NANO LETTERS

2003
Vol. 3, No. 1
55−58

Alumina–Pepsin Hybrid Nanoparticles with Orientation-Specific Enzyme Coupling

Ju Li, Jianquan Wang, Vasilis G. Gavalas, David A. Atwood, and Leonidas G. Bachas*

Department of Chemistry, University of Kentucky, Lexington, Kentucky 40506-0055

Received August 30, 2002; Revised Manuscript Received November 20, 2002

ABSTRACT

Hybrid alumina nanoparticles with pepsin were prepared in a controlled and efficient manner. Phosphorylated pepsin can be coupled to alumina through the interaction between phosphoserine on pepsin and the alumina surface in an orientation-specific manner. A comparison of data obtained with nanoparticles and microsized alumina particles reveals that the conjugated pepsin retained much higher enzymatic activity when it was immobilized on nanoparticles mainly because of the lack of diffusion limitations of the substrate. Additionally, upon attachment to the alumina nanoparticles, the thermal stability of pepsin is enhanced. The coupled enzyme can be quantitatively released by simply incubating the hybrid nanoparticles with phosphate buffer.

Introduction. Nanotechnology has emerged as a powerful tool in the fabrication of materials having superior and often unique properties.[1−4] The incorporation of biological molecules into these materials should expand the range of potential applications to include nanoscale biosensors and biocatalysts.[5] Previous studies on the conjugation of nanoparticles with biomolecules such as proteins and DNA used mainly gold-, silver-, silica-, and nickel-based nanoparticles as well as quantum dots.[6] Several methods have been employed for the attachment of biomolecules on nanoparticles including binding through a thiol group to gold[7,8] and maleimido-modified fullerenes,[9] through amino groups to carboxyl-functionalized paricles,[10] through a polyhistidine tag to nickel,[11] or through electrostatic interactions to charged nanoparticles.[12] Herein, we report a new class of hybrid nanoparticles composed of nanosized alumina functionalized with an enzyme. We demonstrate, using pepsin as a model enzyme, an orientation-specific, efficient, and reversible means to couple phosphorylated proteins to alumina nanoparticles through the interaction between the phosphoryl group and the alumina surface. When alumina nanoparticles are used in this manner, the decrease in enzymatic activity is minimal. A unique property of these hybrid nanoparticles is the ability to release the coupled pepsin quantitatively (98%) in a controlled manner.

Pepsin is an enzyme essential to the digestion process in animals. The optimal pH for its activity is 2.0, which is compatible with that of alumina. The enzyme has a total of 326 amino acid residues and a maximal cross-section length of 6.5 nm based on its crystal structure.[13] One phosphoryl group is attached to the serine 68 residue in the native porcine pepsin. The crystal structure of the protein indicates that this residue is on the surface and away from the catalytic residues Asp32 and Asp215.[14] Thus, the phosphoryl group on serine 68 is properly positioned to allow oriented coupling to alumina from a single point on the enzyme.

Experimental Details. Porcine pepsin (3200−4500 units/mg, confirmed to be 3500 units/mg in our laboratory), alumina WA-1, alumina WA-4, hemoglobin, and formic acid were obtained from Sigma Aldrich (St. Louis, MO). Alumina nanoparticles (lot # I20G14, predominantly γ alumina, 10−20 nm in diameter) were purchased from Alfa Aesar (Ward Hill, MA). Trichloroacetic acid and potassium phosphate were bought from Fisher Scientific (Pittsburgh, PA). Deionized water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA). The purity of the enzyme was determined by SDS-PAGE on a PhastSystem (Pharmacia Biotech, Uppsala, Sweden). Protein concentrations were determined with a Pierce BCA protein assay kit (Pierce Chemical, Rockford, IL). Scanning electron micrographs (SEM) were obtained using a Hitachi S-900 scanning electron microscope (Hitachi Instrument, San Jose, CA) after the particles were precoated with a layer of gold/palladium. Dark-field scanning transmission electron microscopy (DF-STEM) studies were carried out on a JEOL JEM-2010FX microscope equipped with an energy-dispersive X-ray spectroscopy (EDS) analysis system. Particles were placed on carbon-coated copper grids (300 mesh, Ted Pella, Redding,

* Corresponding author. E-mail: bachas@uky.edu. Phone: (859) 257-6350. Fax: (859) 323-1069.

10.1021/nl025778s CCC: $25.00 © 2003 American Chemical Society
Published on Web 12/06/2002

CA) for analysis. FT-IR analysis was performed using an Avatar 360 E. S. P. FT-IR spectrometer (Thermo Nicolet, Madison, WI).

For coupling, porcine pepsin (10 mg) was first dissolved in 8 mL of 1% (v/v) formic acid. Then, different amounts of pepsin (3000−50000 units) in solution were mixed by stirring with 0.1 g of alumina particles at room temperature for 2 h. The mixture was then centrifuged at 12000*g* for 3 min to separate the particles from the supernatant. The particles were washed successively with 1% (v/v) formic acid, 0.1 M sodium acetate along with 0.5 M sodium chloride, and 1% (v/v) formic acid until there was no evident enzyme activity in the wash solution. The particles were finally resuspended in 1% (v/v) formic acid. The enzymatic activity on the hybrid alumina particles, in the supernatant, and in the wash solutions was measured separately as described below.

To measure enzyme activity, 200 μL of a pepsin solution or a particle suspension was mixed with 1 mL of 2.0% (w/v) hemoglobin and incubated at 37 °C by shaking for 10 min. Then, 2 mL of 5% (w/v) trichloroacetic acid was added to the mixture and incubated at 37 °C for 5 min. The solution was centrifuged at 3000*g* for 5 min, followed by filtration through a Whatman syringe filter (0.2 μm). The supernatant was transferred to a quartz cuvette, and the absorbance at 280 nm was measured using an HP UV−visible Chemstations model 8453 spectrophotometer (Hewlett Packard, Palo Alto, CA). The amount of enzyme was calculated in enzyme units. (One unit is defined as the amount of enzyme that produces a $\Delta A_{280\ nm}$ of 0.001/min at pH 2.0 and 37 °C).[15]

To probe the reversible nature of the coupling process, the alumina−pepsin nanoparticles were incubated with 0.2 M potassium phosphate (pH 6.5) at room temperature for 2 h with gentle stirring. After that, the nanoparticles were separated from the supernatant by centrifugation. The enzyme activities on the nanoparticles and in the supernatant were measured.

For the thermostability test, hybrid alumina nanoparticles were resuspended in 1% (v/v) formic acid solution. Both the free-enzyme solution and the nanoparticle suspension were incubated in a water bath for a fixed period of time at a set temperature ranging from 50 to 80 °C. Then, the enzyme solutions were immediately transferred to an ice bath to stop the denaturation of the enzyme, and the residual enzyme activity in each of the solutions was determined as described above.

Results and Discussion. Hybrid nanomaterials were prepared by orienting pepsin on alumina nanoparticles. Two types of microsized alumina particles (WA-1, 95 ± 21 μm and WA-4, 56 ± 20 μm; average size ± standard deviation, $n = 100$, as determined by SEM micrographs) were also coupled with pepsin for comparison purposes. In all cases, the amount of enzyme bound to the particles increases in a Langmuir-type fashion (Figure 1). The curves in Figure 1 were fit to a generalized Langmuir isotherm equation and were used to estimate the capacity of each particle type. The maximum amount of enzyme that could be coupled per milligram of alumina nanoparticles (capacity) is 450 units,

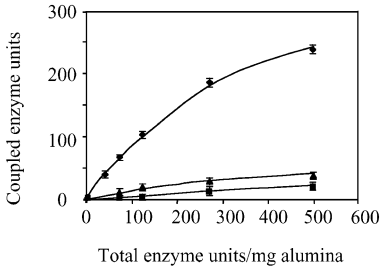

Figure 1. Coupled enzyme units on the particles as a function of the total enzyme units/mg alumina particles used in the immobilization reaction. Pepsin molecules were conjugated on nanoalumina particles (◆) and two types of microsized alumina particles, WA-1 (■) and WA-4 (▲). Data are average ± standard deviation ($n = 3$) values.

which is about 20-fold larger than for the other alumina particles (20 units/mg of WA-1 and 24 units/mg of WA-4 alumina). The difference in the binding capacity for pepsin can be explained by the superior surface-to-volume ratio of nanoparticles. Given that the surface area of alumina nanoparticles is 100 m^2/g (manufacturer's data, which were confirmed in our laboratory), the maximum amount of pepsin that could be used to form a monolayer on the surface of the alumina nanoparticles is ∼1400 units/mg alumina (0.4 mg enzyme/mg alumina). This was estimated by first calculating the area that each pepsin molecule occupies when oriented on alumina particles through serine 68, which was determined from the corresponding cross-section of the molecule from X-ray crystallographic data.[14] The calculated capacity of the alumina nanoparticles derived from fitting the data in Figure 1 to the Langmuir isotherm (450 units/mg alumina) corresponds to 32% coverage on the particle surface with pepsin whereas the highest experimental data point in Figure 1 (232 units/mg alumina) equals ∼17% coverage.

If the particle surface does not affect the enzyme activity, then the activity of the enzyme on the particles should be expected to be equal to the difference between the total units of enzyme used in the coupling reaction and those left in the wash solutions/supernatant after the reaction. Apparently, this is not the case. However, the ratio of the observed units of the coupled enzyme on the alumina nanoparticles to the expected enzyme units was almost a constant value, herein termed as α (Table 1); 100(1 − α) indicates the percentage of activity lost due to the interaction between the alumina surface and the enzyme. The α value for alumina nanoparticles is 0.61 ± 0.01 ($n = 5$) whereas the corresponding values for the microsized alumina are 0.048 ± 0.004 for the WA-4 type and 0.014 ± 0.003 for the WA-1 type. This difference can be explained by the porosity of the microsized alumina, which may allow some of the pepsin molecules (∼35 kDa) to be attached inside the pores. The substrate hemoglobin has a molecular mass of 65 kDa and a length of 6 nm.[16] Thus, diffusional limitations of the substrate may play a role in reducing the apparent enzyme activity. On the contrary, when pepsin is coupled onto alumina nanoparticles, because of the lack of large-sized pores, it is present with

Table 1. Effect of the Total Enzyme Concentration on Pepsin Immobilization on Alumina Nanoparticles[a]

	enzyme units/mg alumina		
units/mg alumina	expected	observed	α^b
31	26	16	0.62
65	53	32	0.61
115	89	54	0.61
265	180	112	0.62
495	232	136	0.59

[a] The first column refers to the total units of enzyme/mg of alumina employed in the protein immobilization step. The expected enzyme units on the nanoparticles were calculated as the difference between the total units of enzyme and the units of enzyme in the wash solutions/supernatant after immobilization. The observed enzyme units on the particles were calculated from the enzyme assay. [b] α = (observed units)/(expected units).

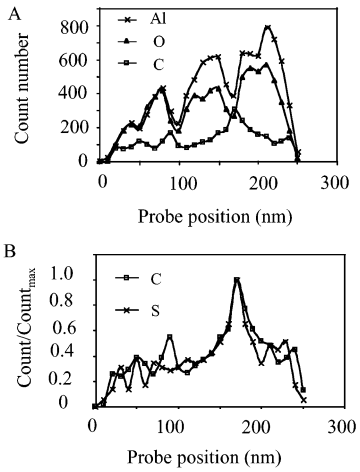

Figure 2. EDS analysis of hybrid alumina nanoparticles under TEM. Scanning was performed across a group of nanoparticles. (A) Count number vs probe position for carbon, oxygen, and aluminum. (B) Relative count number vs probe position for carbon and sulfur.

its active site highly exposed on the surface. This allows the free diffusion of substrate molecules to the enzyme active site.

The hybrid nanoparticles were characterized by using transmission electron microscopy and FT-IR. The sizes of the nanoparticles on the TEM images are consistent with the data from the manufacturer. Energy-dispersive X-ray spectroscopy (EDS) analysis was performed while scanning across a group of hybrid nanoparticles viewed under the DFSTEM (Figure 2). As depicted in Figure 2A, the curves for oxygen and aluminum follow the same pattern, which is expected for alumina. As the analysis beam scans across the hybrid nanoparticles, it is reasonable to assume that EDS will give higher signals for aluminum and oxygen near the center of the particles. However, the profile for carbon (a major element from the enzyme molecules) shows the opposite pattern. As the enzyme is immobilized on the surface of the particles, EDS analysis will give a higher signal for carbon near the edge of the particle, where the cross section of the EDS beam interacts with a larger amount of enzyme. Interestingly, the signal from sulfur follows a similar pattern to that of carbon (Figure 2B). This further confirms the attachment of enzyme molecules on the nanoparticles,

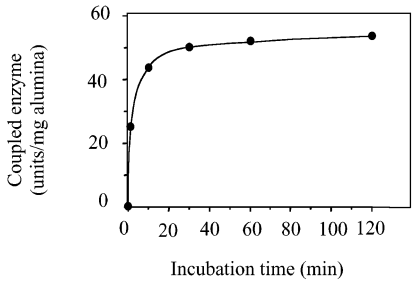

Figure 3. Coupling of pepsin on alumina nanoparticles as a function of immobilization time. The total pepsin used for immobilization is 65 units/mg alumina.

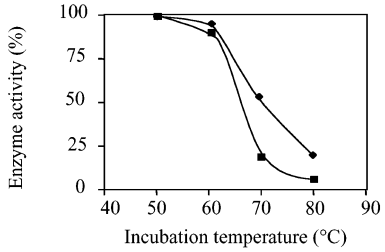

Figure 4. Residual enzyme activities vs incubation temperature. Enzyme activities of both the immobilized pepsin on alumina nanoparticles (◆) and the free pepsin in solution (■) were converted to activity percentages at 50 °C.

as sulfur is a component of the enzyme. In that regard, there are 10 sulfur atoms from cysteine and methionine residues per pepsin molecule. The FT-IR spectrum of the alumina nanoparticles exhibits strong absorption in the range of 500−900 cm^{-1}, characteristic for Al_2O_3.[17] It also has peaks at 1630 and 3422 cm^{-1} that can be attributed to physically adsorbed water molecules.[18] The pepsin FT-IR spectrum exhibits a peak at 1655 cm^{-1}, characteristic of an amide I bond.[19] This peak also appears in the spectra of the hybrid nanoparticles, verifying the attachment of pepsin.

To evaluate the kinetics of the coupling process, pepsin (65 units/mg alumina) was incubated with alumina nanoparticles for different time periods. A 1-min incubation was sufficient to reach ~50% occupancy of the sites available for pepsin attachment (Figure 3). Incubation for 20 min resulted in >90% occupancy whereas 120-min incubation completed the coupling process.

Protein unfolding causes denaturation at higher temperatures.[20] Since immobilization may stabilize the protein structure, we investigated whether the attachment of pepsin on alumina nanoparticles is associated with any improvement in its stability. Studies were carried out that exposed the hybrid alumina−pepsin nanoparticles to different incubation temperatures for 5 min (Figure 4). Pepsin molecules on hybrid alumina nanoparticles were found to have higher thermostability than free pepsin in solution. Indeed, the former retained 54% of its activity after a 5-min incubation at 70 °C whereas free pepsin retained only about 20% under the same conditions. The improved stability for the nanoparticle-coupled enzyme is likely due to the increased conformational stability upon immobilization. These results are consistent with those from a prior study involving enzymes immobilized on microsized alumina particles.[21]

Aqueous phosphate competes with phosphorylated proteins for the alumina surface.[22] This presents an interesting opportunity for the assembly of hybrid nanoparticles, which under controlled conditions (i.e., phosphate) are induced to release the coupled biological component. This may be beneficial for the use of nanoalumina as a drug-delivery vehicle that releases a biomolecule (e.g., a biopharmaceutical protein) in vivo. Indeed, it was found that the binding of pepsin through the phosphorylated serine 68 on alumina is reversible at high phosphate concentrations. By using the hybrid nanoparticles with variable amounts of pepsin (26−231 units/mg alumina), it was found that 93−98% of the enzyme units could be released from the hybrid nanoparticles upon exposure to phosphate. The reversibility of the process is due to the competition between phosphate groups in the solution and the phosphoryl group on pepsin for alumina. In the presence of a high concentration of phosphate ions, the pepsin molecules can be replaced from the hybrid nanoparticles by the inorganic phosphate ions.

In summary, hybrid alumina−protein nanoparticles have been synthesized in a simple and controlled manner. The observed enzyme activities on nanoparticles per milligram of enzyme were much larger than those on microsized alumina particles. The process was reversible, with ∼93−98% of bound pepsin being released from the hybrid nanoparticles with phosphate buffer. This approach should be applicable to the preparation of hybrid alumina nanoparticles with other phosphorylated biomolecules. Thus, since phosphoryl groups can be introduced into a variety of biomolecules, this efficient and reproducible coupling method can be of general applicability. Alumina has been shown to be a bioinert material causing minimal necrosis and fibrosis when implanted into the human body.[23] Thus, nanoparticulate alumina with attached biological components should find potential applications in clinical diagnostics, nanosensors, localized delivery of biopharmaceuticals, and biological recognition systems.

Acknowledgment. We thank the Office of Naval Research (ONR) for funding this research. We also thank Dr. Alan Dozier for his assistance in testing the samples in TEM.

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NL025778S