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# Novel Core-Shell Nanoparticles and Their Application in High-Capacity Immobilization of Enzymes

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## Abstract

Novel core-shell nanoparticles consisting of poly(methyl methacrylate) (PMMA) cores coated with synthetic polymer and biopolymer (polyethyleneimine, chitosan, and casein) shells were synthesized via direct graft copolymerization of methyl methacrylate from hydrophilic polymers in the absence of surfactant. Average hydrodynamic diameters of the nanoparticles ranged from 163 to 263 nm. High-capacity (up to 530 mg/g) immobilizations of enzymes and high-activity retained percentage ($E_{spe}$) (up to 90%) were achieved.

**Index Entries:** Core shell; poly(methyl methacrylate); nanoparticles; immobilized cellulase.

## Introduction

Progress in bioimmobilization has resulted in a revolution of the use of biomolecules for applications in pharmaceutical and food industries as well as in clinical and chemical analyses *(1,2)*. These applications typically

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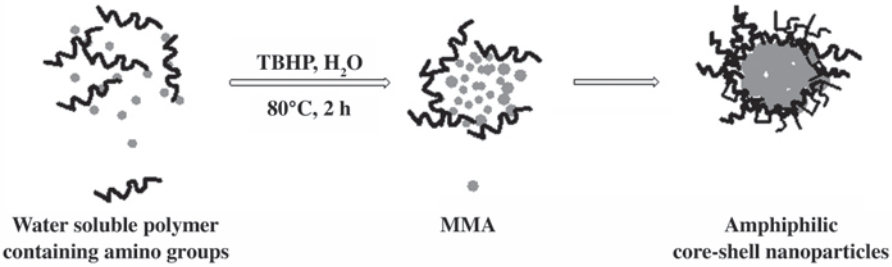


Scheme 1. Schematic representation of formation of amphiphilic core-shell nanoparticles.

rely in large part on the successful immobilization of intact biomolecules onto or within a suitable host *(3,4)*. Over the last decade there have been immense efforts to fabricate core-shell colloidal materials with tailored structural, optical, and surface properties *(5–10)*. Investigations have largely been spurred by the applicability of such colloids in modern materials science, and by their technological importance. For example, composite colloids are utilized in the areas of catalysis, separations, and diagnostics. Core-shell particles often exhibit improved physical and chemical properties over their single-component counterparts and, hence, are potentially useful in a broader range of applications *(11)*. The potential applications of colloidal particles with attached biologic molecules (e.g., enzymes, antibodies, and antigens) have long been recognized *(12–15)*.

Various immobilization procedures have been utilized for the coupling of biomolecules to particles such as polystyrene, polyacrylamide, and azalactone *(16)*. However, small substances do not adhere very well to, e.g., hydrophobic polystyrene surfaces through physical adsorption *(16)*. In contrast to planar substrates *(15)*, the layer-by-layer approach has been utilized to assemble protein multilayer architecture on collidal particles, but this method is extremely complicated and time-consuming. Furthermore, the shell layer is very sensitive to pH and salt changes *(17)*. These disadvantages may greatly restrict their future applications.

In this article, we report three kinds of novel core-shell nanoparticles with amphiphilic core-shell nanostructures, which are synthesized according to our previously described method *(17)* (Scheme 1). These nanoparticles contain polyethyleneimine [PEI], chitosan, and casein as shells as shown in Fig. 1.

The nanoparticles show high affinity to enzymes, and the best specific capacity can reach 530 mg/g, approximately eight times larger than that of conventional support *(18–20)*. Moreover, the highest retained percentage of activity ($E_{spe}$) is also up to 90%.

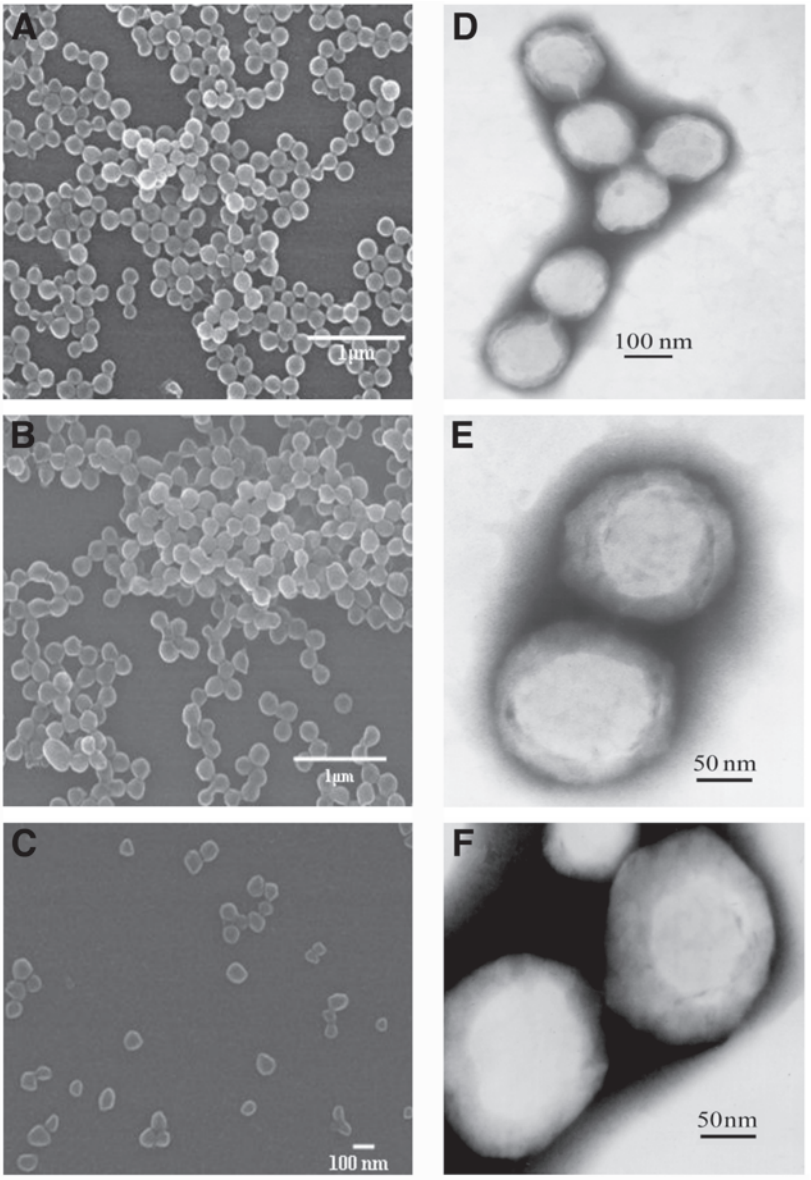


Fig. 1. SEM and TEM micrographs of core-shell nanoparticles: **(A)** PEI/PMMA (SEM); **(B)** chitosan/PMMA (SEM); **(C)** casein/PMMA (SEM); **(D)** PEI/PMMA (TEM); **(E)** chitosan/PMMA (TEM); **(F)** casein/PMMA (TEM).

## Materials and Methods

### *Chemicals*

Bovine serum albumin (BSA) (protease free); PEI, branched (50% solution in water, mol wt of 50,000–60,000); and *tert*-butyl hydroperoxide (TBHP) (70% solution in water) were all obtained from Acros. Chitosan

(low molecular weight, 77% deacetylation determined by an elemental analysis) was purchased from Aldrich. All of these chemicals were used without further purification. Casein (Acros) was purified by mixing it with 1% EDTA solution at 50°C for 48 h in order to remove metal ions in the casein. The phenolic inhibitor in methyl methacrylate (MMA) (Aldrich) was removed by washing three times with 10% NaOH solution and then with deionized water until the pH of the water layer dropped to 7.0. The MMA was further purified by vacuum distillation. Glutaraldehyde solution (25% solution in water) was purchased from Sinopharm. Carboxymethylcellulose (CMC) (ultra low viscosity) was obtained from Fluka-Riedel. Cellulase (*Aspergillus niger*) and 3,5-dinitrosalicylic acid (DNS) were supplied by Sigma. Freshly deionized and distilled water was used as the dispersion medium. All results represent averages of at least three experiments. Moreover, in all cases the experimental error was less than 5%.

### *Synthesis of Core-Shell Nanoparticles*

In a typical experiment to synthesize core-shell nanoparticles, hydrophilic polymers (0.25 g) were dissolved in 23.5 mL of water, followed by the addition of purified MMA monomer (1.0 g) in a water-jacketed flask equipped with a thermometer, a condenser, a magnetic stirrer, and a nitrogen inlet. The stirred mixture was purged with nitrogen for 30 min. Dilute TBHP (0.25 mL, $1.0 \times 10^{-2}$ *M*) was added to the mixture, and the solution was stirred at 80°C for 2 h under nitrogen. After the reaction, the unbound polymers were removed by repeated centrifugation at 20,442*g* for 30 min and decantation until the conductivity of the supernatant was close to that of the water used.

### *Characterization of Nanoparticles*

Zeta potentials were measured with a Zetasizer (Malvern 3000 HSA) in 1 mL KCl solution. The particle size and its distribution were determined with a particle size analyzer (Coulter LS 2300). Transmission electron microscopy (TEM) micrographs were obtained using a JEM-2010HR transmission microscope at an accelerating voltage of 200 kV. The sample was prepared by wetting either a formvar-coated or a carbon-coated grid with a small drop of dilute latex solution. On drying, it was stained with a small drop of 2% phosphotungstic acid for 30 min and dried at room temperature before analysis. Scanning electron microscopy (SEM) micrographs were obtained using a JEOL FEM6330F scanning microscope. Particular dispersions with a concentration of 150 ppm (w/w) in distilled water were dispersed using ultrasonic bath for 2 h. Then the sample was prepared by adding a small drop of the dilute latex solution onto a slide and dried under vacuum. It was coated with gold-palladium under vacuum before analysis.

### Bioimmobilization of Cellulase onto Nanoparticles

#### Physical Bioimmobilization

Enzymes were placed in contact with 500 µL of nanoparticle solution at pH 7.86 and shaken for 4 h.

#### Crosslinking Bioimmobilization

Enzymes were placed in contact with 500 µL of nanoparticle solution at pH 7.86 and shaken for 2 h. Then glutaraldehyde solution was added to the ultimate concentration (2%) and the mixture was shaken for 2 h.

#### Covalent Bioimmobilization

Activation of nanoparticle activation with glutaraldehyde (2%) was performed by shaking the solution for 2 h and then washing the particles with distilled water three times to remove the unreacted glutaraldehyde by centrifugation. The particles were then contacted with enzyme solution by shaking the mixture for 2 h. All of the final samples were washed with distilled water three times before analysis.

### Protein Assay

Protein concentration was estimated by the Bradford protein assay method using BSA as a standard *(21)*.

### Determination of Free and Immobilized Cellulase Activity

Enzyme activity on CMC was determined using DNS reagent as color-developing agent according to the literature *(22)*. The following formula was used to calculate the activity:

$$\text{Activity of cellulase } (\ \mu\text{mol}/[\text{min}\cdot\text{g}]) = \frac{\text{Amount of produced glucose}}{20E_W}$$

in which $E_w$ is the amount of enzyme (mg or mL). One international unit is the amount of enzyme that releases 1 µmol of reducing glucose/min from the CMC under the assay conditions.

## Results and Discussion

### Synthesis and Characterization of Core-Shell Nanoparticles

Alkyl hydroperoxides such as TBHP have been extensively used with metal ions to form redox pairs and initiate polymerization at low temperature *(23)*. Low molecular weight polyamines also form redox pairs with alkyl hydroperoxides that decompose to alkoxy radicals (RO·) and amine radicals *(24)*. These radicals can initiate polymerization under mild conditions *(25)*. In this work, TBHP was used to react with the amino groups of PEI, thereby generating radicals on nitrogen atoms, which subsequently initiate the graft copolymerization of the vinyl monomers *(17)*. Further-

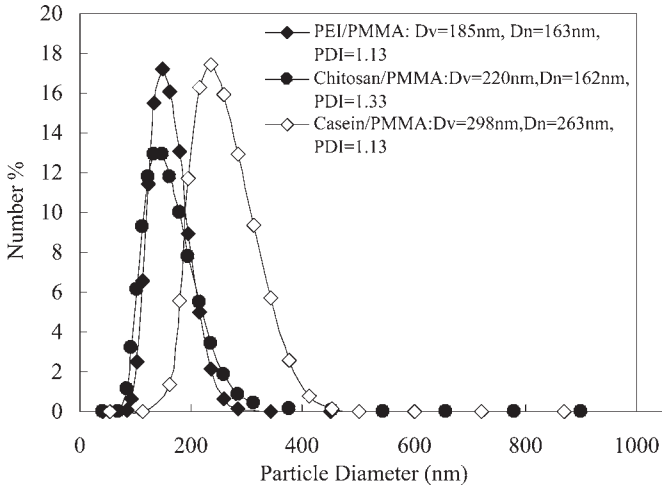


Fig. 2. Particle size distribution of three types of nanoparticles (PEI/PMMA, chitosan/PMMA, and casein/PMMA).

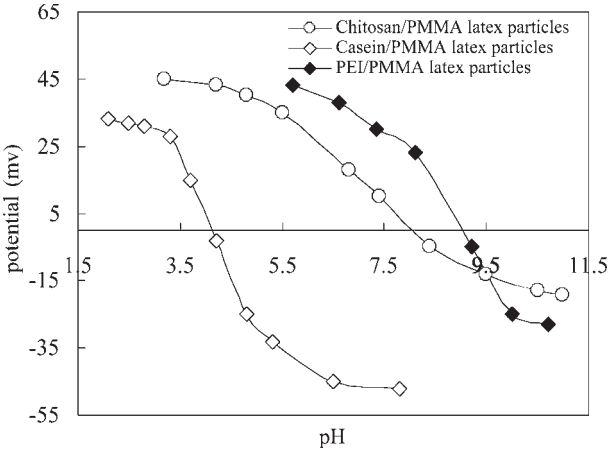


Fig. 3. pH dependence of zeta potential of nanoparticles (PEI/PMMA, chitosan/PMMA, and casein/PMMA).

more, the amphiphilic macroradicals generated *in situ* not only self-assemble to produce micelle-like microdomains that promote the aqueous emulsion polymerization of vinyl monomers, but also act as electrosteric stabilizers to provide stability to the developing nanoparticles. Therefore, the process can be carried out in the absence of surfactants.

Figure 1 shows SEM and transmission electron microscopy (TEM) images of PEI/PMMA, chitosan/PMMA, and casein/PMMA nanoparticles that were produced using this approach. The nanoparticles have a spherical shape. With careful staining using dilute phosphotungstic acid solution, the nanostructure of the particle was clearly revealed, which consisted

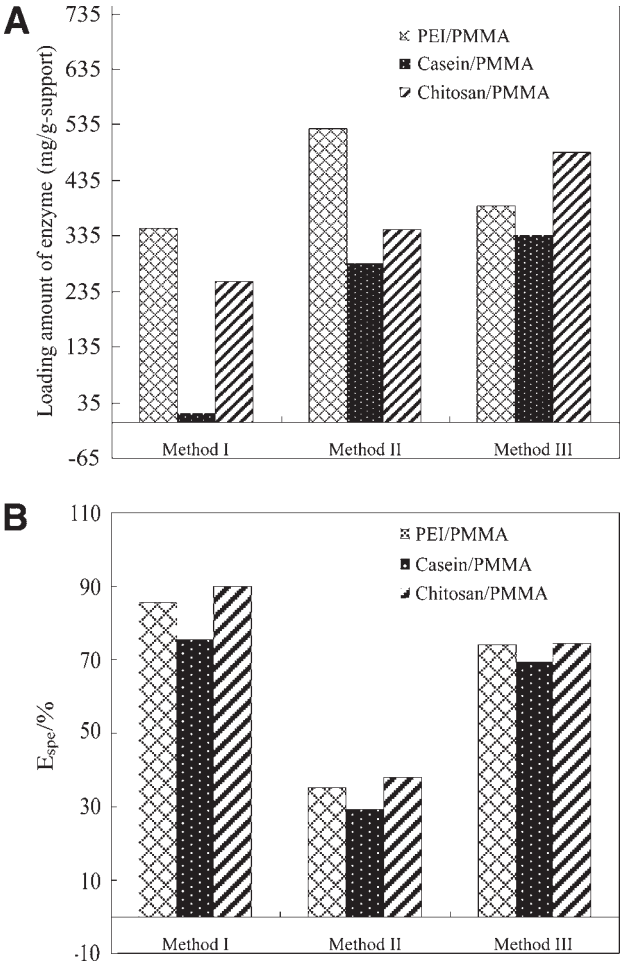


Fig. 4. Comparison of loading amount of **(A)** enzyme and **(B)** $E_{spe}$ using different immobilization methods: method I, physical adsorption; method II, crosslinking method; method III, covalent method.

of a well-defined PMMA core coated with a thick hydrophilic polymer shell.

Dynamic light-scattering measurement indicated that average number diameters of the nanoparticles were between 162 and 263 nm, with narrow particle size distributions (PDI = $D_v/D_n$ = 1.13–1.33) (Fig. 2). $D_n$ and $D_v$ are the number and volume average particle diameters, respectively. $D_v/D_n$ is the polydispersity index of the particle distribution. Cellulase was used to examine the bioimmobilization ability of the three types of nanoparticles, and the goal was to reduce the cost of using cellulase enzymes in the bioethanol process by employing cutting-edge and efficient biochemical technologies *(26,27)*.

The core-shell nanoparticles were very stable, with no flocculation observed even after 12 mo. The presence of hydrophilic polymer shell was confirmed with zeta-potential analysis. Figure 3 shows the zeta potential of the nanoparticles as a function of pH in 1 m*M* KCl solution at 25°C. The three isoelectric points (p*I*s) of pH were very close to those of relevant polymers. These results indicate that the polymers were located on the surface of the particles. Furthermore, the zeta-potential analysis shows that the PEI/PMMA nanoparticles in distilled water were positively charged and had a zeta potential of about 30 mV, the chitosan/PMMA nanoparticles in acetic solution were also positively charged and had a zeta potential of about 42 mV in acetic solution, and the casein/PMMA nanoparticles in a weak basic medium were negatively charged and the zeta potential was about –55 mV in 1 m*M* KCl solution. The results demonstrate that the core-shell nanoparticles were fairly stable. The stability was mainly from the electrostatic stabilization.

### *Study of Bioimmobilization Ability of Core-Shell Nanoparticles Using Cellulase as a Model*

Three methods of bioimmobilization were utilized to make a comparison: (1) enzyme directly contacted with the nanoparticles (physical adsorption), (2) crosslinking of the enzyme adsorbed onto the nanoparticles (crosslinking method), and (3) activation of the nanoparticles prior to immobilization (covalent method). The p*I*s of three types of nanoparticles were 4.10 (casein/PMMA), 8.20 (chitosan/PMMA), and 9.00 (PEI/PMMA), respectively. Thus, the casein/PMMA nanoparticles were negatively charged but the chitosan/PMMA and PEI/PMMA nanoparticles were positively charged at pH 7.86. That is, the PEI/PMMA nanoparticles possessed positive charges at pH 7.86 whereas the casein/PMMA nanoparticles had a large amount of negative charges. Nevertheless, according to amino acid composition, the p*I* of cellulase is 7.011 *(28)*. Therefore, the enzyme was negatively charged at pH 7.86. Figure 4A shows that in that PEI/PMMA nanoparticles adsorbed the most amount of enzymes and casein/PMMA adsorbed the least, which indicates that the main interaction between enzymes and nanoparticles was the electrostatic effect. The least enzyme loading amount with by casein/PMMA particles was due to the negative charges, which repelled each other (method I).

It has been reported that the bioimmobilization of polysaccharases (i.e., cellulases, amyloglucosidase, pectinase, and α-amylase) was the highest at their p*I* when the enzymes had zero net charge. Therefore, it was suggested that bioimmobilization had taken place through a van der Waals type of force *(29)*. In the present study, we examined the effect of pH on the loading amount and found that the lowest value was obtained near the p*I* of nanoparticles (see supplemental materials) and that higher values were

gained at a pH above the p*I* of nanoparticles. These results indicated that the nanoparticles interacted with enzymes mainly by electrostatic interaction. Hydrogen bonding also played an important role in the bioimmobilization, which could be proved by the effect of salt and urea on the loading amount of protein (see supplemental materials).

Regarding methods II and III, the enzyme was adsorbed to the nanoparticles in the optimal conditions (at pH 7.86), and the optimal glutaraldehyde concentration was found to be 2% (v/v). When smaller amounts of glutaraldehyde were charged, a lower loading amount of enzyme resulted owing to the release of the enzyme into the washing water. But enzymic activity of higher enzyme loading using a higher concentration of glutaraldehyde was actually lower, because there was a greater density of bonds per enzyme molecule and, consequently, molecular structure deformation could occur. In the case of the crosslinking method, the use of glutaraldehyde eventually led to the formation of covalent bonds on the particle surface, which should be taken into account, and this formation would affect the entrapment of enzyme.

Figure 4 shows a comparison of the loading amount of enzyme and retained percentage of activity ($E_{spe}$) using different immobilization methods. The highest loading amount was up to 530 mg/g, and most of them were more than 235 mg/g (<200 mg/g in other work) *(18–20)*. Such impressive improvement of enzyme loading amount may be attributed to the fact that reducing the particle size to near nanometer scales could increase the surface area and concentration of external functional groups. Furthermore, the hydrophilic outer shells of the nanoparticles and well-dispersed particles in solutions also played important roles in their bioimmobilization performance. This high bioimmobilization ability was also unusual even compared with that of porous supports *(30)*. However, the loading amount using casein/PMMA as support by method I was fairly low because of the electrostatic repulsion, as discussed earlier. The different $E_{spe}$ values of the three methods can be explained by the effect of glutaraldehyde. The highest $E_{spe}$ (up to 90%) was gained in the absence of glutaraldehyde solution, and the $E_{spe}$ was the lowest using the crosslinking method was introduced (Fig. 1B). Regarding the physical method, the conformations of enzyme barely changed by means of electrostatic interaction. On the other hand, the crosslinking method could deform the conformation of outer enzymes on the particles and, at the same time, the inner enzymes could not contact the substrates in the solution. Thus, the remaining activity was fairly low. However, regarding the covalent method, only inner enzymes were affected by reacting with glutaraldehyde, and little structure deformation occurred on the outer enzymes. Overall, among the three methods, the covalent method should be the optimum one (335–375 mg/g of protein loading with 70% of retained enzyme activity) for the formation of stable enzyme-host conjugates and higher-capacity bioimmobilization of enzymes.

## Conclusion

High-capacity (up to 530 mg/g) immobilization of enzymes onto core-shell nanoparticles and high activity retained percentage ($E_{spe}$) (up to 90%) were demonstrated, which indicates that nanometer-scale particles with well-defined amphiphilic core-shell morphology are superior support for effective immobilization of enzyme. The novel core-shell nanoparticles have better bioimmobilization performance than the porous ones because of easy access to the enzymatic shell on the particle surface. Thus they overcome the greater diffusional limitation associated with using porous materials *(31)*. Moreover, this strategy permits the preparation of functional films on the spherical nanoparticles with a high density of biomolecules. These biomultilayer nanoparticles are expected to find new applications in biologic and other areas.

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