Interfacing biology with nanoparticles
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Abstract
New approaches are constantly being developed for both the synthesis of inorganic nanomaterials and their surface modification for sensing and electronic applications. Insofar as surface modification of gold nanoparticles is concerned, thiol chemistry is the most popular approach to bind ligands to their surface. We have been pursuing the possibility of using amine functionality to bind ligands to the surface of gold nanoparticles and have found that amine binding is as strong as thiol binding. The advantages of using amine chemistry for surface modification of nanogold are many, the possibility of complexing a large variety of biomolecules such as amino acids and proteins being one of the most important. In this article, we review the work from this laboratory on the stabilization of gold nanoparticles using amino acids as well as using amino acids as reducing agents to obtain stable aqueous solutions of gold nanoparticles of variable size. We also discuss the possibility of forming bioconjugates of enzymes with gold nanoparticles decorating the surface of polymer microspheres and their application as reusable biocatalysts. There is much to be gained by marrying nanomaterials with biology with considerable spin-offs likely in both nanotechnology and biotechnology.
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1. Introduction
Nanomaterials have wide-ranging implications in a variety of areas, including chemistry, physics, electronics, optics, materials science and the biomedical sciences. Metal nanoparticles are of considerable interest as they exhibit unique electronic, optical, and catalytic properties, due to the quantum size effects [1–4]. These nanoparticles are presently under intensive study for applications in optoelectronic devices [5], ultrasensitive chemical and biological sensors [6–8], and as catalysts in chemical and photochemical reactions [9,10]. A key challenge in the application of these materials is prevention of agglomeration of the nanoparticles, which can be overcome through surface functionalization/stabilization [11]. Therefore, the development of synthesis protocols for nanomaterials over a range of chemical compositions, sizes and indeed shapes constitute a steadily evolving branch of nanotechnology. The synthesis of metal nanoparticles has, in particular, received considerable attention, and potential applications in catalysis [12], single electron tunneling [13], non-linear optical devices [14] and DNA sequencing [15] have been demonstrated. Like metal nanoparticles, magnetic and semiconductor nanoparticles are also being viewed with interest from a fundamental point of view [16] as well as in applications such as magnetic memory storage devices [17], magnetic resonance image enhancement [18] and magnetic refrigeration [19]. The fact that the optical absorption/emission properties of semiconductor nanoparticles can be tuned by simple variation in nanoparticle size is particularly attractive in the facile band-gap engineering of materials [20] and the growth of quantum dot lasers [21]. More recently, nanoscale matter has been
looked at with interest for potential application in nano-computers, synthesis of advanced materials, energy storage devices, electronic and optical displays, chemical and biosensors as well as biomedical devices [22]. It is expected that some of the more immediate applications of nanoparticles will be in medical diagnosis and therapeutics. Exciting examples include detection of genetic disorders using gold nanoparticles [23,24], color-coded fluorescent labeling of cells using semiconductor quantum dots [8] and cell transfection for gene therapy and drug delivery [25].

While the properties of individual (non-interacting) nanoparticles is well documented and understood, in many situations it would be desirable to assemble the nanoparticles either in solution or on suitable substrates. In more scientific terms, tailoring the collective properties of nanoparticle ensembles is also an important aspect of nanotechnology. There is increasing scientific interest in the growth of ordered two-dimensional (2D) and three-dimensional (3D) structures assembled from nanoparticles. Much of the work in this direction is stimulated by the potential use of ordered 2D and 3D nanostructures in advanced technologies such as photonics and plasmonics. The different techniques for the assembly of inorganic nanoparticles into materials with higher-order architecture are collectively termed nanotectonics. These include the shape-directed assembly [26] and programmed assembly [15] of nanoparticles comprising surface attached molecules, ligands, and recognition sites, as well as the formation of complex hybrid nanostructures by in situ transformation of unstable nanoparticle-based precursors [27]. The template-directed assembly includes the use of nanoparticles, which are spatially confined within organized interiors such as tobacco mosaic virus (TMV) [28], carbon nanotubes [29], colloidal crystals [30], coated polymer beads [31] and bacterial membranes [32]. Synthesis of hierarchical ordered inorganic framework materials using different templates is of great importance because of their application in catalysis, separation techniques and materials chemistry. Electrostatic interactions and hydrogen bonding are the driving force for assembly of inorganic nanoparticles into superstructures of varying topological complexity in solution. Recently researchers have also used biological molecules as templates for the generation of inorganic structures and materials. Biological systems form sophisticated mesoscopic and macroscopic structures with tremendous control over the placement of nanoscopic building blocks within extended architectures. Both biotechnology and materials science meet at the same length scale because biomolecular components have typical size dimensions in the range of about 5–200 nm. To exploit and to utilize the concepts administered in natural nanometer-scale systems, the development of nanochemistry is crucial. On the other hand, microorganisms including viruses, bacteria, and fungi, possess unique, structurally interesting motifs, and can reproduce quickly and inexpensively, which makes them attractive targets for use in directing materials synthesis. The fabrication of inorganic nanoscale materials has been dominated by studies of biomineralization processes, during which biological structures provide templates for, or even catalyze, naturally occurring as well as artificially induced reactions involving inorganic precursors [33–39]. Examples in nature of inorganic materials synthesis directed by microorganisms include the formation of iron oxides by bacteria, calcification cyanobacteria, silica deposition in diatoms, and the formation of various other biogenic minerals by bacteria, lichen, algae, and fungi [38–41]. Other examples include the binding of metal ions to surface-layer proteins [42], DNA [33], virus protein cages [34], or ordered protein assemblies [35] to initiate sites of nucleation for nanoparticle growth, and the synthesis of metallic nanocrystals through bioreduction of metal ions inside bacterial [36], virus [37], and fungal species [38]. Typically, wet-chemical preparation of nanoparticles is carried out in the presence of stabilizing agents (often citrate, phosphanes or thiols), which bind to the atoms exposed at the surface of the nanoparticles. This capping leads to stabilization and prevents uncontrolled growth and aggregation of the nanoparticles. Amino acids [43,44] and proteins/enzymes [45–47] are some of the biomolecules that can directly be linked with the metal nanoparticle.

In Section 2 we will present results from this laboratory on the use of amino acids as a capping and reducing agents in the synthesis of stable metal nanoparticles in an aqueous medium. In Section 3 we will use the propensity of gold nanoparticles to bind to amino acids to immobilize enzymes with the nanoparticles thereby creating a new class of biocatalyst.

2. Interfacing nano with amino acids: capping and reducing agents

There are a number of synthesis procedures for obtaining gold nanoparticles over a range of sizes and shapes that may be broadly classified into two sections depending on whether the nanoparticles are grown in a non-polar organic medium or a polar medium such as water. The gold particles capped and stabilized with thiol-derivative monolayers may be obtained as a powder (by evaporation of the organic solvent) that is readily re-dispersible in non-polar and weakly polar organic solvents. Murray and co-workers have shown that such monolayer-protected clusters (MPCs) may be viewed as novel chemical reagents wherein the gold nanoparticle core plays the role of a support for the reactive molecules constituting the shell [48]. Development of synthesis protocols for realizing monolayer-protected
water-dispersible gold nanoparticles, on the other hand, have received considerably less attention and would have immediate application in catalysis, sensors, molecular markers, and in particular, biological applications such as biolabelling and drug delivery. Recognizing that the solubility of gold (and in some instances silver) MPCs in water/strongly polar organic solvents would be dominated by the terminal functionality of the capping monolayer, a number of different thiol-coordinated functional groups such as tiopronin [49], glutathione [50], succinic acid [51], sulfonic acid [52], and ammonium ions [53] have been shown to result in MPCs that are readily dispersible in water. Relatively more recent is the demonstration that amine derivatives complex with gold nanoparticles in a manner similar to that of thiol derivatives. Using amine chemistry for surface modification, we have shown that aqueous gold nanoparticles may be phase-transferred into non-polar organic solvents by complexation with alkylamine molecules [54,55]. This new approach opens up the exciting possibility of surface modification of gold nanoparticles with amino acids where binding with the gold nanoparticle surface may be accomplished through the amine functionality. In this section, we report our finding that capping aqueous gold nanoparticles with the amino acid lysine stabilizes the particles in solution electrostatically and also render them water-dispersible. The lysine capped gold nanoparticles may be obtained in the form of dry powder after evaporation of the aqueous component, this powder being extremely stable in air and readily dispersible in water. Development of protocols for the synthesis of water-dispersible nanoparticles has immense application in a variety of fields, but clearly more so in bio-related areas such as bio-labeling and biosensing. This study represented the first step in the possible application of amino acid-derivatized gold nanoparticles as novel aqueous reagents in amide and esterification reactions in a manner similar to that demonstrated by Murray and co-workers for functionalized gold nanoparticles in an organic environment.

Fig. 1A shows the UV–VIS absorption spectra of the lysine capped gold hydrosol at different stages of preparation. Curve 1 in the figure corresponds to the spectrum of gold colloidal solution obtained by borohydride reduction of aqueous chloroauric acid; curve 2 is the spectrum of gold colloidal solution after capping with lysine, and curve 3 the spectrum of the redispersed purified powder of lysine-capped gold nanoparticles in water at pH 7. The spectra have been shifted vertically for clarity. A strong absorption in curve 1 at ≈510 nm is observed that corresponds to excitation of surface plasmon vibrations in the gold nanoparticles. When the gold nanoparticles are capped with lysine, a broadening and red shift of the surface plasmon band are observed (curve 2) and indicate some aggregation of the gold nanoparticles consequent to surface modification. However, the lysine-capped gold colloidal solution was stable for months with little evidence of further aggregation. What is interesting and particularly germane to this study is the fact that the spectrum recorded from the redispersed lysine-capped gold nanoparticle solution (curve 3) shows only slight broadening relative to that recorded from the as-capped gold colloidal solution (curve 2). This clearly shows that repeated centrifugation, washing, and finally drying of the lysine-capped solution and long-term storage of the powder thus obtained lead to a tolerable degree of aggregation of the gold nanoparticles. The above results show that it is indeed possible to stabilize gold nanoparticles in water by surface complexation with the amino acid lysine and also render them water-dispersible.

Fig. 1B shows the UV–VIS spectra recorded from the redispersed lysine-capped gold nanoparticles as a function pH of the hydrosol, curves 1, 2, and 3 in the figure corresponding to spectra recorded at pH 3, 7, and 10, respectively. In all the spectra, a strong absorption band ca. 525 nm is observed that corresponds to excitation surface plasmon vibrations in the lysine-capped gold nanoparticles. On comparison of curve 1 (pH 3) with curves 2 (pH 7) and 3 (pH 10), one observes a broadening of the surface plasmon resonance at the two higher pH values. The isoelectric point (pI) of pure lysine is 9.74. However, when lysine binds to gold nanoparticles through the amine group, its isoelectric point is expected to change. Consequent to binding of one of the amine groups with the gold nanoparticle surface, the new isoelectric point will be the average of the pK\text{a} of the carboxylic acid group and the pK\text{b} of the terminal amine.
amine) group and, therefore, \( \approx 6.35 \). Below \( \text{pH} \ 6.35 \), the surface-bound lysine molecules exist in cationic form due to the formation of ammonium ions. The ammonium ions prevent formation hydrogen bonds between neighboring gold nanoparticles illustrated in Scheme 1. Above \( \text{pH} \ 6.35 \), surface-bound lysine molecules are negatively charged due to the formation of carboxylate ions, which readily form hydrogen bonds with surface-bound amine groups of neighboring gold nanoparticles (Scheme 1). Hence, lysine-capped gold nanoparticles at \( \text{pH} \ 7 \) and 10 show broadening of the surface plasmon resonance in comparison with the gold nanoparticle solution at \( \text{pH} \ 3 \). The broadening of the surface plasmon resonance is due to hydrogen-bonding induced aggregation of the gold nanoparticles is easily shown by heating the high-\( \text{pH} \) solution at 90 °C for 10 min (curve 4). It is observed that this spectrum is almost identical to that of the lysine-capped gold colloidal solution at \( \text{pH} \ 7 \), showing that particles may be peptized by this heating process as observed in our earlier work on cysteine capped silver colloidal solutions [43].

Fig. 2A and B show the representative TEM images recorded from as-prepared borohydride-reduced gold nanoparticles and lysine-capped gold nanoparticles re-dispersed in water at \( \text{pH} \ 7 \) deposited in the form of films onto carbon-coated TEM grids, respectively. Due to the fact that there is no stabilization of the gold nanoparticles in the borohydride-reduced nanoparticles, they aggregate to yield clusters in which the individual particles are difficult to distinguish (Fig. 2A). On the other hand, capping of the gold nanoparticles with lysine stabilizes the particles and prevents their physical contact (Fig. 2B). From Fig. 2B, it is seen that the gold nanoparticles are fairly monodisperse and adequately protected by the amino acid monolayer. Furthermore, it is observed that the lysine-capped gold nanoparticles assemble into a network with a very uniform separation between the nanoparticles. Negligible sintering of the nanoparticles is observed, clearly indicating that the surface coating of lysine molecules stabilizes the particles in solution and in thin film form. It is clear that this layer of lysine also enables redispersion of the nanoparticles in water.

Fig. 2C and D show representative TEM images recorded from lysine-capped gold nanoparticles re-dispersed in water at \( \text{pH} \ 3 \) and \( \text{pH} \ 10 \), respectively. At \( \text{pH} \ 10 \) (Fig. 2D), it is clearly seen that the particles aggregate into large superstructures in which the individual gold particles are difficult to distinguish. On the other hand, the lysine-capped gold nanoparticles at \( \text{pH} \ 3 \) are well-separated from each other (Fig. 2C). Thus, the TEM results of Fig. 2C and D provide direct and unequivocal support to the UV–VIS conclusions that the particles aggregate (reversibly) at \( \text{pH} \ 10 \) while they are fairly well-dispersed at \( \text{pH} \ 3 \) (Scheme 1).

We have discussed above how amino acids could act as capping agents that stabilize and render gold nanoparticles water-dispersible. We have also used amino acids as reducing agents for synthesizing stable metal
nanoparticles in an aqueous medium. For reduction of aqueous gold ions we have used the amino acids aspartic acid [56] and tryptophan [57] as reducing agents. Fig. 3A shows the UV–VIS spectra recorded from the gold colloidal solution as a function of time of reduction of chloroaurate ions with aspartic acid under boiling conditions. A monotonic increase in the surface plasmon resonance intensity with time is observed, the reaction terminating after /C24 10–12 min of reaction. The inset of Fig. 3A is a plot of the absorption intensity at 530 nm as a function of time and clearly shows how the reaction proceeds. This measurement thus establishes the optimum conditions for the preparation of gold colloids, which were stable for months. Fig. 3B shows a representative TEM image recorded from the aspartic acid synthesized gold nanoparticle solution. It is seen that the gold nanoparticles are extremely uniform in size (ranging in size from 24 to 27 nm), fairly monodisperse and adequately protected by the amino-acid monolayer. Therefore the use of biomolecules such as amino acids for surface modification of metal nanoparticles and the resulting water-dispersible nanoparticles have important implications for the application of this methodology to the formation of other medically important bioconjugates and for novel reagents in reactions such as peptide bond formation, esterification and so forth in aqueous media.

3. Gold nanoparticles assembled on polyurethane microspheres: novel template for enzyme immobilization

Gold nanoparticles have been used successfully for the binding of enzymes such as pepsin [45], fungal protease [46] and endoglucanase [47]. It is now fairly well accepted that the interaction of amine groups with gold nanoparticles is as strong as that of the more commonly used thiols and thus, amino acids and proteins may be immobilized on gold nanoparticles without modification. The binding of the enzymes to gold nanoparticles occurs through the amine groups and the cysteine residues present in the enzyme. While the gold nanoparticle bioconjugates showed excellent catalytic activity, a major drawback was that their reusability was extremely poor [45–47]. This was primarily due to the difficulty in separating the bioconjugate material from the reaction medium even under ultracentrifugation conditions. To overcome the problem, we have assembled gold nanoparticles on the submicron polyurethane (PU) microspheres [58] (Fig. 4A). Binding of the gold nanoparticles to the polymer surface occurs through the nitrogen present in the polymer backbone. The gold nanoparticles present on the surface of the polymer microspheres are used for the bioconjugation of the enzyme pepsin [58]. The polyurethane gold enzyme bioconjugates were easily separated from the reaction mixture, and showed excellent reuse characteristics. Such methods can be extended to the assembly of gold nanoparticles on the surface of amine-functionalized zeolites and thereafter used for the bioconjugation of the enzymes [59,60].

The PU microspheres of 2 μm mean diameter were synthesized as described elsewhere [61,62]. 10 mg of nanogold-PU 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 ≈10 min yielded an emulsion-like phase that rapidly phase separated upon cessation of shaking. The PU powder had turned purple

![Fig. 3](https://example.com/fig3.png)
is shown in Fig. 4A. The nanogold coated PU microspheres and accumulated at the hexane-water interface. The surface plasmon resonance in the as-prepared colloidal gold solution (curve 1) and the gold solution after addition of PU spheres and filtration (curve 2). The inset shows the UV–VIS spectrum recorded from a film of gold nanoparticle shell-PU core spheres on a quartz substrate. (Reprinted with permission from Ref.[58], ©2003 American Chemical Society.)

and accumulated at the hexane-water interface. The binding of gold nanoparticles on the PU microspheres is shown in Fig. 4A. The nanogold coated PU microspheres were separated by filtration, washed with double-distilled water, and dried in air. Thereafter, 10 mg of nanogold-PU powder was dispersed in 2 ml KCl-HCl buffer. To this solution, 100 μl of the stock solution consisting of 10 mg/ml of pepsin was added under vigorous stirring. After 1 h of stirring the bioconjugates were separated by centrifugation. The enzyme bound to the gold nanoparticles on polyurethane surface is shown in Fig. 4A. Fig. 4B 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 ≈520 nm (curve 1). 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 (as shown in Fig. 4A). The purple-colored 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 Fig. 4B 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 ≈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 (Fig. 4A). The amount of the gold nanoparticles in the nanogold-PU material was estimated by atomic absorption spectroscopy (AAS) and was ~20 wt.%.

Moreover, the presence of gold nanoparticles was also confirmed by spot profile EDAX measurements on nanogold-PU conjugate material (data not shown for brevity).

Fig. 5A and B show representative TEM micrographs of the Au-PU composite on a carbon-coated copper grid. At lower magnification (Fig. 5A), a number of PU spheres over a range of sizes in close contact can clearly be seen. At higher magnification (Fig. 5B), 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 [63] and primary amines [64] 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.

Fig. 5C show representative SEM images of drop-cast films of the PU-nanogold core-shell material and Fig. 5D and E the pepsin-nanogold-PU bioconjugate material on Si (1 1 1) substrates. While the surface texture of the PU spheres capped with gold nanoparticles is quite smooth (Fig. 5C, the gold nanoparticles are not clearly visible at the resolution of the SEM measurement), after conjugation with pepsin, thin sheets of presumably the enzyme are seen together with smooth PU microspheres (Fig. 5D and E). Spot profile EDAX analysis of the sheets (marked by an ‘X’ in Fig. 5D) 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 [65]. Spot profile EDAX analysis of the smooth nanogold-capped PU spheres away from the pepsin sheets (marked by a ‘+’ in Fig. 5D) also showed the presence of sulfur indicating binding of the enzyme to the gold nanoparticles even though not visible by SEM imaging. The sulfur signal was absent in the nanogold-PU core-shell material as expected.

The most important aspect of this study concerns retention of the biocatalytic activity of pepsin after adsorption onto the nanogold-PU surface. Since the amount of pepsin bound to the bioconjugate could be estimated from UV–VIS measurements (the loss in absorbance at 280 nm 280 nm arising from π to π transitions in tryptophan and tyrosine residues in the enzyme) in the supernatant after centrifugation of the bioconjugates, 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 amount of pepsin bound to 10 mg of nanogold-PU was estimated 0.18 mg. The biocatalytic activity of the identical amount of the 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 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 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 [45–47]. 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 [45–47]. 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

Table 1
Biocatalytic activities obtained from assaying the pepsin-gold nanoparticles-PU bioconjugate material over six sequential reuses

<table>
<thead>
<tr>
<th>System</th>
<th>Biocatalytic activity (IU/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin in solution</td>
<td>11.2</td>
</tr>
<tr>
<td>Pepsin-gold-PU spheres, run 1</td>
<td>13.2</td>
</tr>
<tr>
<td>Pepsin-gold-PU spheres, run 2</td>
<td>10.2</td>
</tr>
<tr>
<td>Pepsin-gold-PU spheres, run 3</td>
<td>8.0</td>
</tr>
<tr>
<td>Pepsin-gold-PU spheres, run 4</td>
<td>7.2</td>
</tr>
<tr>
<td>Pepsin-gold-PU spheres, run 5</td>
<td>4.3</td>
</tr>
<tr>
<td>Pepsin-gold-PU spheres, run 6</td>
<td>3.6</td>
</tr>
</tbody>
</table>

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* 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 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 non-existent in this case.

Table 1 shows the results of six 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 \( \approx 28\% \) of the starting activity after six 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 [66]. 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-Au nano-PU bioconjugate material as a function of reuse of cycles is due to the leaching of the weakly bound enzyme in the form of aggregates as seen in the SEM images. UV-vis spectroscopy measurements were carried out on the supernatant from 10 mg of the pepsin-Au nano-PU bioconjugate material immersed in 2 ml of pH 2 buffer solution at 37°C. A 1 ml of sample of the supernatant was analyzed in intervals of 1 h, which is characteristics of the reaction times in the reuses. 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 arising from \( \pi \) to \( \pi^* \) transitions in tryptophan and tyrosine residues in the enzyme). 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). 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-Au nano-PU bioconjugate (Fig. 5D) correspond to the weakly bound enzyme that leaches out in the first reaction cycle. Hence to confirm the leaching of the weakly bound pepsin to bioconjugates during the biocatalytic reaction, SEM images of bioconjugates after one cycle of reuse were recorded. It is clearly seen from the Fig. 5F that the percentage of the 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 [67]. 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. While 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. Fig. 6A shows the

![Fig. 6.](image-url)
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 2–10. 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 (Fig. 6A). A small biocatalytic activity was observed in the pepsin-nanogold-PU bioconjugate even at pH 10.

Fig. 6B 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 ºC. Three separate measurements at each temperature were performed to check the reproducibility of the data. While the free enzyme in solution is intolerant to even a 10 ºC increase in temperature of the reaction medium (biocatalytic activity at 60 ºC is only 4% of the value at 50 ºC), pepsin in the bioconjugate material showed only a 32% fall in biocatalytic activity under similar temperature rise conditions (Fig. 6B). Indeed, the bioconjugate material showed a significantly high biocatalytic activity even at 70 ºC (Fig. 4B, ≈14% of the specific biocatalytic activity at 50 ºC) 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.

So far, we have discussed the possibility of using inorganic nanoparticles for linking with biomolecules. We have also pursued the possibility of using microorganisms such as fungi in the synthesis of metal [68,69] and metal sulfide [70] nanoparticles. This green chemical approach to the synthesis of inorganic nanomaterials draws its inspiration from nature where inorganic nanostructures of exquisite structure are routinely synthesized. The interested reader is directed to a recent review for further details [71].

4. Conclusions

In this article, we have attempted to outline some of the new approaches developed in this laboratory on the synthesis and assembly of inorganic nanoparticles such as gold using biomacromolecules. We have shown that gold nanoparticles may be stabilized by lysine, this amino acid also rendering the particles water-dispersible. Amino acids such as aspartic acid and tryptophan are also good reducing agents and yield stable gold nanoparticles of controllable size and monodispersity in an aqueous medium. The fact that amino acids bind effectively to gold nanoparticles through the amine groups has been used in the immobilization of proteins and enzymes on gold nanoparticles. As a means of developing a simple biocatalyst with good reuse characteristics and pH and temperature stability, we have decorated polyurethane microspheres with gold nanoparticles and used these core-shell structures as scaffolds in the immobilization of enzymes such as pepsin. It is clear that both nanotechnology and biotechnology can benefit much from effectively interfacing nanoparticles and biology.

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