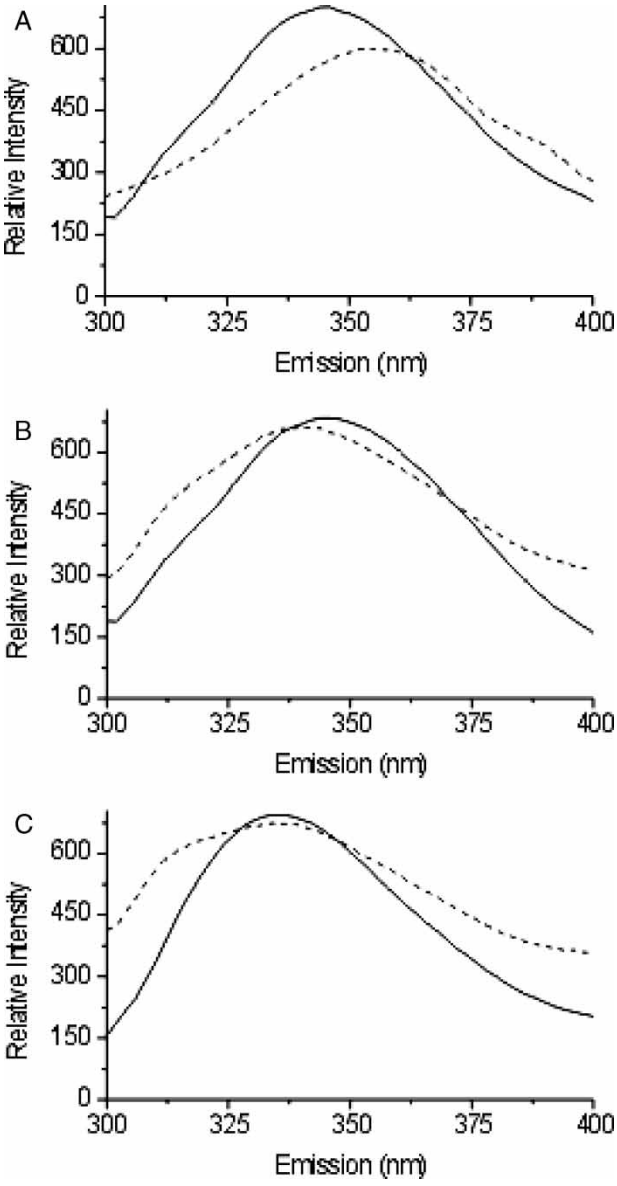

FIGURE 5 Fluorescence emission spectra of native (A) and immobilized trypsin on βCD- (B) and γCD-gold colloids (C) before (—) and after (. . . . .) 1 h incubation at 55°C.

higher for the nanocatalyst prepared with the γCD-gold colloids, indicating higher interaction between this nanomaterial and the enzyme protein structure.

In conclusion, we have reported the formation of supramolecular complexes between bovine pancreatic trypsin and CD-coated gold nanoparticles in aqueous solution. This interaction conferred an enhanced catalytic activity and thermal stability to the enzyme and is in agreement with previous results from our groups on the stabilization of enzymes by covalent modification or addition of CDs-containing systems. Our results open a new perspective to the possible application of CD-modified gold nanospheres as water-soluble carriers for enzyme immobilization.

Acknowledgements

We thanks Prof. Yasuhisa Asano for provide us fluorescence spectroscopy facilities. This research was supported by The Third World Academy of Sciences, through a grant to R. Villalonga (Grant 01-279 RG/CHE/LA). Financial support from the International Foundation for Science, Stockholm, Sweden, and the Organization for the Prohibition of Chemical Weapons, The Hague, The Netherlands, through a grant to R. Villalonga (Grant F/3004-1) is also gratefully acknowledged.

References

[1] Kumar, C. V.; McLendon, G. L. *Chem. Mater.* **1997**, *9*, 863.
[2] Gole, A.; Dash, C.; Soman, C.; Sainkar, S. R.; Rao, M.; Sastry, M. *Bioconjugate Chem.* **2001**, *12*, 684.
[3] Schuler, C.; Caruso, F. *Macromol. Rapid Commun.* **2000**, *21*, 750.
[4] Schultz, D. A. *Curr. Opin. Biotechnol.* **2003**, *14*, 13.
[5] Fernández, M.; Fragoso, A.; Cao, R.; Baños, M.; Villalonga, R. *Enzyme Microb Technol.* **2002**, *31*, 543.
[6] Fernández, M.; Fragoso, A.; Cao, R.; Villalonga, R. *J. Mol. Catalysis B* **2003**, *21*, 133.
[7] Villalonga, R.; Fernández, M.; Fragoso, A.; Cao, R.; Di Pierro, P.; Mariniello, L.; Porta, R. *Biotechnol. Bioeng.* **2003**, *81*, 732.
[8] Villalonga, M. L.; Fernández, M.; Fragoso, A.; Cao, R.; Villalonga, R. *Prep. Biochem. Biotechnol.* **2003**, *33*, 53.
[9] Fernández, M.; Villalonga, M. L.; Caballero, J.; Fragoso, A.; Cao, R.; Villalonga, R. *Biotechnol. Bioeng.* **2003**, *83*, 743.
[10] Fragoso, A.; Caballero, J.; Almirall, E.; Villalonga, R.; Cao, R. *Langmuir* **2002**, *18*, 5051.
[11] Szejtli, J. *Chem. Rev.* **1998**, *98*, 1743.
[12] Schwert, G. B.; Takenaka, Y. *Biochim. Biophys. Acta* **1955**, *16*, 570.
[13] Liu, J.; Ong, W.; Román, E.; Lynn, M. J.; Kaifer, A. E. *Langmuir* **2000**, *16*, 3000.
[14] Liu, J.; Alvarez, J.; Ong, W.; Román, E.; Kaifer, A. E. *J. Am. Chem. Soc.* **2001**, *123*, 11148.
[15] Templeton, A. C.; Pietron, J. J.; Murray, R. W.; Mulvaney, P. *J. Phys. Chem.* **2000**, *104*, 564.
[16] Nath, N.; Chilkoti, A. *Anal. Chem.* **2002**, *74*, 504.
[17] Klibanov, A. M. *Adv. Appl. Microbiol.* **1983**, *29*, 1.
[18] Moskvichyov, B. V.; Komarov, E. V.; Ivanova, G. P. *Enz. Microb. Technol.* **1986**, *8*, 498.
[19] Cho, Y.; Gu, W.; Watkins, S.; Lee, S. P.; Kim, T. R.; Brady, J. W.; Batt, C. A. *Protein Eng.* **1994**, *7*, 263.
[20] Privalov, P. L.; Gill, S. J. *Adv. Protein Chem.* **1988**, *39*, 191.
[21] Park, Y. D.; Wu, B. N.; Tian, W. X.; Zhou, H. M. *Biochemistry (Moscow)* **2002**, *67*, 914.