Articles

Direct Assembly of Gold Nanoparticle "Shells" on Polyurethane Microsphere "Cores" and Their Application as Enzyme Immobilization Templates

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The assembly of aqueous gold nanoparticles on the surface of polyurethane (PU) spheres leading to [gold nanoparticle shell]−[polyurethane core] structures is demonstrated. The assembly of gold nanoparticles on the polymer microspheres occurs through interaction of the nitrogens in the polymer with the nanoparticles. Such direct assembly obviates the need to perform additional surface modification of the polymer microspheres, which is an important step in other polymer-based core−shell structure protocols. The nanogold−PU material is then conjugated with the enzyme pepsin, leading to the formation of a new class of biocatalyst. In relation to the free enzyme in solution, the new bioconjugate material exhibited a slightly higher biocatalytic activity and significantly enhanced pH and temperature stability. The use of gold nanoparticle-labeled polymer microspheres in pepsin bioconjugation enables easy separation from the reaction medium and reuse of the bioconjugate over six reaction cycles.

Introduction

Nanotechnology is currently witnessing impressive advances in aspects such as synthesis of nanoparticles, understanding their fundamental physical and chemical properties,[1] and organization of nanoscale matter using weak, noncovalent interactions.[2] One fascinating area with important application in the areas of coatings, electronics, photonics, and catalysis is the controlled engineering of colloidal surfaces to yield composite "core−shell" structures.[3] A number of different colloidal "cores" such as TiO_2[3] and SiO_2[4] have been investigated, and spheres of polystyrene (PS) of submicron and micron sizes have also received considerable attention.[5] The capping of PS spheres with different nanoparticles has been accomplished using a layer-by-layer (lbl) assembly protocol employing polyelectrolytes as the glue between the PS core and nanoparticle "shell".[5b,c]

Immobilization of biomolecules on different surfaces is an area of great interest. Such biomaterials have numerous applications in the areas of biotechnology, immunosensing, and biomedical applications.[6] Biomolecules such as proteins have been immobilized on various supports such as phospholipid bilayers,[7] self-assembled monolayers,[8] Langmuir−Blodgett films,[9] polymer matrix,[10] and galleries of α-zirconiumphosphate[11] and in thermally evaporated fatty lipid films.[12] The use of nanoparticles with their extremely high surface-to-volume ratio in the immobilization of biomolecules dates back to the early 1980s.[13] Both polymer[14−16] and inor-

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(1) Edelstein, A. S., Cammarata, R. C., Eds. *Nanomaterials: Synthesis, Properties and Applications*; Institute of Physics Publishing: Philadelphia, PA, 1996.

(2) (a) Fendler, J. H.; Meldrum, F. C. *Adv. Mater.* **1995**, *5*, 607. (b) Sastry, M. *Curr. Sci.* **2000**, *78*, 1089. (c) Shipway, A. N.; Katz, E.; Willner, I. *ChemPhysChem* **2000**, *1*, 19.

(3) Davies, R.; Schurr, G. A.; Meenan, P.; Nelson, R. D.; Bergna, H. E.; Brevett, C. A. S.; Goldbaum, R. H. *Adv. Mater.* **1998**, *10*, 1264.

(4) Velikov, K. P.; van Blaaderen, A. *Langmuir* **2001**, *17*, 4779.

(5) (a) Radichenko, I. L.; Sukhorukov, G. B.; Gaponik, N.; Kornowski, A.; Rogach, A. L.; Mohwald, H. *Adv. Mater.* **2001**, *13*, 1684. (b) Caruso, F.; Lichtenfeld, H.; Mohwald, H.; Giersig, M. *J. Am. Chem. Soc.* **1998**, *120*, 8523. (c) Ji, T.; Lirtsman, V. G.; Avny, Y.; Davidov, D. *Adv. Mater.* **2001**, *13*, 1253.

(6) (a) Tischer, W.; Wedekind, F. *Top Curr. Chem.* **1999**, 95 and references therein. (b) Rembaum, A.; Dreyer, W. J. *Science* **1980**, *208*, 364. (c) Wilchek, M.; Bayer, E. A. *Methods Enzymol.* **1990**, *184*, 5. (d) de Wildt, R. M. T.; Mundy, C. R.; Gorick, B. D.; Tomlinson, I. M. *Nat. Biotech.* **2000**, *18*, 989.

(7) Hamachi, I.; Fujita, A.; Kunitake, T. *J. Am. Chem. Soc.* **1994**, *116*, 8811.

(8) (a) Fang, J.; Knobler, C. M.; *Langmuir* **1996**, *12*, 1368. b) Mrksich, M.; Sigal, G. B.; Whitesides, G. M. *Langmuir* **1995**, *11*, 4383.

(9) (a) Boussaad, A.; Dziri, L.; Arechabaleta, N. J.; Tao, R.; Leblanc, R. M. *Langmuir* **1998**, *14*, 6215. (b) Nicolini, C.; Erokhin, V.; Antolini, F.; Catasti, P.; Facci, P. *Biochim. Biophys. Acta* **1993**, *1158*, 273.

(10) (a) Yang, Z.; Mesiano, A. J.; Venkatasubramanian, S.; Gross, S. H.; Harris, J. M.; Russel, A. J. *J. Am. Chem. Soc.* **1995**, *117*, 4843. (b) Franchina, J. G.; Lackowaski, W. M.; Dermody, D. L.; Crooks, R. M.; Bergbreiter, D. E.; Sirkar, K.; Russell, R. J.; Pishko, M. V. *Anal. Chem.* **1999**, *71*, 3133.

(11) (a) Kumar, C. V.; McLendon, G. L. *Chem. Mater.* **1997**, *9*, 863. (b) Kumar, C. V.; Chaudhari, A. *J. Am. Chem. Soc.* **2000**, *122*, 830.

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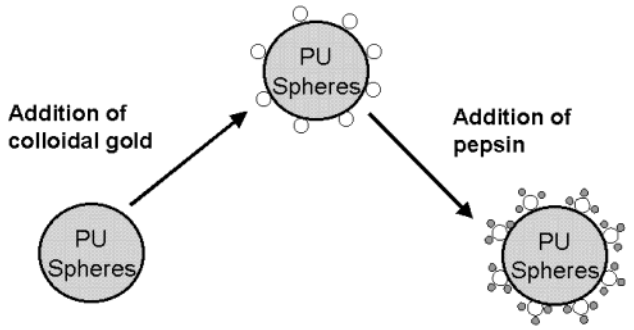
Scheme 1. Illustration of the Direct Assembly of Gold Nanoparticles on Polyurethane Spheres and Thereafter, Immobilization of the Enzyme Pepsin on the Gold Nano−PU Conjugated Material



ganic nanoparticles such as gold and silver[17−19] have been successfully conjugated with proteins/enzymes. Gold nanoparticles, in particular, are excellent candidates for bioconjugation with proteins because amine groups and cysteine residues in the proteins are known to bind strongly with gold colloids.[19] Although the gold nanoparticle bioconjugates showed excellent catalytic activity, a major drawback was that their reusability was extremely poor.[19] This was primarily due to the difficulty in separating the bioconjugate material from the reaction medium even under ultracentrifugation conditions.

In this report, we demonstrate for the first time the direct assembly of gold nanoparticle "shells" on polyurethane microsphere "cores" (PU, mean diameter 2 μm, Scheme 1). Binding of the gold nanoparticles to the polymer surface occurs through nitrogens in the polymer backbone and thus obviates the need for surface-modification of the polymer spheres, which is required in other polymer microsphere-based nanoparticle immobilization methods.[15,16] Bioconjugation of the nanogold-labeled PU microspheres with the enzyme pepsin (Scheme 1) leads to a new class of immobilized enzyme material that enjoys the advantages of both the nanoparticle and polymer microsphere-based immobilization methods. In addition to the simplicity of the protocol for their preparation, the pepsin−nanogold−PU bioconjugates exhibit biocatalytic activity marginally higher than that of the free enzyme in solution and significantly enhanced temperature and pH stability. A salient feature of the work is the reuse characteristics of the pepsin−nanogold−PU bioconjugates − they may be easily separated from the reaction mixture by mild centrifugation and show significant catalytic activity over 6 cycles of reuse. Presented below are details of the investigation.

Experimental Section

Chemicals. Pepsin (porcine gastric mucosa) and casein were obtained from Sigma Chemicals and used as-received. Chloroauric acid and sodium borohydride were obtained from Aldrich. All buffer salts were prepared from standard commercial sources.

Gold Nanoparticle Synthesis. A 100-mL sample of a 1.25×10^{-4} M concentrated aqueous solution of chloroauric acid ($HAuCl_4$) was reduced by 0.01 g of sodium borohydride ($NaBH_4$) at room temperature to yield a ruby-red (surface plasmon absorption maximum at ca. 520 nm) solution containing 35 ± 7 Å diameter gold nanoparticles.[20]

Formation of Nanogold−Polyurethane Composites. The PU microspheres of 2-μm mean diameter were synthesized as described elsewhere.[21] A 10-mg portion of the PU microsphere powder was dispersed in 10 mL of hexane, following which the dispersion was taken in a separating funnel along with 10 mL of the colloidal gold solution. Vigorous shaking of the biphasic mixture for ca. 10 min yielded an emulsion-like phase that rapidly phase-separated upon cessation of shaking. The originally ruby-red colloidal gold solution was now colorless (as is the hexane phase), and the PU powder had turned purple and accumulated at the hexane−water interface. The nanogold-coated PU spheres were separated by filtration, washed with double-distilled water, and dried in air.

Formation of Pepsin−Nanogold−PU Conjugates. A 10-mg aliquot of the nanogold−PU powder was dispersed in 2 mL of KCl−HCl buffer (0.02 M, pH 2), and to this dispersion 100 μL of ethanol was added to aid in the dispersion of the PU spheres. To this solution, 100 μL of a stock solution consisting of 10 mg/mL of pepsin in KCl−HCl buffer (0.02 M, pH 2) was added under vigorous stirring. After 1 h of stirring (Scheme 1), the pepsin−nanogold−PU spheres were separated by centrifugation. The loss in absorbance at 280 nm in the supernatant (arising from $\pi-\pi^*$ transitions in tryptophan and tyrosine residues in proteins)[22] was used to quantify the amount of pepsin bound to the nanogold−PU spheres for biocatalytic activity (in IU/μg) determination. The powder so obtained was rinsed several times with KCl−HCl buffer (0.02 M, pH 2) solution, re-suspended in buffer (pH 2) solution, and stored at 4 °C prior to further experimentation.

UV−Vis Spectroscopy Studies. The binding of colloidal gold to the polyurethane spheres, as well as pepsin to the nanogold−PU microspheres, was monitored by UV−vis spectroscopy on a Shimadzu dual-beam spectrometer (model UV-1601 PC) operated at a resolution of 1 nm. The decrease in intensity of the surface plasmon resonance in the aqueous colloidal gold solution arising from binding of the gold nanoparticles to the PU microspheres (resonance at ca. 520 nm)[20] was used to estimate the percent coverage of the PU spheres by the gold nanoparticles.

Scanning Electron Microscopy (SEM) and Energy Dispersive Analysis of X-ray (EDAX) Measurements. Samples for SEM and EDAX measurement were prepared by drop-coating a film of the nanogold−PU and pepsin−nan-

(12) (a) Gole, A.; Chaudhari, P.; Kaur, J.; Sastry, M. *Langmuir* **2001**, *17*, 5646. (b) Gole, A.; Dash, C.; Rao, M.; Sastry, M. *J. Chem. Soc., Chem. Commun.* **2000**, 297. (c) Gole, A.; Dash, C.; Mandale, A. B.; Rao, M.; Sastry, M. *Anal. Chem.* **2000**, *72*, 4301. (d) Gole, A.; Vyas, S.; Sainkar, S. R.; Lachke, A. L.; Sastry, M. *Langmuir* **2001**, *17*, 5964. (e) Gole, A.; Sastry, M. *Biotechnol. Bioeng.* **2001**, *74*, 172. (f) Phadtare, S.; Parekh, P.; Gole, A.; Patil, M.; Pundle, A.; Prabhune, A.; Sastry, M. *Biotechnol. Prog.* **2002**, *18*, 483.

(13) Rembaum, A.; Dreyer, W. J. *Science* **1980**, *208*, 364.

(14) (a) Caruso, F.; Mohwald, H. *J. Am. Chem. Soc.* **2000**, *121*, 6039. (b) Schuler, C.; Caruso, F. *Macromol. Rapid Commun.* **2000**, *21*, 750.

(15) Schmitt, A.; Fernandez-Barbero, A.; Cabrerizo-Vilchez, M.; Hidalgo-Alvarez, R. *Prog. Colloid Polym. Sci.* **1997**, *104*, 144.

(16) Elgersma, A. V.; Zsom, R. L. J.; Norde, W.; Lyklema, J. *J. Colloid Interface Sci.* **1990**, *138*, 145.

(17) Keating, C. D.; Kovaleski, K. M.; Natan, M. J. *J. Phys. Chem. B* **1998**, *102*, 9414.

(18) (a) Stonehuerner, J. G.; Zhao, J.; O'Daly, J. P.; Crumbliss, A. L.; Henkens, R. W. *Biosens. Bioelectron.* **1992**, *7*, 421. (b) Zhao, J.; O'Daly, J. P.; Henkens, R. W.; Stonehuerner J. G.; Crumbliss, A. L. *Biosens. Bioelectron.* **1996**, *11*, 493. (c) Crumbliss, A. L.; Perine, S. C.; Stonehuerner J. G.; Tubergen, K. R.; Zhao, J.; O'Daly, J. P. *Biotechnol. Bioeng.* **1992**, *40*, 483.

(19) (a) Gole, A.; Dash, C.; Ramakrishnan, V.; Sainkar, S. R.; Mandale, A. B.; Rao, M.; Sastry, M. *Langmuir* **2001**, *17*, 1674. (b) Gole, A.; Dash, C.; Soman, C.; Sainkar, S. R.; Rao, M.; Sastry, M. *Bioconjugate Chem.* **2001**, *12*, 684. (c) Gole, A.; Vyas, S.; Phadtare, S.; Lachke, A.; Sastry, M. *Colloids Surf. B* **2002**, *25*, 129.

(20) Patil, V.; Malvankar, R. B.; Sastry, M. *Langmuir* **1999**, *15*, 8197.

(21) (a) Ramanathan, S.; Shukla, P. G.; Sivaram, S. *Pure Appl. Chem.* **1998**, *70*, 1295. (b) Shukla, P. G.; Sivaram, S. U.S. patent 5859,-075 CA 1999; 129: 276913.

(22) Stoscheck, C. M. *Met. Enzymol.* **1990**, *182*, 50.

ogold−PU solutions on a Si (111). SEM measurements of pepsin−nanogold−PU bioconjugates after the first reaction cycle were done to confirm the leaching of weakly bound pepsin molecules in the bioconjugate material. These measurements were performed on a Leica Stereoscan-440 scanning electron microscope equipped with a Phoenix EDAX attachment.

Transmission Electron Microscopy (TEM) Measurements. TEM measurements were performed on a JEOL model 1200EX instrument operated at an accelerating voltage of 120 kV. Samples for TEM analysis were prepared by placing a drop of the nanogold−polyurethane solution onto a carbon-coated TEM copper grid. The drop was allowed to dry for 1 min, following which the extra solution was removed using a blotting paper.

Biocatalytic Activity Measurements. The biocatalytic activity of free pepsin in solution and of the pepsin−nanogold−PU bioconjugate in KCl−HCl buffer (0.02 M, pH 2) was determined by reaction with 0.6% casein at 37 °C for 1 h. In the latter case, the pepsin−nanogold−PU bioconjugate material was separated from the reaction medium by mild centrifugation for recycling studies (see below). In a typical experiment to estimate the biocatalytic activity (IU/μg) of the conjugate solution, 1 mL of a carefully weighed amount of the pepsin−nanogold−PU bioconjugate in water was reacted with 1 mL of casein solution at 37 °C for 1 h. After the incubation time, an equal volume of 1.7 M perchloric acid was added to the reaction solution to precipitate the residual casein. After 1 h, the precipitate was removed by centrifugation and the optical absorbance of the filtrate was measured at 280 nm. Pepsin digests casein and yields acid soluble products (tryptophan and tyrosine residues) that are readily detected by their strong UV signatures at 280 nm.[12b] The amount of pepsin in the pepsin−Au−PU bioconjugate material was quantitatively estimated during preparation of the bioconjugate material as briefly mentioned earlier. For comparison, the biocatalytic activity of an identical concentration of the free enzyme in solution under identical conditions was recorded. To determine the confidence limits of the biocatalytic activity measurements for the pepsin−nanogold−PU bioconjugate material, separate measurements of the biocatalytic activity (IU/μg) were made as described above for 6 different pepsin−nanogold−PU bioconjugate solutions. The stability of the pepsin−nanogold−PU bioconjugate was checked by preincubating it for 1 h in the temperature range 50−80 °C and compared with that of an identical amount of free enzyme in the KCl−HCl buffer (0.02 M, pH 2) under similar conditions. The pH dependence of the biocatalytic activity of the bioconjugate was studied by preincubating the pepsin−nanogold−PU bioconjugate for 1 h at five different pH values (pH 2, KCl−HCl buffer; pH 5, sodium phosphate buffer; pH 8 and 10, glycine−NaOH buffer) at 37 °C. Three separate biocatalytic activity measurements were done in each of the above experiments to check the reproducibility of the assays.

Reuse characteristics of the pepsin−nanogold−PU bioconjugate material were tested over six successive reaction cycles. The bioconjugate material was separated from the reaction medium by mild centrifugation and washed with copious amount of water prior to each reaction. A small loss in biocatalytic activity of the pepsin−nanogold−PU materials was observed with reuse. To understand the reasons for the loss in catalytic activity, control experiments were done to ascertain whether this was due to detachment of gold nanoparticles from the PU microsphere surface or leaching out of the enzyme alone. The supernatant was obtained after centrifugation of the bioconjugates after each reuse and was analyzed by a Varian Spectra AA 220 atomic absorption spectrometer (AAS) for the presence of gold nanoparticles.

Results and Discussion

Our earlier successful investigation into the immobilization of pepsin in thermally evaporated lipid films[12b] motivated us to study its immobilization and biocatalytic activity on gold nanoparticles bound to the surface of polyurethane microspheres.

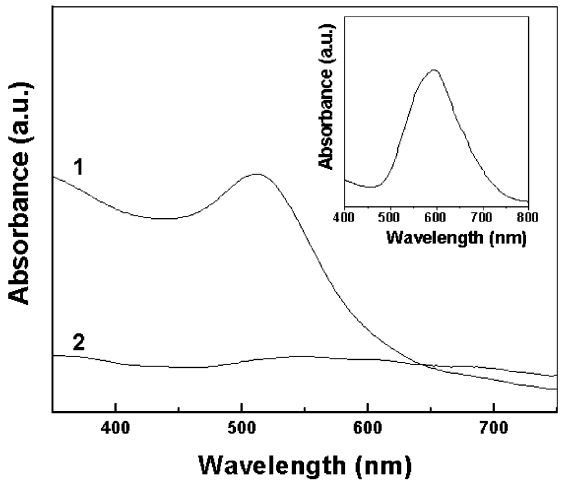


Figure 1. UV−vis spectrum of the as-prepared colloidal gold solution (curve 1) and the gold solution after addition of PU spheres and filtration (curve 2, text for details). The inset shows the UV−vis spectrum recorded from a film of gold nanoparticle shell−PU core spheres on a quartz substrate.

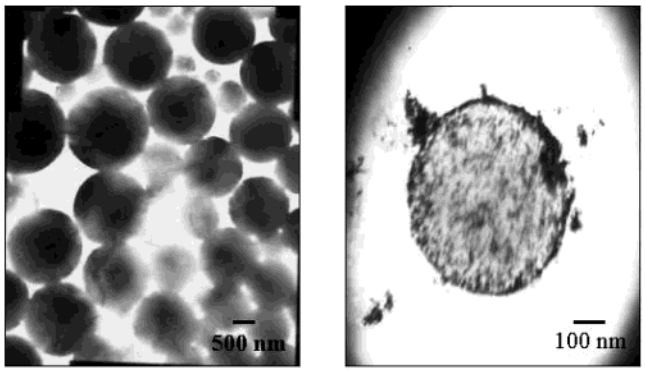


Figure 2. Low (A) and high (B) magnification TEM images of gold nanoparticle shell−PU core microsphere monolayers on a carbon-coated TEM grid.

Figure 1 shows UV−vis spectra of the as-prepared colloidal gold solution (curve 1) and the gold solution after addition of PU spheres and filtration (curve 2). The surface plasmon resonance in the as-prepared colloidal gold solution can be clearly seen at ca. 520 nm (curve 1).[20] After shaking the colloidal gold solution with the PU dispersed in hexane, there is loss in intensity of the surface plasmon resonance due to a decrease in the concentration of gold nanoparticles in the aqueous solution (curve 2). This indicates binding of colloidal gold particles to the PU microspheres through nitrogen atoms in polyurethane. The purple polyurethane spheres capped with gold nanoparticles were observed to assemble at the interface between the two liquids and could be separated and cast in the form of a film. The inset of Figure 1 shows the UV−vis spectrum recorded from a film of the PU spheres taken from the hexane−water interface after drying the film. A broad absorption band centered at ca. 600 nm is observed and arises from the gold nanoparticle "shell" surrounding the PU "core" spheres. The shift in the resonance wavelength indicates considerable aggregation of the gold nanoparticles consequent to binding with the PU surface (Scheme 1).[20,23]

Figure 2A and B shows TEM micrographs of the Au−PU composite on a carbon-coated copper grid. At lower

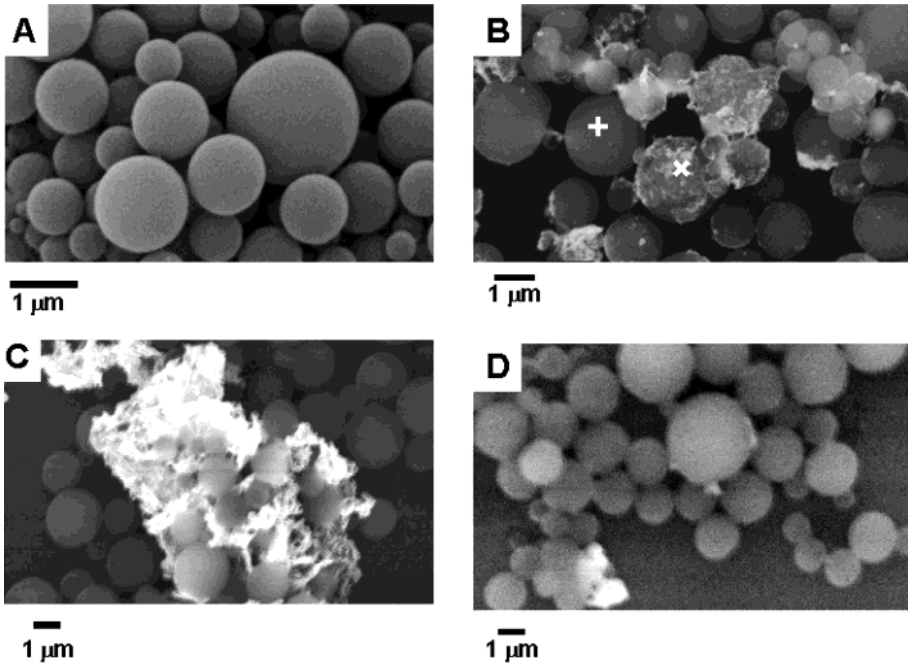


Figure 3. SEM images of the (A) nanogold−PU core−shell material, (B) and (C) the pepsin−nanogold−PU bioconjugate material; and (D) the pepsin−nanogold−PU bioconjugates after one cycle of reuse cast as films onto Si (111) wafers (see text for details).

magnification (Figure 2A), a number of PU spheres over a range of sizes in close contact can be seen clearly. At higher magnification (Figure 2B), the presence of gold nanoparticles (smaller dark spots) bound to the surface of the underlying PU sphere is visible. It is well-known that pyridine[24] and primary amines[25] bind to colloidal gold through nitrogen atoms. We believe a similar mechanism involving nitrogen atoms in PU in the entrapment of gold nanoparticles on the microspheres is operative in this study.

Figure 3 shows SEM images of drop-cast films of the PU−nanogold core−shell material (A) and the pepsin−nanogold−PU bioconjugate material (B and C) on Si (111) substrates. Although the surface texture of the PU spheres capped with gold nanoparticles is quite smooth (Figure 3A, the gold nanoparticles are not clearly visible at the resolution of the SEM measurement), after conjugation with pepsin, thin sheets of, presumably, the aggregated enzyme are seen together with smooth PU microspheres (Figure 3B and C). Spot profile EDAX analysis of the sheets (marked by an "X" in Figure 3B) confirmed that they were composed of only the enzyme (through a strong sulfur signal from cysteine residues of pepsin). These sheets thus correspond to highly aggregated pepsin molecules, such protein aggregation having been observed by Caruso et al in multilayer films of polymer−anti-IgG composites.[26] Spot profile EDAX analysis of the smooth nanogold-capped PU spheres away from the pepsin sheets (marked by a "+" in Figure 3B) also showed the presence of sulfur, indicating binding of the enzyme to the gold nanoparticles even though this was not visible by SEM imaging. The sulfur signal was absent in the nanogold−PU core−shell material as expected.

Table 1. Biocatalytic Activities Obtained from Assaying the Pepsin−Gold Nanoparticles−PU Bioconjugate Material over Six Sequential Reuses

system	biocatalytic activity (IU/μg)[a]
pepsin in solution	11.2
pepsin−gold−PU spheres, run 1	13.2
pepsin−gold−PU spheres, run 2	10.2
pepsin−gold−PU spheres, run 3	8.0
pepsin−gold−PU spheres, run 4	7.2
pepsin−gold−PU spheres, run 5	4.3
pepsin−gold−PU spheres, run 6	3.6

[a] One unit of protease activity is measured as a change in absorbance at 280 nm of 0.001 per minute at pH 2 and 37 °C measured as acid soluble products using casein as the substrate.

The most important aspect of this study concerns retention of the biocatalytic activity of pepsin after adsorption onto the nanogold−PU surface. Because the amount of pepsin bound to the bioconjugate could be estimated from UV−vis measurements, it is possible to compare the biocatalytic activities of enzyme (IU/μg) in the pepsin−nanogold−PU bioconjugate and the free enzyme in solution under identical assay conditions. The activity of free pepsin in solution was determined to be 11.2 IU/μg, and that of the enzyme in the pepsin−nanogold−PU bioconjugate surface was determined to be 13.2 IU/μg (Table 1). It is clear from the above measurement that the biological activity of the enzyme is not compromised consequent to immobilization on the gold nanoparticles bound to PU. Indeed, there is a

(23) (a) Storhoff, J. J.; Lazarides, A. A.; Mucic, R. C.; Mirkin, C. A.; Letsinger, R. L.; Schatz, G. C. *J. Am. Chem. Soc.* **2000**, *122*, 4640. (b) El-Sayed, M. *Acc. Chem. Res.* **2001**, *34*, 257.

(24) (a) Blatchford, C. G.; Campbell, J. R.; Creighton, J. A. *Surf. Sci.* **1982**, *120*, 435. (b) Galletto, P.; Brevet, P. F.; Girault, H. H.; Antoine, R.; Broyer, M. *J. Phys. Chem. B* **1999**, *103*, 8706.

(25) (a) Leff, D. V.; Brandt, L.; Heath, J. R. *Langmuir* **1996**, *12*, 4723. (b) Brown, L. O.; Hutchison, J. E. *J. Am. Chem. Soc.* **1999**, *121*, 882. (c) Sastry, M.; Kumar, A.; Mukherjee, P. *Colloids Surf. A* **2001**, *181*, 255. (d) Kumar, A.; Mukherjee, P.; Guha, A.; Adyantaya, S. D.; Mandle, A. B.; Kumar, R.; Sastry, M. *Langmuir* **2000**, *16*, 9775. (e) Sastry, M.; Kumar, A.; Mukherjee, P. *Colloids Surf. A* **2001**, 181, 255. (f) Selvakannan, PR.; Mandal, S.; Pasricha, R.; Adyanthaya, S. D.; Sastry, M. *Chem. Commun.* **2002**, 1334.

(26) Caruso, F.; Furlong, D. N.; Ariga, K.; Ichinose, I.; Kunitake, T. *Langmuir* **1998**, *14*, 4559.

marginal enhancement in the biocatalytic activity in the bioconjugate material that is outside experimental uncertainty.

Earlier studies on immobilization of enzymes directly on gold nanoparticles in solution yielded excellent catalytic activity of the enzymes, and in many cases, enhancement in the enzyme thermal stability as well.[18,19] However, one major drawback of enzyme−gold nanoparticle bioconjugates is that they are not easily separated from the reaction medium even under ultracentrifugation, rendering their reuse almost impossible.[19] The protocol described herein where the gold nanoparticles ride "piggy-back" on the considerably more massive polymer spheres enables easy separation of the gold nanoparticle−polymer spheres from the reaction medium by mild centrifugation. Often sedimentation under gravity is sufficient to accomplish the separation. Furthermore, the advantages associated with gold nanoparticle−enzyme bioconjugates is not lost by virtue of immobilization of the gold nanoparticles on the polymer microsphere surface, which in itself, has a high surface curvature. In effect, the immobilized enzyme molecules in the pepsin−nanogold−PU bioconjugate material behave almost like free enzyme molecules in solution, thus rendering the mass transport problem commonly associated with enzyme immobilization strategies within solid matrixes virtually nonexistent in this case.

Table 1 shows the results of 6 cycles of reuse of the pepsin−nanogold−PU bioconjugate. It is seen that there is a small, monotonic decrease in the biocatalytic activity of the enzyme with reuse, the biocatalytic activity falling to ca. 28% of the starting activity after 6 cycles of reuse. This excellent retention of biocatalytic activity of pepsin in the nanogold−PU bioconjugate with reuse is to be contrasted with the almost complete loss in activity of the same enzyme immobilized in thermally evaporated fatty amine films after just 3 cycles of reuse.[12b] Clearly the blockage of diffusion pathways of substrate molecules implicated in the earlier study for loss in activity with recycling is not operative in the bioconjugation strategy presented in this paper. This is a salient feature of the work with immense commercial implications. However, the monotonic and perceptible loss in catalytic activity of the pepsin−nanogold−PU bioconjugate material as a function of reuse cycles needs elaboration. It is possible that the gold nanoparticles detach from the surface of the PU microspheres during successive reaction cycles. Another possibility is the leaching out of pepsin from the bioconjugate material in successive reactions. To distinguish between the two mechanisms, atomic absorption spectroscopy (AAS) measurements were performed on the supernatant obtained after centrifugation of the reaction medium during each of the reaction cycles. Gold could not be detected by AAS [detection sensitivity ∼ parts per million (ppm)] in any of the reaction cycles clearly showing that the nanoparticles are strongly bound to the underlying PU microsphere template. This results points to loss of enzyme from the nanoparticle surface during reaction. UV−vis spectroscopy measurements were carried out on the supernatant from 10 mg of the pepsin−nanogold−PU bioconjugate material immersed in 2 mL of pH 2 buffer solution. A 1-mL sample of the supernatant was analyzed in intervals of 1 h, which is

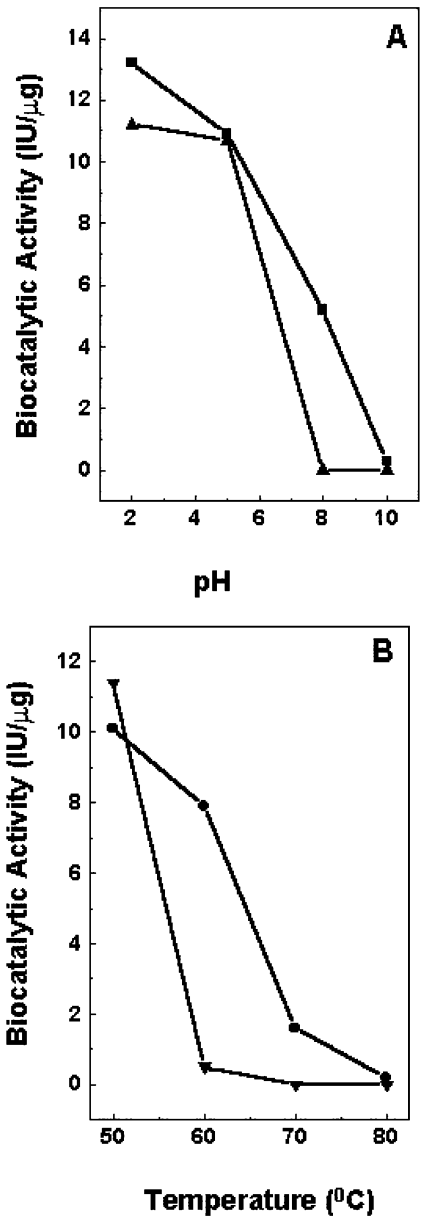


Figure 4. (A) pH-Dependent biocatalytic activity of free pepsin in solution (triangles) and pepsin−nanogold−PU bioconjugate material (squares) preincubated for 1 h at different pHs. (B) Temperature-dependent biocatalytic activity of free pepsin in solution (triangles) and pepsin−nanogold−PU bioconjugate material (circles) preincubated for 1 h at different temperatures. The solid lines in all cases are aids to the eye and have no physical significance.

characteristic of the reaction times in the reuse measurements. After each measurement, the analyte was added back to the original buffer solution to simulate the reaction conditions precisely. It was observed that after 1 h of immersion, roughly 35% of the total pepsin loading was released into solution (estimated from the absorbance at 280 nm).[22] Thereafter, no further loss of enzyme occurred for longer times of immersion of the bioconjugate material. This percentage loss of enzyme correlates well with degree of loss of biocatalytic activity during the first reuse cycle (Table 1). We believe the initial loss of pepsin corresponds to loss of weakly bound enzyme from the bioconjugate. It is likely that the sheets of aggregated pepsin molecules observed in the SEM images of the pepsin−nanogold−PU bioconjugate (Fig-

ure 3B and C) correspond to the weakly bound enzyme that leaches out in the first reaction cycle. That this is indeed the possible mechanism is indicated by the SEM image recorded from the pepsin−nanogold−PU bioconjugate material after one cycle of reuse (Figure 3D). It is clearly seen from this figure that the percentage of aggregated pepsin sheets observed in the as-prepared bioconjugate material has reduced drastically.

Many applications of immobilized enzymes require their operation under pH and temperature conditions far removed from optimum operating conditions. We have recently showed that the enzyme endoglucanase, when immobilized in fatty lipid films, exhibited significant catalytic activity under highly alkaline conditions as well as enhanced temperature stability.[12d] This feature is tremendously exciting for application of this enzyme in the paper pulp and fabric treatment industries where such harsh conditions are normally encountered.[12d] Although enhanced biocatalytic activity of the enzyme pepsin over a large pH range may not conceivably have an immediate application, such a variation in reactivity of the enzyme under different conditions of immobilization would shed some light on the nature of interaction of the enzyme with the host matrix. Figure 4A shows the biocatalytic activities (IU/μg) of the pepsin−nanogold−PU bioconjugate material (squares) along with that of the free enzyme in solution (triangles) as a function of solution pH in the range 2 to 10. The optimum biocatalytic activity in both cases is at pH 2 with a marginal fall in activity observed at pH 5. The most dramatic observation, however, is retention of nearly 40% of the optimum biocatalytic activity by the pepsin−nanogold−PU bioconjugate at pH 8. Under these pH conditions, free pepsin in solution showed no catalytic activity at all (Figure 4A). A small biocatalytic activity was observed in the pepsin−nanogold−PU bioconjugate even at pH 10. We are unable to explain the stability of pepsin immobilized on the gold nano-PU surface as a function of pH at present. Further studies on other enzymes are currently underway that may throw some light on this interesting observation.

Figure 4B shows the temperature variation in biocatalytic activity (IU/μg) of free pepsin in solution (triangles) and the pepsin−nanogold−PU bioconjugate material (circles) determined at pH 2 in the temperature range 50−80 °C. Three separate measurements were performed at each temperature to check the reproducibility of the data. Although the free enzyme in solution is intolerant to even a 10 °C increase in temperature of the reaction medium (biocatalytic activity at 60 °C is only 4% of the value at 50 °C), pepsin in the bioconjugate material showed only a 32% fall in biocatalytic activity under similar temperature rise conditions (Figure 4B). Indeed, the bioconjugate material showed a significantly high biocatalytic activity even at 70 °C (Figure 4B, ca. 14% of the specific biocatalytic activity at 50 °C) under conditions where free pepsin showed no biocatalytic activity at all. Thus, complexation of the pepsin molecules with gold nanoparticles capping PU microspheres considerably increases the temperature and pH stability of the enzyme.

Control experiments on the immobilization of pepsin molecules directly onto PU microspheres were performed. It was observed that pepsin binding to the polymer was below detection limits of our experimental procedures. This clearly underlines the important role of the gold nanoparticle shell surrounding the polymer core in binding and stabilizing the enzyme.

Conclusion

In this study, we have demonstrated the direct assembly of colloidal gold particles on polyurethane spheres by interaction of the urethane nitrogens with the gold particles. The gold nanoparticle shell−PU core structures may be used to immobilize enzymes such as pepsin. Bioconjugation of the nanogold−PU spheres with pepsin leads to a new class of biocatalyst that combines the utility of immobilization in terms of reuse with easy access of substrates to the enzyme normally reserved for enzymes present directly in solution. The pepsin−nanogold−PU biocomposite material exhibited outstanding reuse capability and temperature/pH stability. The use of such gold nanoparticle shell−PU core structures in DNA immobilization and detection methodologies is currently being envisaged.

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