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Research Article

Immobilization of Urease onto Nanochitosan Enhanced the Enzyme Efficiency: Biophysical Studies and *in Vitro* Clinical Application on Nephropathy Diabetic Iraqi Patients

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Immobilization of enzymes is an effective method for improving the properties and applications of modern enzymes. There are several supports for enzyme immobilization. Because of its unique features, such as inertness and high surface area, chitosan was widely used to immobilize enzymes. Immobilization of urease onto chitosan is a promising approach to treating high urea levels in the blood, however, the immobilization conditions for the best kinetics and enzyme efficiency are still challenging. Herein, we tried to immobilize urease onto nanochitosan (chitosan NPs) through a cross-linker and study the kinetics (k_m and v_{max} values) and thermodynamics (E_a, ΔH, ΔS, and ΔG) parameters of the enzyme reaction before and after immobilization at different substrate concentration (50, 100, 150, 200, and 250 mg/dl) and incubation temperature (15, 20, 25, 30, 35, and 40°C) under selected optimum conditions. The immobilized urease chitosan NPs was characterized in our previous work using Fourier transform infrared infrared(FTIR), Atomic force forcemicroscopy (AFM), and andimaged here by scanning electron microscopy microscopy(SEM). Results revealed that the highest efficiency % of immobilization (70.38%) was observed at 750 mg/ml chitosan NPs and phosphate buffer pH 7 at 40°C. With an increase of K_m value for the immobilized enzyme, however, the efficiency of the enzyme was significantly higher than the free enzyme, $p < 0.001$. In addition, the activation energy of the reaction catalyzed by the immobilized enzyme was lower than that of the free enzyme, which suggests that the active site geometry of the immobilized enzyme was more favorable to accommodate the substrate and thus required less energy than that of the free enzyme. The reaction was endothermic by means of positive ΔH. The immobilized urease enzyme was *in vitro* applied to blood samples of Iraq nephropathy diabetic patients ($n = 35$) to investigate the effect on serum urease activity and urea level compared to healthy volunteers. Interestingly, the activity of serum urease significantly increased after adding the immobilized enzyme and the level of urea significantly decreased ($p < 0.0001$) by ~1.5 folds. Thus, applying an immobilized ureaseurease to remove urea from blood could be effective in the blood detoxification or dialysis regeneration system of artificial kidney machines.

1. Introduction

Urease (EC 3.5.1.5), a metalloenzyme [1], catalyzes the hydrolysis of urea into ammonia and carbon dioxide [2]. Removal of urea and its ammonium ion plays an essential role in the analytical, clinical, and chemical treatments associated with several processes, such as blood and urine analysis, renal failure, artificial reinforcement, as well as wastewater treatment, food, and drug analysis [3]. Urease has been employed for the direct removal of urea from the blood for detoxification [4]. Urease immobilization has been performed on many matrices for clinical analytical purposes [5, 6]. Several strategies can be effectively used to immobilize enzymes [7], including surface adsorption, encapsulation, or entrapment [8]. However, most procedures of immobilization cause a reduction of the enzyme activity [9, 10] or

alter the urease structure (e.g., when immobilization happens on the nanoceria surface) [11]. There is no single approach or support that retains the enzymatic activity to be suitable for applications, yet. This is due to the different chemical properties and compositions of the enzymes, as well as the diverse qualities of the substrates and products. The major advantages of immobilization techniques are the stability of the enzymatic activity after immobilization and the reversibility of the process, which allows the enzyme and support materials to be reused for multiple applications. Immobilization of enzymes is critical not just for their reuse but also for their more efficient utilization. Immobilized enzymes are more robust and more resistant to environmental changes compared to free enzymes in the solution [12, 13]. Chitosan, a poly-N-acetyl glucosamine, is the N-deacetylated derivative of chitin (the primary component of crustacean shells), which is the second most prevalent biopolymer after cellulose [14]. Chitosan has a primary amine at the C-2 position of glucosamine residues. The presence of many high amines is a unique feature that provides chitosan with important functional properties [15]. Chitosan has numerous important biological and chemical features due to a unique collection of traits such as biocompatibility, biodegradability to harmless products, nontoxicity, physiological inertness, antibacterial activity, heavy metal ion chelation, gel-forming ability, hydrophilicity, and extraordinary attraction to proteins [16]. Nanoparticles (NPs) are nano-sized ultrafine colloidal drug carrier systems between 10 and 100 nm, consisting of different functional organic and/or inorganic backbones [17]. In the past 15 years, using of NPs becomes exponentially important. Those particles have been extensively explored due to their exciting unique properties compared to microparticles such as sustainability, controlling of drug release, site-specific targeting, and higher surface area to volume ratio than microparticles. These exceptional properties found a wide range of applications in biomedical and pharmaceutical fields such as biomolecule detection, vaccines, tissue engineering, drug delivery, cancer therapy, and high and quick accuracy diagnosis [18]. Moreover, chitosan NPs are more stable, soluble in aqueous acidic solution, biodegradable, less toxic, and fully biocompatible natural polymer. These chitosan NPs are approved by GRAS (generally recognized as safe by the United States Food and Drug Administration (US FDA)) [19]. Functionalization of NPs surface is to improve and/or add extra properties to enable those NPs to be applicable in medical or pharmaceutical fields. In this regard, different types of nanomaterials with characteristic properties and specific functional groups located on the surface are the priority of the functionalization process. Some molecules are used to functionalize the NPs surface such as small proteins, fragments of peptides, antibodies, DNA, enzymes, and oligosaccharides [20]. Interestingly, this biochemical modification of the surface of NPs is often caused less toxicity and high stability in biological fluids [21, 22]. Herein, we aimed to increase the efficiency of the urease enzyme by immobilizing onto chitosan NPs and thus functionalize their surfaces and deliver the enzyme into the site-cell target without degradation. The population target chosen for this study was diabetes patients with nephropathy diagnosed with high level of blood urea. The immobilized enzyme polymer was characterized using scanning electron microscopy (SEM), Fourier transform infrared (FTIR), and atomic force microscopy (AFM). Kinetics and thermodynamic behaviors of the enzyme were investigated before and after immobilization events.

2. Experimental

2.1. Materials. Urease enzyme was obtained from enzymatic colorimetric test with urea kit (human; Germany); chitosan NPs (>95% degree of deacetylation and viscosity 80 maps) was obtained from Shaanxi Sangherb Bio-Tech, China; glutaraldehyde (25%) was from Avonchem (UK); glacial acetic acid and sodium tripolyphosphate were from ALPHA CHEMIKA (India); sodium phosphate buffer was from ChemicalPoint (Germany); sodium acetate were from Thomas Baker (India); and ammonium chloride and ammonia were from Merck, Instruments: pH meter (TZ397A Orion), Spectrophotometer (CRCIL. England), SEM (Philips XL-30S FEG, Netherlands).

2.2. Methods

2.2.1. Immobilization of Urease onto Chitosan NPs. Preparation of chitosan NPs was carried out according to the ionic gelation method described by Vaezifar [23]. Briefly, 750 mg of chitosan NPs (average molecular weight and degree of deacetylation, 90%) were dissolved in 1% acetic acid. Sodium tripolyphosphate solution (1 mg/ml) was added continuously to the chitosan NPs solution and stirred at room temperature until chitosan NPs nanoparticles formed spontaneously. After 24 h, a viscous pale-yellow chitosan solution was formed. Glutaraldehyde solution (1%) was added to the viscous pale-yellow solution of chitosan NPs and stirred for 24 h at room temperature to become increasingly viscous. The enzyme 1.2 IU (0.1 mL solution in 0.1 mM phosphate buffer, pH 7) was immobilized on chitosan NPs. After that, the solutions were combined and agitated for 1 h at room temperature.

2.2.2. Urease Activity (Free and Immobilized). Urease activity was measured before and after immobilization using a spectrophotometric method [24]. Briefly, urea (30 μl, 50 mg/dL) was incubated with 0.25 ml of urease solution (1.2 μl for free and 1.56 μl for immobilized enzyme) at 37°C for 10 min. A volume of 0.3 ml of Berthelot's reagent A (5.0 gm phenol + 25 mg of sodium nitroprusside in 500 mL of phosphate buffer) was added and mixed well, and then 0.15 ml of Berthelot's reagent B (2.5 g sodium hydroxide + 4.2 mL of sodium hypochlorite in 500 mL of phosphate buffer) was added; the mixture was mixed well and incubated at dark for 30 min to allow the enzymatic hydrolysis of urea into ammonia and water. The resulted ammonia was measured at 670 nm. The activities of free and immobilized urease were expressed as μmol·min^{-1} [25]. One unit of enzyme activity

was defined as the amount of enzyme that converts 1 μmol ammonia into product per minute at pH 7 and 37 °C.

2.2.3. Effect of pH and Temperature on the Enzyme Activity. The effect of pH on immobilized urease activity was studied at different pH values ranging from 3.6 to 10.9, using 0.1 M sodium acetate (pH 3.6–6.0), 0.1 M sodium phosphate (pH 6.0–8.0), and 0.1 M ammonium chloride-NaOH (pH 8.0–11.0). The activity of the enzyme was measured as previously described [24] to indicate the optimum pH that gives the highest activity.

To determine the optimum temperature of the enzymatic reaction, the reaction mixture (enzyme, urea, and reagents A and B) was incubated under different temperatures (20–60°C) and the optimum pH. The activity of the enzyme was measured as previously described [24].

2.2.4. Effect of Concentration of Chitosan NPs on the Enzyme Activity. To determine the best concentration of chitosan NPs to carry the enzyme efficiently, different weights of chitosan NPs (0.25, 0.5, 0.75, and 1.0 gm) were used to immobilize the enzyme using the suitable conditions for the immobilization event. The activity of the immobilized urease was measured as previously described [24].

2.2.5. Kinetic and Thermodynamic Parameters. Kinetics and thermodynamics were determined by using five different concentrations of urea substrate (50, 100, 150, 200, and 250 mg/dL) at different incubation times (10, 20, and 30 min) and temperatures (15, 20, 25, 30, 35, 40, and 45°C). The Michalis–Menten equation (equation (1)) was linearized by the Lineweaver–Burk equation (equation (2)) and used to determine the kinetics parameters $V_{\max}$, K_m, and $V_{\max}/K_m$ [26]:

$$V = \frac{V_{\max}[S]}{K_m + [S]}, \tag{1}$$

where V° = initial velocity, μg·N·g^{-1}·min^{-1}; $[S]$ = urea concentration, mM; $V_{\max}$ = maximum velocity, μg·N·g^{-1}·min^{-1}; and K_m = Michaelis constant, mM.

$$\frac{[S]}{V} = \frac{[S]}{V_{\max}} + \frac{K_m}{V_{\max}}. \tag{2}$$

The linearized version (equation (3)) of the Arrhenius equation (equation (4)) was used by assuming $K \approx V$ to determine E_a and A parameters for the immobilized urease:

$$K = Ae - \frac{E_a}{RT}, \tag{3}$$

$$\ln K = \ln A - \frac{E_a}{R}\frac{1}{T}, \tag{4}$$

where K = velocity constant, min^{-1}; E_a = activation energy, KJ·mol^{-1}; A = pre-exponential factor, μg·g^{-1}·min^{-1}; and $R = 8.314$ K^{-1}·mol^{-1}.

The relationship that links the change of enthalpy (ΔH) to the change of entropy (ΔS), the Eyring–Polanyi equation (equation (5)), was used to determine these parameters [27]:

$$\left(\mathrm{Ln}\frac{V}{T}\right) = \mathrm{Ln}\frac{KB}{h} + \frac{\Delta S}{R} - \frac{\Delta H}{R}\frac{1}{T}. \tag{5}$$

The fraction *(Ln V/T)* was plotted versus [1000/T(k)] to obtain the slope *(−ΔH)/R)* and the intercept was *(Ln KB/h + ΔS/R)*, where KB = Boltzmann constant (1.38×10^{-23} J.K^{-1}). R = General gases constant (8.314 J.K^{-1} mol^{-1}). H = Planck constant (6.62×10^{-34} J.s).

The free Gibbs relationship (equation (6)) was then used to calculate the change in Gibbs energy:

$$\Delta G = \Delta H - T\Delta S. \tag{6}$$

where the temperature (T) was measured in Kelvin at 25°C.

2.2.6. Characterization of the Free and Immobilized Enzyme. Chitosan NPs and the immobilized enzyme were well characterized using FTIR and AFM [28]. They were also characterized here using SEM.

2.2.7. Scanning Electron Microscopy (SEM). SEM was used to characterize the morphology of chitosan NPs and immobilized urease on chitosan NPs. Spraying gold powder was used to apply the gold coating. To create a dilute suspension, samples were sonicated in water for 3 minutes. After drying, a drop of the original chitosan NPs diluted suspension was put on a glass slide and examined.

2.3. Clinical Application of Immobilized Enzymes. For the best utilization of the immobilized enzyme polymer, it was *in vitro* applied to the serum of diabetic nephropathy patients who are supposed to have high urea levels. The results were compared with those obtained from control volunteers. The enzyme activity and urea level were measured before and after adding the immobilized enzyme. Some other parameters such as sex, age, weight, height, and fasting blood sugar (FBS) were collected and compared.

2.3.1. The Study Groups. This study included 70 volunteers in total who visited the National Diabetes Center of Mustansiriyah University in Baghdad. The participants were separated into two groups: the first group consists of 35 patients with diabetic nephropathy aged between 23 and 60 years, and the second group was a control group consisting of 35 healthy participants aged between 18 and 50 years. Research ethics of human rights were followed according to the standard ethics of the National Diabetes Center of Mustansiriyah University. All participants were diagnosed by specialists of the center.

2.3.2. Blood Samples. A volume of 5 ml blood was collected in the morning, left at room temperature for half an hour to coagulate, centrifuged at 3,000 rpm for 10 minutes, and then

the serum was collected. The serum samples were stored at −4°C until further enzyme analyses.

2.3.3. Exclusion Criteria. This study included patients free of pregnancy, chronic complications of the kidney, any kind of tumors, any participants out of the required age limit (18–60 years old), and animals.

2.3.4. Statistical Analysis. Unpaired Student *t*-test (with Welch's correction, two-tailed) and ordinary one-way ANOVA with Brown–Forsythe and Bartlett's tests were performed to indicate statistical descriptive and analyses and significant differences between the studied groups at p significant level <0.05. For statistical analysis, GraphPad Prism 9 software was used.

2.3.5. Determination of Serum Urea Concentration. The immobilized urease was used to hydrolyze urea in the serum of patients and compare the results with those of control *in vitro*. The immobilized urease was incubated in phosphate buffer 50 mM (pH 7.0) at 37°C. The reaction was initiated by adding 90 μl of serum. After 10 minutes, the reaction mixture was taken out; the precipitated proteins were removed by centrifugation; and the urea was estimated from the supernatant [29, 30]. A standard curve was used for the known concentration. The urea estimated in serum samples was also compared with measurements done through Autoanalyzer® (Genolab TEK, USA).

2.3.6. Determination of Serum Urease Activity. Before adding the immobilized enzyme, serum urease activity was calculated by incubating 90 μl of serum sample with 10 μl urea (8.33 mMol) at 37°C for 10 minutes. The reaction was started by adding 100 μl of Berthlos reagent (reagent A). A volume of 50 μl of Berthlos reagent B was then added to the reaction mixture and incubated at 37°C in the dark. The optical density was detected at wavelength 670 nm.

After adding immobilized urease, the activity of the enzyme was calculated by incubating 90 μl of serum sample with 90 μl of immobilized enzyme and 10 μl of urea (8.33 mMol) at 37°C for 10 minutes. The reaction was started by adding adding 100 μl of Berthlos reagent (reagent A). A volume of 50 μl of Berthlos reagent B was then added to the reaction mixture and incubated at 37°C in the dark. The optical density was detected at wavelength 670 nm.

3. Results and Discussion

3.1. Kinetic Parameters

3.1.1. Effect of Concentration of Chitosan NPs on the Immobilization. At a constant urease enzyme concentration, the activity of immobilized urease displayed a typical saturation curve with the increase of the amount of chitosan NPs (Figure 1(a)). When the concentration of chitosan NPS increased up to 1 g·w/w, a gradual increase in the activity of immobilized urease was indicated up to 2701 U/L. This result demonstrated that the optimum concentration of chitosan NPs (750 mg·w/w) and that of urease (1.2 mg/ml) were efficient for the immobilization process. The loading capacity of urease on chitosan NPS decreased with increasing the amount of chitosan NPs at a constant concentration of the enzyme (Figure 1(a)). The high amount of chitosan NPs over 750 mg may distribute urease throughout the chitosan NPs particles. Therefore, it is likely that 750 mg w/w of chitosan NPs provides a significant surface area occupied by urease [31].

3.1.2. Effect of pH on Immobilization Process. The effect of pH on the activity of immobilized urease enzyme is shown in (Figure 1(b)). The optimum pH of the immobilized urease was 7.0. The activity of the enzyme showed a sharp improvement from pH 3.6 to 7.0 due to the production of ammonia, which modulated the acidity of the solution and enhanced the activity [32]. Figure 1(b) also showed that the activity dropped in the basic solution, which may be due to a possible distribution of the protonation status of side chains of the amino acid residues on the surface of the enzyme. This deprotonation in an alkaline medium could negatively affect the intramolecular electrostatic interactions, cause desaturation of the 3D architecture of the enzyme, and reduce its activity [33].

3.1.3. Effect of Temperature on Immobilization Urease Activity. The effect of temperature on immobilized urease activity is shown in Figure 1(c). The activity of the enzyme increased as the temperature increased to 40°C due to urease loading capacity as a large number of potential binding sites on its surface, allowing it to spread more easily on the nanoparticle surface [29, 34]. Over 40°C, there was a decline in the urease activity due to the denaturation of the enzyme.

The velocity of the enzyme and the kinetics was determined using Michaelis–Menten as follows:

$$V = \frac{V\max[S]}{K_m + [S]}, \tag{7}$$

where K_m is the Michaelis–Menten constant and $V_{\max}$ is the maximal reaction rate. Lineweaver–Burk plot (Figure 2(a)) was used to obtain K_m values. The K_m for free and immobilized urease were 10.01 and 11.36 mM, respectively. This result indicated that the free urease enzyme has a higher affinity for its substrate urea when compared to the urease immobilized on chitosan NPs. Increased K_m of urease enzyme after immobilization has similarly been reported by [29, 30, 35]. Furthermore, $V_{\max}$ for free and immobilized urease on chitosan NPs were 61.34 μmol·min^{-1} and 63.69 μmol·min^{-1}, respectively (Figure 2(a)).

3.2. Thermodynamic Parameters. Arrhenius graph between ln K and $1/T$ was used to calculate E_a of free and immobilized urease activity [36], where the relationship between ln (K/T) and $1/T$ was used to calculate the enthalpy (ΔH) and entropy (ΔS). The results in Figure 2(b) indicated that the activation energy of immobilized urease was 27.295 KJ/mol, which was

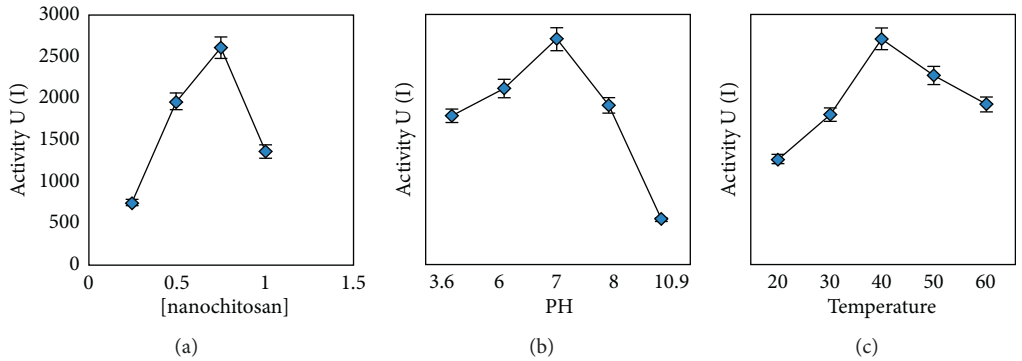


Figure 1: Kinetics of immobilized enzyme. Effect of amount of chitosan NPs on immobilization urease activity (a), the effect of pH on the enzyme activity (b), and the effect of temperature on the activity of immobilized enzyme (c).

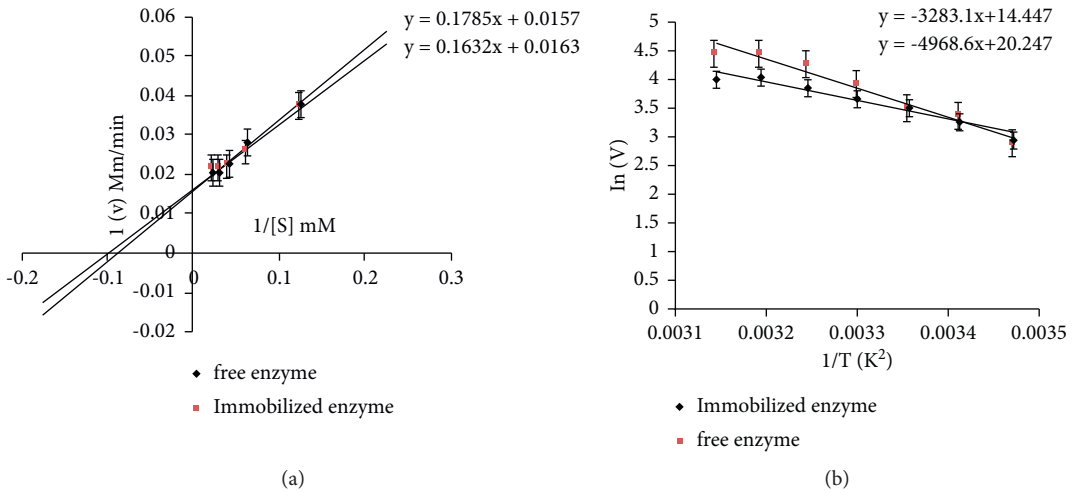


Figure 2: (a) Lineweaver–Burk plots of free enzyme and immobilized enzyme and (b) Arrhenius relationships of free enzyme and immobilized enzyme.

lower than that of free enzyme 41.303 KJ/mol, which confirmed the previous study [37]. Lower E_a for the immobilized enzyme could indicate that the conformation of the active site was more favorable to bind most of the [S] molecules in the reaction mixture, and thus, more [$E-S$] complexes could reach the energy barrier [38].

The thermodynamic parameters of the urease hydrolysis reaction, enthalpy ΔH, entropy ΔS, and Gibbs free energy ΔG were calculated for immobilized urease enzyme and compared with the standard values of urease (see Figure 3). Results of the immobilized enzyme showed $\Delta H = 24.96$ KJ/mol, $\Delta S = -0.132$ KJ/mol, and $\Delta G = 64.29$ KJ/mol. The parameters of free enzyme are $\Delta H = 38.97$ KJ/mol, $\Delta S = -0.084$ KJ/mol, and $\Delta G = 64.0$ KJ/mol) [39]. The lower enthalpy ΔH value of the immobilized enzyme could indicate the efficiency of the transition state or activated complex [$E-S$], when compared with that of the free enzyme. The entropy ΔS values of immobilized and free form were negative. It suggests that the structure of [$E-S$] at the transition state is more ordered than that of the ground state. The ΔG is the best parameter to estimate the feasibility and extent of the chemical reaction. Results indicated low ΔG values for both enzyme forms, which means that the conversion of the [$E-S$] complex to the product was more spontaneous [40].

3.3. Characterization of the Immobilized Enzyme Copolymer. The polymer chitosan NPs loaded enzyme was well characterized in our previous work by atomic force microscope AFM and Furious transfer infrared FTIR.

3.3.1. Scanning Electron Microscopy (SEM). The result of SEM in Figure 4 showed the morphology of the immobilized enzyme before and after immobilization. Figure 4(a) showed that the morphology of chitosan NPs was uniform, spherical, and well dispersed. The morphology of the urease enzyme showed a heterogeneous surface (Figure 4(b)); however, the morphology of the immobilized enzyme was uniform surface when compared to chitosan NPs (Figure 4(c)). This might imply that the enzyme's interaction with chitosan NPs is more efficient. As a result, the compact and spherical form of chitosan NPs may become fluffy in the presence of the urease enzyme.

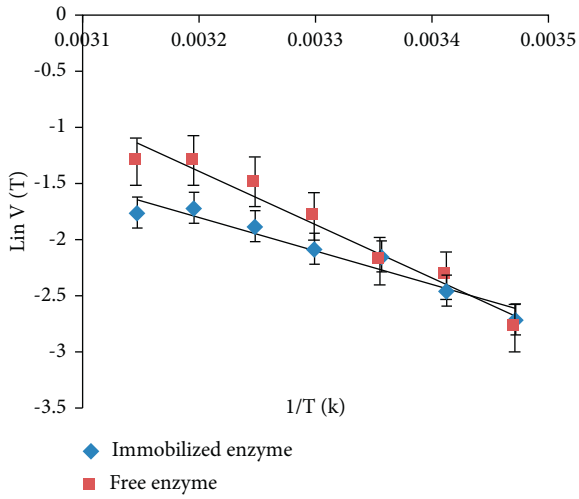
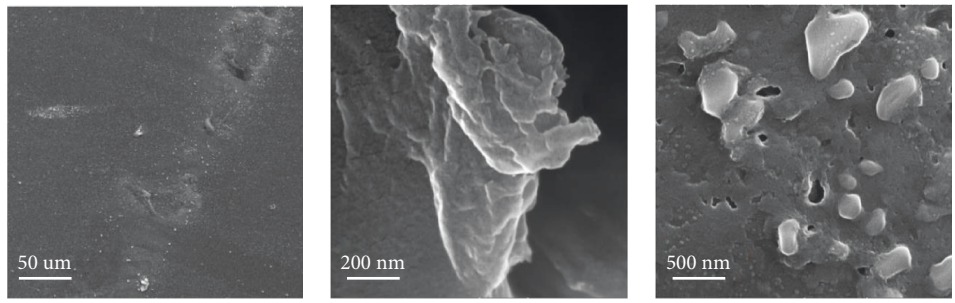


Figure 3: The thermodynamic curve of free urease immobilized urease enzyme.



(a) (b) (c)

Figure 4: SEM images of urease enzyme (a), chitosan NPs particles (b), and immobilized urease-chitosan NPs (c). Scale bars = (a) 50 μm, (b) 200 μm, and (c) 500 nm.

Table 1: A description of the studied groups. Values were expressed by mean ± SD.

	Sex	Age (year)	Weight (Kg)	BMI (Kg/m^2)	Duration of disease (year)	FBS (mg/mL)
N	35	35	35	35	35	35
Mean ± SD (patients)	17 F 18 M	40.34 ± 8.468	80.69 ± 15.08	28.64 ± 4.710	6.343 ± 3.81	165.8 ± 9.55**** ∝
Mean ± SD (control)	19 F 16 M	30.43 ± 7.81	67.80 ± 10.70	24.19 ± 2.61‡	—	89.00 ± 9.97 ∝

‡Calculated for 10 participants only. ****$P < 0.0001$, refers to the difference in the significance level between FBS of patients compared to that of control.

3.4. Clinical Application of Immobilized Enzyme. The immobilized urease was *in vitro* applied to serum of nephropathy diabetic Iraqi patients who suffered hyperaemia to investigate the effect of the released enzyme on the serum urea level. Results were compared with control volunteers. The serum concentration of urea hydrolyzed by the immobilized urease enzyme was compared with those obtained by the Autoanalyzer®. Table 1 showed that the mean age of the studied population ranged from 30 to 40 years old, patients were overweight (BMI = 28.64 ± 4.710 Kg/m^2, <29 Kg/m^2) compared with normal-weight controls (24.19 ± 2.61 Kg/m^2), and they were suffered from nephropathy diabetes for ~6 years ± 3.81. Levels of fasting blood sugar FBS were significantly higher in patients ($p < 0.0001$; Table 1; Figure 5(a)). Nephropathy diabetic patients suffer from hyperglycemia perhaps due to injury to the small blood vessels in the body due to their high levels of sugar, which reduces the ability of the small blood vessels in the kidneys to carry out their function in purifying the blood and filtering it from wastes, causing the accumulation of these wastes in the blood.

Table 2 indicates that applying the embedded enzyme has significantly enhanced the serum activity of urease for patients by ~2-folds and by ~3.5-folds for controls ($p < 0.0001$, see Figure 5). This enhancement could imply the successful release of urease from the platform (chitosan) into the serum within 30 min incubation time. Interestingly, Table 2 also showed that the urea level in patients was

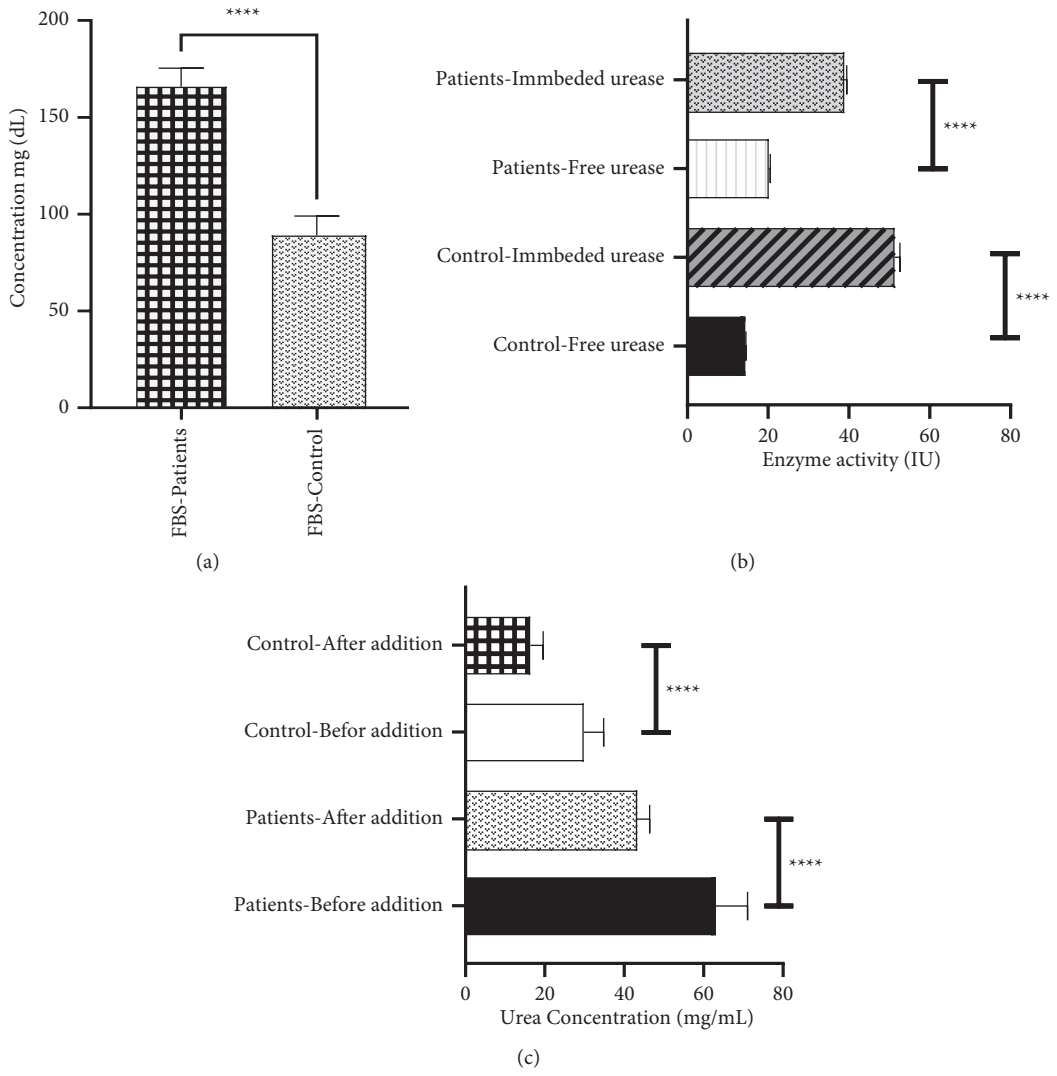


Figure 5: Effect of the immobilized urease enzyme on the activity of serum urease enzyme and urea concentration of nephropathy diabetic patients and control volunteers: (a) levels of FBS in patients compared to that in control, (b) effect of the immobilized urease enzyme on the enzyme activity, and (c) effect of the immobilized urease enzyme on the urea level. All data were collected in triplicates. The black bars refer to the standard error of means SEM. The significance level of differences p was <0.0001.

Table 2: Levels of free and embedded urease in patients compared with controls. Values were expressed by mean ± SD.

	Free urease (IU)	Embedded urease (IU)	Urea (mg/dL) § (before addition)	Urea (mg/dL) § (after addition)
Mean ± SD (patients)$^{***}\xi$	20.15 ± 2.28 ∝	37.88 ± 9.32**** ∝	63.11 ± 7.95 ∝ , ξ	42.97 ± 3.40**** ∝
Mean ± SD (control)$^{***}\xi$	14.34 ± 0.96 ∝	51.32 ± 7.53**** ∝	29.80 ± 5.00 ∝ , ξ	16.34 ± 3.22**** ∝

§Before and after adding the immobilized enzyme. $^{****}p < 0.0001$. $^{***}P < 001$, ξ refers to the significance level of blood urea in patients before adding the embedded enzyme compared to that of control. ∝ refers to the significance level of blood urea in patients after adding the embedded enzyme compared to that of control or to that before the addition.

significantly elevated over that of the control (63.11 ± 7.95 mg/dL vs. 29.80 ± 5.00 mg/dL). However, the urea level of patients was reduced after adding the embedded enzyme by around ~−1.5-fold and ~−2-fold for controls ($p < 0.001$; Figure 5(b)). The reduction of urea level explained that the catalyzing action of urease to hydrolyze urea into bicarbonate and water was highly activated after adding the embedded enzyme and therefore lowered the urea concentration in serum (Table 2). Thus, applying the embedded urease enzyme onto chitosan showed for the first time a successful acceleration of the original serum urease enzyme. Mechanistically, the availability of a larger number of enzyme molecules increased the chance for the available substrate molecules to bind and then be hydrolyzed.

4. Conclusion

This study explored the immobilization efficiency of urease enzyme onto nanochitosan particles, for improving the enzyme prosperities in biotechnological and biomedical applications. The immobilization conditions were optimized. Accordingly, the urease enzyme was successfully immobilized onto chitosan NPs as a support material, confirmed by results of FTIR, AFM, and SEM. The kinetic and thermodynamic parameters revealed that the affinity of urea to the active site of the immobilized enzyme was lower than that of the free enzyme. The enzymatic efficiency after immobilization was higher than the free enzyme, and the reaction was endothermal. Additionally, *in vitro* application of the immobilized urease on sera of nephropathy diabetic patients significantly enhanced the enzyme activity of the serum urease and decreased the urea level ($p < 0.0001$) by ~1.5-folds. To our knowledge, this is the first time to show the effect of immobilized urease enzyme on reducing the blood urea level of nephropathy diabetic patients. Thus, applying an immobilized urease to remove urea from blood could be effective in the blood detoxification or dialysis regeneration system of artificial kidney machines.

Data Availability

All data have been included in the manuscript.

Additional Points

No animals were used in this research. All human research procedures were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) authorized by the National Diabetes Center for Treatment and Research. Although this was an *in vitro* study, the authors have obtained permission from all participants.

Ethical Approval

Ethical approval was given by the ethics committee of Mustansiriyah University (Iraq).

Consent

Not applicable.

Disclosure

This study was registered in the research annual plan of the Chemistry Department at Mustansiriyah University, 2020 https://uomustansiriyah.edu.iq/e-newsite.php.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Zahraa Al-Garawi designed the project, analyzed and interpreted the data, wrote the first draft, and reviewed and edited the final manuscript. Ali A Taha has collected and analyzed the samples and data and wrote the manuscript. Ahmed N Abed has contributed to writing, revising, and editing the manuscript. Noor T has helped with collecting the clinical data.

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Supplementary Materials

Graphical abstract. (*Supplementary Materials*)

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