

Fabrication of Biocompatible Surface through Fixation of Silver Nanoparticles Assembled on Polyurethane

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Abstract

Development of simple and reliable protocols for the immobilization of enzymes is an important aspect of nano biotechnology. Silver nanoparticles are known to bind enzymes, but reuse characteristics of the silver nano-enzyme bioconjugates has hitherto been poor. In this paper, we demonstrate that silver nanoparticles bound at high surface coverage on polyurethane. This material can be washed, dried, and stored for extended periods without the loss of nanoparticles. The performance of the material is as an antibacterial filter. The silver nanoparticle coated on polyurethane then act as templates for the immobilization of the enzyme butyrylcholinesterase (BuChE). BuChE in the bioconjugate system shows enhanced stability toward harsh temperature and pH conditions.

Keywords: Silver Nanoparticles; Polyurethane; Enzyme Immobilization; Nanobiotechnology.

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1. Introduction:

Synthesis of biocompatible surfaces is an area of considerable interest in biotechnology with application in the immobilization of proteins, enzymes, and DNA as well as in biosensing and biomedical areas [1-6]. Problems related to the immobilization of enzymes are of particular relevance in the biosensing and chemical industries. Consequently, a number of processes for enzyme immobilization in silica nanotubes, within phospholipid bilayers, on self-assembled monolayers, in Langmuir Blodgett films, within polymer matrix and galleries of α -zirconium phosphate, in mesoporous MCM-41 (Mobil Composite Material), and in thermally evaporated lipid films have been developed [7-14]. A number of groups have studied the adsorption of proteins on both polymer and inorganic colloidal particles of

oxides/metals [15]. Recently, biochemically functionalized silica nanoparticles have been used for immobilizing enzymes [15]. In this study, both pure and dyedoped silica nanoparticles were prepared and their surfaces were modified by enzymes and biocompatible chemical reagents [16]. In the area of metal nanoparticle-enzyme conjugate materials, Crumbiss, Stonehuerner, and coworkers have studied the formation and enzymatic activity of gold nanoparticles complexed with horseradish peroxidase, xanthine oxidase, and glucose oxidase and carbonic anhydrase molecules [17-19]. A salient feature of their work is the demonstration that enzyme molecules are bound tightly to gold colloidal particles and retain significant biocatalytic activity in the conjugated form while the enzyme molecules denature on adsorption to planar surfaces of gold.

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2. Experimental

2.1. Synthesis of silver nanoparticles

The synthesis of Ag nanoparticles was done according to the literature procedure [11]. Briefly, the synthesis involves the following materials and methods: A solution of silver nitrate (25 mL, 0.005 M) in water was diluted to 125 mL and heated until it begins to boil. 5 mL of 1% sodium citrate solution was then added, heating continued until the color was pale yellow. The solution was cooled to room temperature. The synthesized nanoparticles were characterized by UV-Vis spectroscopy, transmission electron microscopy (TEM).

2.2. Polymer synthesis

Initially, 4 mL of pH = 4 HEPES buffer (150 mM with 50 mM CoCl₂) containing surfactant pluronic L-62 (%1 wt) were placed in a narrow mixing vessel. Then approximately 4 mL of Hypol 5000 prepolymer (preheated to 35 °C to limit problems due to high viscosity) were added to the mixture. This two phase system was mixed for 45 to 50 min. During the reaction period, the solution was poured into a glass vessel. CO₂ evolved during the reaction of water and isocyanate and subsequent curing brings the foam to a final volume of approximately 50 to 60 mL. Bulk foam samples were usually placed in a hood to facilitate the removal of residual water. Foams were stored at -4 °C until use.

2.3. Formation of nanosilver-polymer

Polyurethane (PU) foams were soaked in silver nanoparticle solution for overnight. For the saturated coating of 2 cm ~ 2.5 cm foam of 8 mm thickness ~150 mL of the nanoparticle solution was required. This leads to saturation coverage. Partial coverage can be achieved for shorter exposure times or reduced nanoparticle concentrations. The sheets were washed repeatedly with water to remove any adsorbed ions like citrate and were air-dried.

2.4. Microbiological Experimentation

E. coli ATCC 25922 and *E. coli* MTCC 1302 were selected as indicators. Nutrient broth was used as the growing medium. Bacteria were grown aerobically in nutrient broth at 37 °C for 12 h. The cultures were centrifuged and the cells were washed and suspended in distilled water, reaching a final concentration of 1 × 10⁵–1 × 10⁶ CFU/mL. *E. coli* suspension (10 mL) in distilled water was taken in sterilized test tubes. 1 cm × 9 cm × 0.6 cm pieces of the foam were put into the test tubes (one piece in each test tube). After 5 min and 10 min, the foam samples were taken out from the test tubes and were put into an empty test tube. They were squeezed to get the treated water. Plating was done with this treated water by series dilution method

for 10⁰, 10⁻² and 10⁻⁴ dilutions with nutrient agar and m-Endo agar. Plating was also done for the initial CFU count and with uncoated PU-treated solution. For every dilution, 10 µL of the solution was plated. Plating was done by the pour plate method. Plates were incubated at 37 °C for 24h.

2.5. Formation of BuChE-silver nano-polyurethane bioconjugates

The nanosilver-polyurethane (3 g) was dispersed in KCl-HCl buffer (2 mL, 0.02 M, pH = 2). To this solution, a solution of BuChE (100 mL, 10 mg/mL) in KCl-HCl buffer (0.02 M, pH = 2) was added under vigorous stirring. After 1 h of stirring, the BuChE-silver nanopolyurethane bioconjugate material was separated. Loss in absorbance at 280 nm was used to quantify the amount of BuChE bound to the nanosilver polyurethane for specific activity determination. The polymer thus obtained was rinsed several times with KCl-HCl buffer (0.02 M, pH 2) solution, resuspended in buffer solution (pH 2), and stored at 4 °C for further experiments.

2.6. Biocatalytic Activity Measurements

The biocatalytic activity of free BuChE in solution and of BuChE-silver nano-polyurethane bioconjugate in KCl-HCl buffer (0.02 M, pH = 2) was determined by reaction with 0.6% BuChE at 37 °C for 1 h. Control experiments on the biocatalytic activity of BuChE immobilized directly onto polyurethane were also performed under identical conditions as described above.

The BuChE silver nano-polyurethane and BuChE-polyurethane bioconjugate materials were separated from the reaction medium.

In typical experiments to estimate the biocatalytic activity of the bioconjugates, a carefully measured amount of the BuChE – silver nano-polyurethane/ BuChE-polyurethane bioconjugate in buffer was incubated with casein solution (1 mL, 0.6%) at 37 °C for 1 h. After the incubation time, an equal volume of perchloric acid (1.7 M) was added to the reaction mixture to precipitate the residual BuChE. After 1 h, the precipitate was removed by centrifugation and the optical absorbance of the filtrate was measured at 280 nm. The amount of BuChE in the bioconjugate material was quantitatively estimated during the preparation of the bioconjugate.

3. Results and Discussion

3.1. Synthesis of silver nanoparticles assembled on polyurethane

The use of metal nanoparticles for disinfection is relatively new. Because of their high reactivity due to the large surface to volume ratio, nanoparticles are expected to play a crucial role in purification, when environment becomes an important

commodity. Several investigations have been carried out on the bactericidal effect of nanoparticles and their applications in the plastics, health, textile, and paint industry. Experiments showed that the treated *Escherichia coli* cells were damaged, showing formation of pits in the cell wall of the bacteria. TEM image was used to characterize the synthesized silver nanoparticles. This image can be well indexed to spherical structure of nanosilver (Figure 1).



Figure 1. TEM image of nanosilver colloids

3.2. Synthesis of polyurethane

Results of elemental analysis obtained are as follows:

Table 1. Elemental analysis for polyurethane

Polymer	%C	%H	%S	%N
Calculated	58.75	6.17	13.05	5.71
Found	59.01	6.67	13.45	5.98

As shown in figure 2, the FTIR spectra of polymer showed the characteristic absorption of $\nu(\text{C}=\text{O})$ bond of urethane groups at $1780\text{-}1600\text{ cm}^{-1}$, $\nu(\text{-NH})$ stretching of urethane groups at $3500\text{-}3100\text{ cm}^{-1}$, $\nu(\text{-NH})$ bending vibration of the urethane group at $1600\text{-}1510\text{ cm}^{-1}$ and $\nu(\text{CH}_2)$ stretching at 2960 cm^{-1} .

1.1. Morphology of silver nanoparticles coated polyurethane

Color of polyurethane foam changes from white to yellow when it is soaked in silver nanoparticles on polyurethane nanoparticles solution overnight. The saturated binding of gives a silver yellow color to it. There was no loss of nanoparticles after several (4–7 times) washing and drying operations and after keeping it for several months in a closed environment.

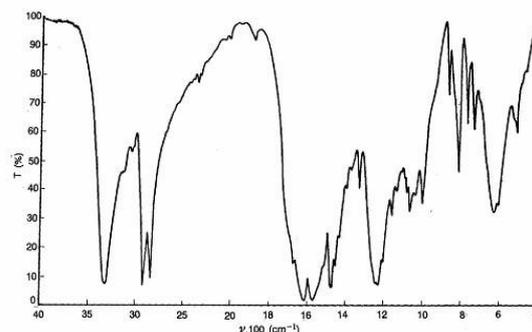


Figure 2. FTIR spectra of polyurethane

Washing was done with distilled water and each time foam was kept in the water for ~ 20 min. We have shown below that binding is due to interaction between nitrogen of the -N(H)- of polyurethane and silver nanoparticles. Nanoparticles are stable on the foam and are not washed away by water. Morphology of the foam was retained after coating. The results of antibacterial test showed that output count of *Escherichia coli* was nil when the input water had a bacterial load of 10^5 colony-forming units (CFU) per mL.

The UV-Vis spectrum of the silver nanoparticles solution (Figure 3) shows a peak around 420 nm. The data show that silver nanoparticles are not undergoing any change after coating onto the polyurethane surface.

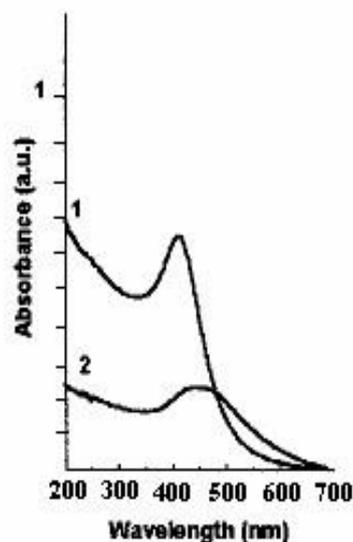


Figure 3. UV-Vis spectra of colloidal silver solution (curve 1) & the silver solution after mixing with polyurethane for 12 h (curve 2)

Preparation of the nanosilver - polyurethane followed by UV-vis spectra recorded from the as prepared colloidal silver solution and the silver solution after stirring with polyurethane for 1 h and filtration. After the colloidal silver solution is

stirred with the polyurethane for 12 h, it was seen that there is a loss in intensity of UV-vis spectra due to a decrease in the concentration of silver.

SEM image of coated polyurethane is shown in (Figure 4). The PU maintained its morphology upon coating with silver nanoparticles. It appears that the nanoparticle binding is on the surface. We could not observe silver nanoparticles on the surface, probably due to specific surface characteristics of the foam. The surface of the foam is known to be highly porous which results in a large surface area. It is likely that the surface roughness of the foam is comparable to the dimensions of the nanoparticles and topography of the surface may not reflect the presence of nanoparticles. At much larger magnifications, one could have observed the particles.

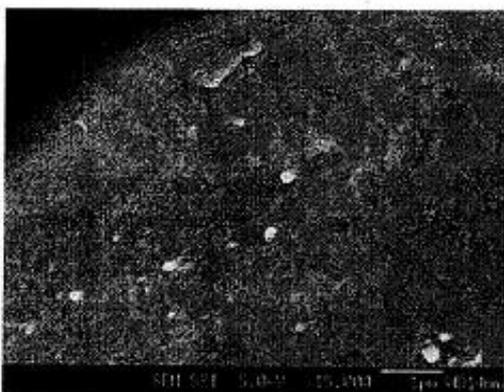
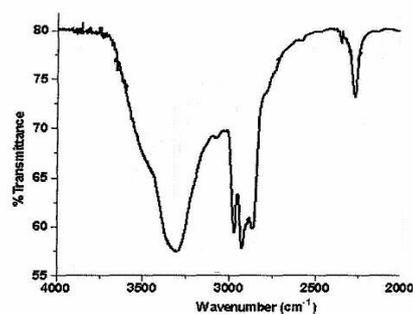


Figure 4. SEM image of polyurethane coated with silver nanoparticles

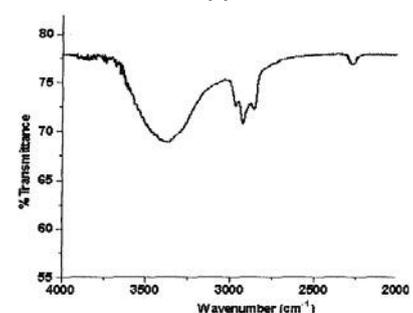
FTIR studies (Figure 5) show that binding is due to the interaction between the nitrogen of the $-N(H)-$ bond of polyurethane and silver nanoparticles. There is a significant shift in the $-N-$ peak upon nanoparticle coating, while all the other peaks remain unchanged. The shift observed for this peak is 60 cm^{-1} . It appears that not all the PU is involved in bonding as part of the $-NH-$ stretch is unaffected.

1.1. Microbiological Results

After a contact time of 5 and 10 min with the PU coated with silver nanoparticles, there was no bacterium detected in the treated water. For both *E. coli* strains, the output count was zero for all the dilutions. There was no bacterium in the output water after passing through the coated foam for *E. coli* for a continuous and constant flow rate of 0.5 L/min . This was checked for input loads of 1×10^3 and $1 \times 10^5\text{ CFU/mL}$. There was no growth below the PU coated with nanoparticles while growth was seen in case of pure PU, which again confirms the antibacterial property of PU coated with silver nanoparticles.



(a)



(b)

Figure 5. FTIR spectra of PU without (a) & with (b) silver nanoparticles

1.2. Immobilization of enzyme on nanosilver coated polyurethane

The high efficiency of the immobilization technique can be partly attributed to the mild conditions, which minimize enzyme denaturation. We have found that silver nanoparticles form excellent bioconjugates with enzymes. However, one of the major drawbacks of a silver nanoparticle based method for enzyme immobilization is that reuse of the biocatalyst is difficult to achieve. Amine groups bind very strongly to silver nanoparticles and therefore, we have attempted to entrap aqueous silver nanoparticles on the surface of polymer with amine groups and thereafter use the silver-nano-decorated polymer to immobilize the enzyme BuChE.

1.3. Biocatalytic Activity Measurements

The most important goal in enzyme immobilization strategies is complete retention of biocatalytic activity of the bioconjugate. To compare quantitatively the biocatalytic activity of BuChE immobilized on the nano silver polyurethane with the BuChE in solution, the amount of immobilized BuChE was estimated from UV-vis measurements (table 2). It is clear that the biological activity of the enzyme in the bioconjugate system is not compromised after immobilization on the surface of the nano silver polyurethane particles. The apparent marginal enhanced activity of the bioconjugate system is within experimental uncertainty, and therefore, we hesitate in making claims to enhanced biocatalytic activity of the immobilized enzyme on silver coated polyurethane.

Table 2. Biocatalytic Activities of the theBuChE in solution and BuChE-Nano Silver Polyurethane Bioconjugate

No. of cycles	Biocatalytic activity of BuChE in solution	Biocatalytic activity of BuChE immobilized on nanosilver- Polyurethane (IU/g)
1	10.3	14.6
2	6.5	12.9
3	4.1	12
4	2.5	10.7
5	0	9.3
6	0	8.2

While the exact reasons for the better reuse characteristics of the BuChE-silver nanopolyurethane system are not clear at the moment, we believe the BuChE molecules are bound much more strongly to the silver nanoparticles than to the polyurethane polymer. This would significantly reduce leaching out of the enzyme during the successive reaction cycles and thus lead to improved retention of biocatalytic activity.

It is seen that optimum biocatalytic activity (Figure 6) in both the cases is at pH 6, with a marginal loss in biocatalytic activity at pH 10. At pH 10.5, however, free enzyme molecules in solution retain only 2% of the biocatalytic activity recorded at pH 6, while the BuChE molecules immobilized on the silver nano-polyurethane template retain as much as 50% of the catalytic activity recorded at pH 6. This highlights the important role of the silver nanoparticles in enhancing and stabilizing the catalytic activity of BuChE in the immobilized state. At higher temperatures, dramatic differences in the biocatalytic activity of the enzyme in the two cases are observed (Figure 7). At 50 °C, the free enzyme has lost almost all of its biocatalytic activity (only 4% of the biocatalytic activity at 40 °C), while BuChE immobilized on the silver nanopolyurethane template retained 90% of the initial biocatalytic activity (40 °C measurement).

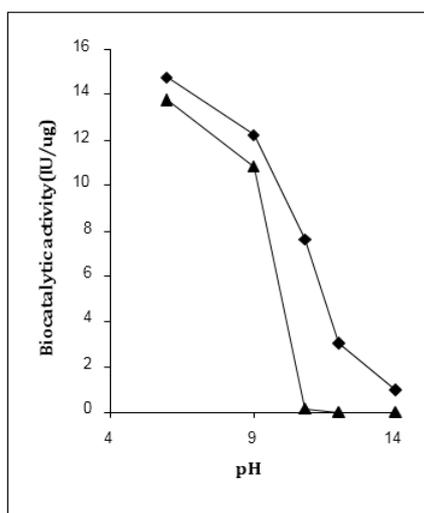


Figure 6. pH dependent enzymatic activity of BuChE in solution (triangles) and BuChE in the silver-PU (circles)

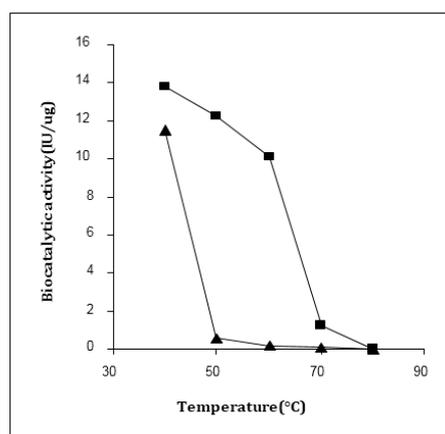


Figure 7. Temperature dependent enzymatic activity of BuChE in solution (triangles) and BuChE in the silver-PU (squares)

2. Conclusions

In this study, we have demonstrated the assembly of silver nanoparticles on polyurethane, the binding of the nanoparticles occurring via complexation with free amine groups present in the polyurethane. The silver nanoparticle coated on polyurethane then act as templates for the immobilization of the enzyme BuChE. This enzyme in the bioconjugate system shows enhanced stability toward harsh temperature and pH conditions.

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