Adsorption and Desorption Behaviors of Bovine Serum Albumin on Gelatin/Chitosan Sponge

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Abstract

Gelatin (Gel) and chitosan (CTS) have several biomedical applications because of their biodegradability and biocompatibility. Crosslinking of Gel and Gel/CTS systems was evaluated using *N*-acetyl-D-glucosamine (GlcNAc) formed into sponges by lyophilization. The prepared sponges were used to study the adsorption and desorption of fluorescein isothiocyanate (FITC) labeled bovine serum albumin (BSA) as a model instead of a growth factor. The effect of FITC-BSA concentration and temperature on the adsorption behavior of Gel/CTS sponges was investigated. The Langmuir adsorption isotherm model was used on the basis of the assumption that monolayer adsorption occurs on the surface; the results fit with the experiment data. The adsorption constants were 5.77 and 9.68 mL/mg for Gel and Gel/CTS sponges, respectively. The adsorption thermodynamic constants were found; adsorption onto sponges was an exothermic reaction. In particular, Gibbs free energy (ΔG) exhibited negative values in the range of 283 - 343 K for both Gel and Gel/CTS sponges, demonstrating the spontaneous nature of adsorption reaction. In addition, desorption behavior was evaluated for different concentrations and pH values of the FITC-BSA solution. The high adsorbed amounts of FITC-BSA on sponge resulted in high desorbed amounts in sponge, up to 55% from 3.5 mg/mL adsorbed concentration (around 1.5 mg from 3 mg adsorb amount). Desorption decreased following the buffer solution pH decrease, from 7.4 to 4 and 2 in Gel and Gel/CTS sponges, respectively. Based on the results of this preliminary study, these composite sponges could have significant application in biomedical materials.

1. Introduction

Gelatin (Gel) is a mixture of peptides and proteins produced by partial acid or alkaline hydrolysis of collagen. Gel is biocompatible and shows low antigenicity [1], in contrast to collagen, which has greater antigenicity because of its animal origin [2] [3]. In addition, chitosan is well investigated by the biomaterial, food, and chemical industries because of its good biocompatibility, biodegradability, hemostasis, and other properties [4]-[9]. It is commonly used in nanofibers, gels, scaffolds, membranes, filaments, powders, granules, and sponges or as a composite [10]. Moreover, Gel/CTS sponges are successfully prepared for wound dressing [11], tissue engineering [12], bone tissue engineering [13], and vital organ engineering [14].

In this study, we examined Gel/CTS sponges developed by crosslinking with GlcNAc. According to the Maillard reaction, crosslinking GlcNAc and amino group produces browning compounds due to the interaction between carbonyl group, reducing sugar, and the amino compound [1]. Protein adsorption is very important in biomedical research. The interaction between the protein and surface material that occurs during adsorption can determine (a) changes in the hydration of protein molecules and the material's surface, (b) electrostatic interaction, and (c) structure rearrangement in the adsorbing protein molecule [15] [16]. Fluorescein isothiocyanate (FITC)-bovine serum albumin (BSA) was used for protein adsorption as a model instead of a growth factor. FITC is among the simplest and most commonly used reagents for labeling proteins. The isothiocyanate group (–N=C=S) of FITC can form bonds with amino groups on proteins. Furthermore, the effect of concentration, temperature, and pH on the adsorption and desorption behavior of sponge was investigated. The adsorption constant and thermodynamic parameters were also evaluated to use the data as basic information on Gel/CTS sponges in order to apply or improve the properties of biomaterials.

2. Experimental Procedures

2.1. Materials

CTS (FM-80; DAC 88.3, viscosity 30 mPa·s) was supplied by Koyo Chemical Co. Ltd (Japan). Gel (PGS 250-WIA; Pig skin type B) and BSA (fraction V, 96%) were purchased from Koei Chemical (Japan) and Sigma-Aldrich Co (USA), respectively. GlcNAc, Phosphate buffer saline (PBS, pH 7.4), and FITC (≥95%) were purchased from Wako Pure Chemical Industries, Ltd. (Japan).

2.2. Preparation of Gel Sponges

First, 5.6 wt% CTS solution in 4 wt% acetic acid was prepared. Next, Gel and GlcNAc was dissolved in distilled water and the CTS solution was added to the mixture. As the result, the content of Gel, GlcNAc, and CTS in the mixture was 5, 5, and 0.04 wt%, respectively. The mixture was covered and put in a water bath at 50°C ± 2°C for 10 h to remove entrapped air and obtain a homogeneous solution.

Following this, the samples were allowed to cool at room temperature. Then, they were freeze-dried to obtain the sponge. Later, samples were heated for 24 h at 100˚C to obtain Gel/CTS sponge. Moreover, Gel sponge without CTS was also prepared from same procedure.

2.3. Preparation of FITC-BSA

BSA was labeled with FITC to be used as a protein adsorption model in order to avoid the effect of Gel adsorption at the same wavelength as BSA. First, 3 mg/mL BSA solution was prepared in 0.1 mol/L carbonate buffer (pH 9.0). After mixing with 1 mg/mL FITC solution in DMSO (BSA solution 1 mg/mL: FITC solution 0.10 mg), the solution was kept overnight at 4˚C. To remove any uncoupled FITC, dialysis was used against water [17]. The light absorption at 494 nm dropped below 0.003 for the supernatant, as indicated by the UV-Vis spectrum. The concentration and FITC: protein (F:P) ratio were determined according to the methods described by the manufacturer (Thermo Scientific, Tech tip #31). All preparation steps were performed avoiding light.

Calculation of protein molarity:

$$\text{protein concentration}\ (\text{M}) = \frac{A_{280} - (A_{\max} - \text{CF})}{\varepsilon} \times \text{dilution factor} \qquad (1)$$

ε = protein molar extinction coefficient (BSA ~43,824 $M^{-1} \cdot cm^{-1}$ at 280 nm)

$A_{\max}$ = absorbance (A) of a dye solution measured at maximum wavelength (FITC = 494 nm)

CF = correction factor; adjusts for the amount of absorbance at 280 nm caused by dye (FITC = A_{280}/A_{494} nm)

Calculation of the degree of labeling:

$$\text{moles dye per mole BSA} = \frac{A_{\max}\ \text{of labeled BSA}}{\varepsilon' \times \text{BSA concentration}\ (\text{M})} \times \text{dilution factor} \qquad (2)$$

ε' = molar extinction coefficient of the fluorescent dye (BSA ~68,000 $M^{-1} \cdot cm^{-1}$ at 494 nm)

2.4. Adsorption Behaviors of FITC-BSA on Sponges

Adsorption was determined by immersing ~0.1 g of sponge in FITC-BSA in PBS until reaching adsorption equilibrium. The solution was sampled, and the concentration was measured using a spectrofluorometer at excitation and emission wavelengths of 494 and 565 nm, respectively. A predetermined standard concentration-intensity calibration curve was used to characterize the absorbance peaks and estimate the BSA concentration. FITC-BSA varied at 0.1 - 4.0 mg/mL in PBS (pH 7.4, room temperature), and it was investigated to determine the effect of the solution concentration. The experiment was repeated for the same concentration of FITC-BSA at 10˚C, 25˚C (room temperature), 37˚C, 50˚C, and 70˚C to study the effect of temperature on adsorption efficiency. Finally, the adsorption constant and thermodynamic parameters were evaluated.

2.5. Desorption Behavior of FITC-BSA from Sponges

Similarly, a release study was also performed for the same FITC-BSA loaded on the sponge after washing the sponge in deionized water and freeze-drying. The adsorbed sponge was immersed in 25 mL of PBS (pH 7.4, 37°C). The solution was sampled and measured using a spectrofluorometer with the same method as that in the adsorption experiment. The effect of the concentration adsorbed on the sponge as well as the solution pH (7.4, 4.0, and 2.0) on the desorption rate (%desorption) was investigated.

$$\%\text{desorption} = \frac{\text{released concentration at time}}{\text{FITC-BSA amount absorbed in the sponge}} \times 100 \qquad (3)$$

3. Results

3.1. Morphology of Sponges

Figure 1 shows the prepared sponge. The brown color appeared after heat treatment, in accordance with the Maillard reaction, which produces browning compounds because of the interactions between the carbonyl group of GlcNAc and the amino compounds of CTS and Gel. The surface morphology of the composite sponges appeared in the SEM image. The entire sample surface showed an interconnected porous structure in the micrometer scale with an average diameter of 130 and 150 μm for Gel and Gel/CTS sponges, respectively. CTS increased the mean pore size in the sponge composite.

3.2. Structure of FITC-BSA

A typical UV-Vis spectrum of FITC in PBS is shown in **Figure 2**. To determine the FITC correction factor, the FITC maximum peaks were determined at 494 and 280 nm corresponding to the absorption wavelength of the BSA protein. Therefore, the correction factor for the calculated value of FITC-BSA was calculated by eliminating the amount absorbed by the dye, which is 0.12014/0.34737 = 0.345. The protein concentration and the ratio of labeling were 2.965×10^{-5} M (Equation (1)) and 4.025 (Equation (2)), respectively.

3.3. Absorption Behavior of FITC-BSA

3.3.1. Effect of Concentration
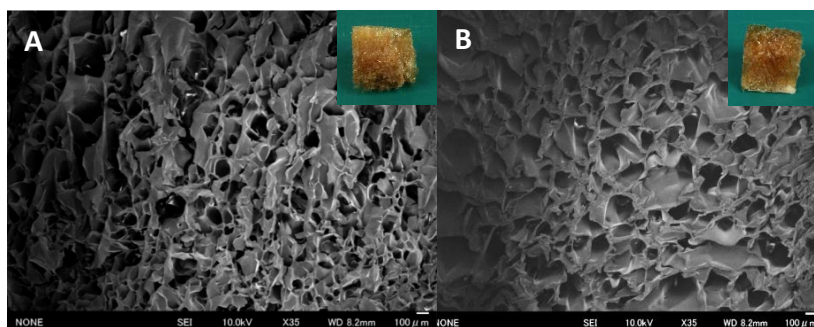As shown in **Figure 3**, when the FITC-BSA concentration increased, the amount

Figure 1. SEM images of (A) Gel and (B) Gel/CTS sponges: Scale bar is 200 μm.
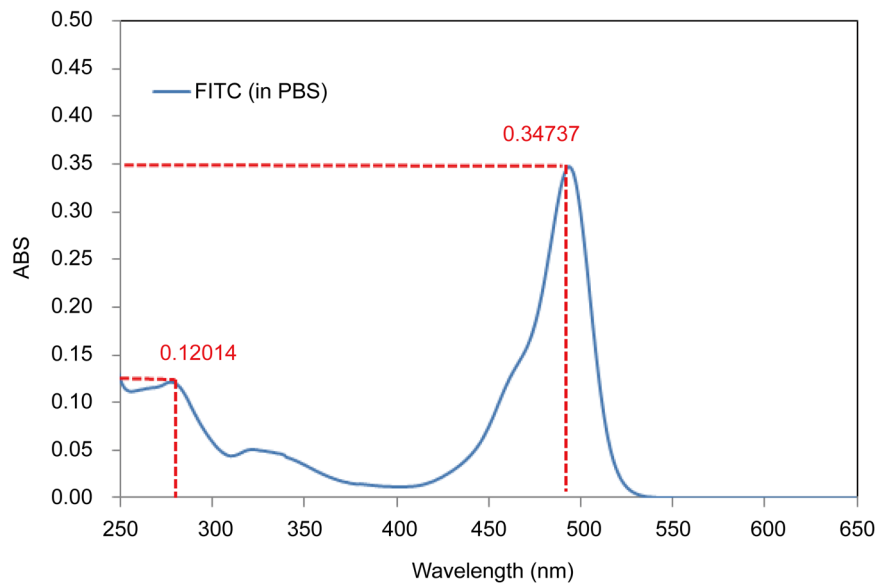
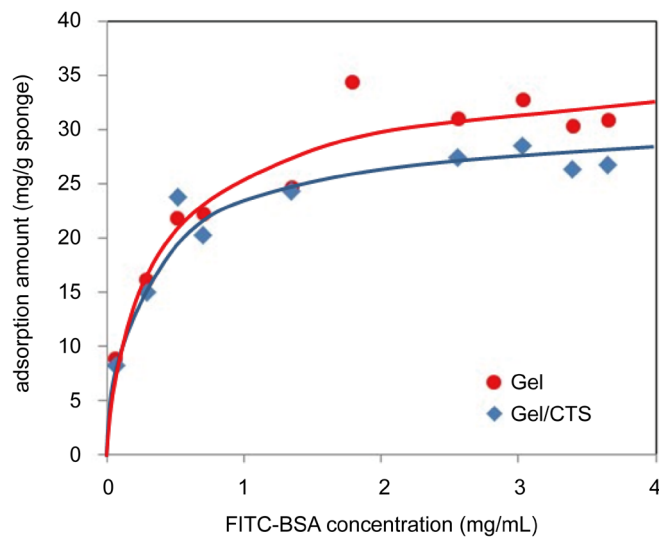**Figure 2.** Typical UV-Vis spectrum of FITC in PBS.

Figure 3. The effect of FITC-BSA concentration to adsorbed amount on the sponges.

absorbed on the Gel sponge increased, until equilibrium was reached at around 30 mg/g sponge, and it showed a little higher adsorption amount than the Gel/CTS sponge.

The Langmuir and Freundlich equations were usually used to model the adsorption system. In this study, the Langmuir adsorption isotherm model was used based on the assumption that the surface adsorption occurs in a single layer. The Hanes-Woolf plot was commonly used in several studies because of the minimized deviation from the fitted equation resulting in optimum error distribution [18].

Langmuir's Hanes-Woolf plot equation:

$$\frac{C_e}{q_e} = \frac{1}{K_L q_m} + \frac{C_e}{q_m} \qquad (4)$$

where q_e (mg/g) is the adsorbed amount at equilibrium, q_m (mg/g) is the maximum adsorbed amount of FITC-BSA on the sponges, C_e (mg/mL) is the equilibrium concentration of adsorbent in the solution, and K_L (mL/mg) is the equilibrium adsorption constant.

Figure 4 shows the linear correlation between C_e/q_e and C_e based on the experiment data and following the Langmuir's Hanes-Woolf plot equation. K_L and q_m were calculated from the slope of the plot; the value for each sponge is given in **Table 1**. The experimental data fit well with the isotherm in both sponges ($R^2 \geq 0.99$). The maximum adsorption capacities (q_m) for Gel and Gel/CTS sponges were 32.679 and 27.932 mg/g, respectively. The equilibrium adsorption constants (K_L) for Gel and Gel/CTS sponges were 5.773 and 9.675 mL/mg, respectively. K_L of the Gel/CTS sponge was greater than that of the Gel sponge, indicating more favorable adsorption on the Gel/CTS sponge, a result which was confirmed by the fluorescein microscope observations (**Figure 5**). Following this,
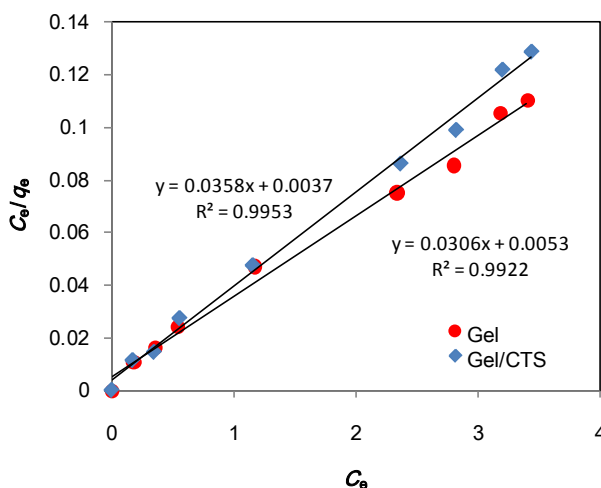

Figure 4. Linearized Langmuir isotherms (Equation (4)) for FITC-BSA at 25˚C.

Table 1. Thermodynamic parameter for FITC-BSA adsorption on sponges.

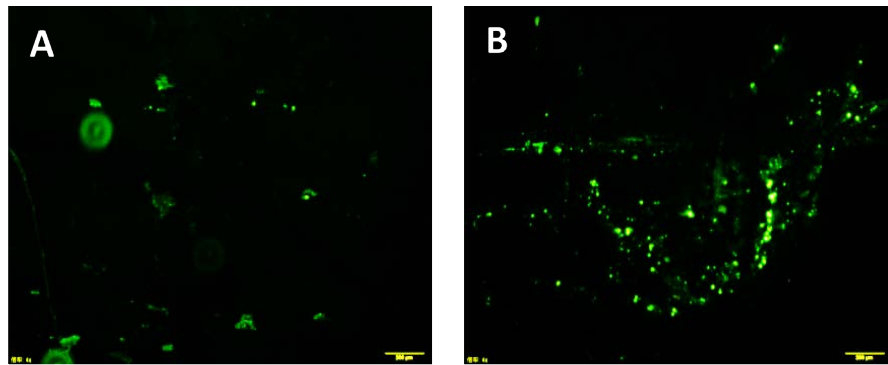| Sponge	Gel	Gel/CTS
Linear equation	y = 1115.2x − 0.7771	y = 655.67x + 0.8334
R^2	0.8207	0.9513
ΔS° (kJ/mol)	−6.46	6.93
ΔH° (kJ/mol)	−9.272	−5.451
ΔG (kJ/mol)		
283 K (10˚C)	−7.443	−7.412
298 K (25˚C)	−7.346	−7.516
310 K (37˚C)	−7.269	−7.599
323 K (50˚C)	−7.185	−7.689
343 K (70˚C)	−7.056	−7.828

Figure 5. Fluorescein microscope images of (A) Gel sponge and (B) Gel/CTS sponge: Scale bar is 200 μm.

in the adsorption experiment, washed and freeze-dried sponge was used to compare the surface morphology at the same initial adsorption concentration (0.3 mg/mL). Figure 5 shows the comparative amounts of FITC-BSA adsorbed on the Gel and Gel/CTS sponges.

3.3.2. Effect of Temperature

Figure 6 shows the FITC-BSA absorbed amounts for 1.8 mg/mL FITC-BSA concentration in PBS (pH 7.4) with changing temperature. For both sponges, the adsorbed amount decreased when the temperature increased; in particular, at 70°C, the Gel sponge adsorbed a slightly lower amount than the Gel/CTS sponge.

Based on the results, the thermodynamic parameter can calculated with the following equation [19] [20]:

$$\ln K_d = \frac{\Delta S^\circ}{R} - \frac{\Delta H^\circ}{RT} \tag{5}$$

where

$$K_d \left(\text{mL/g} \right) = \frac{\text{amount of FITC-BSA in absorbent}}{\text{amount of FITC-BSA in solution}} \times \frac{V}{m} \tag{6}$$

V is the volume of the solution (mL) and m is the weight of the sponge (g). ΔS°, ΔH°, R, and T are entropy (J/mol·K), enthalpy (kJ/mol), gas constant (8.314 J/mol·K), and temperature (K), respectively. ΔH° and ΔS° correspond to the slope and Y–intercept of the plot between $\ln K_d$ and $1/T$, respectively (Figure 7). The Gibbs free energy (ΔG) is calculated using the following well-known equation:

$$\Delta G = \Delta H^\circ - T\Delta S \tag{7}$$

The thermodynamic parameter values calculated from the experiment data appear in Table 1. The negative values of ΔG for both sponges under all temperature conditions indicated the spontaneous nature of the adsorption reaction. For the Gel sponge, ΔH° and ΔS° were negative values, referring to an exothermic reaction; thus, the reaction proceeded at low temperatures The Gel/CTS sponge had negative ΔH° and positive ΔS°, indicating an exothermic reaction, whose entropy spontaneously increases at all temperatures.
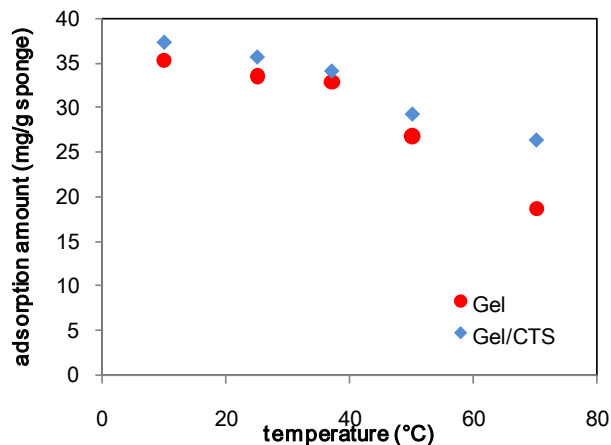
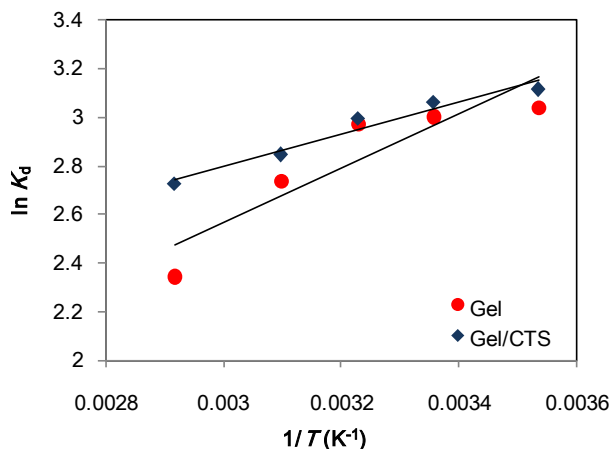**Figure 6.** The effect of temperature to adsorbed amount of FITC-BSA on the sponges.

Figure 7. The effect of temperature on the thermodynamic behavior for adsorption of FITC-BSA on the sponges.

3.4. Desorption Behavior of FITC-BSA

3.4.1. Effect of Concentration

Figure 8 shows the spectrofluorometer results for the desorbed FITC-BSA in PBS at the same wavelength as the adsorption test. The initial rate of FITC-BSA release from the composite was rapid, from 0 h to 6 h, and it remained constant up to 12 h. The high adsorbed amount of FITC-BSA on sponge resulted in high desorption (in PBS at 37°C), around 25% from 0.3 mg/mL (~0.5 mg from adsorbed amount ~2 mg) and 55% from 3.5 mg/mL (~1.5 mg from adsorbed amount ~3 mg adsorbed concentration in sponge). The Gel/CTS sponge had just little higher %desorption than the Gel sponge.

3.4.2. Effect of pH

Figure 9 shows the results of pH against the %desorption FITC-BSA in PBS. For the same adsorbed amount, 1.6 mg/mL, the sponge samples were separated to test the desorption in different buffer pH solution (2, 4, and 7.4) at 37°C, after 12 h, as measured using a spectrofluorometer. The results showed a %desorption
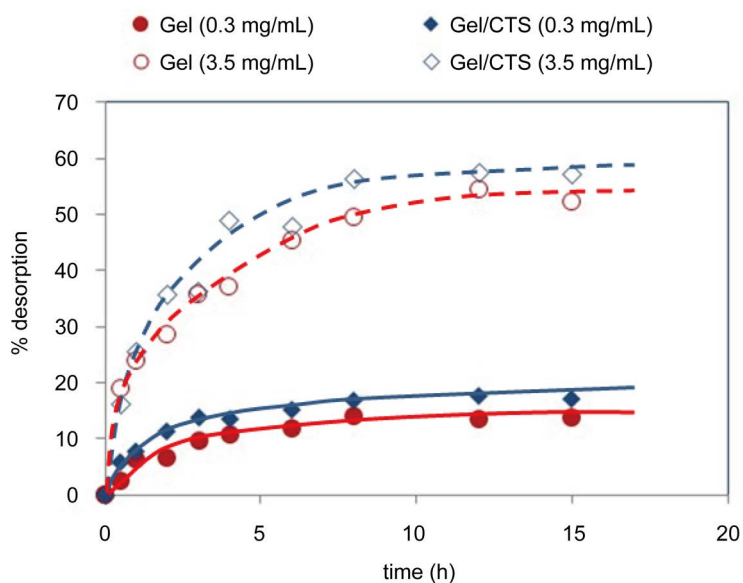
Figure 8. The effect of FITC-BSA at different concentration to %desorption of the sponges in PBS at 37˚C.
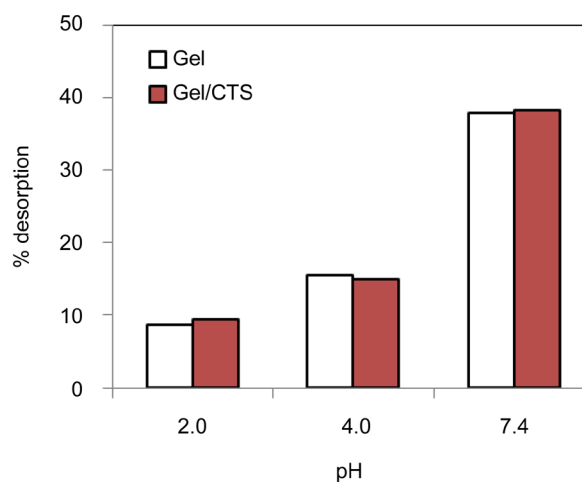

Figure 9. The effect of pH to %desorption of FITC-BSA on the sponges in PBS at 37˚C.

decrease with changing buffer solution pH (from 7.4 to 4 and 2); Gel and Gel/CTS sponge data exhibited the same trend. **Figure 10** shows fluorescein microscopic images after desorption of Gel/CTS sponge. The results confirmed the sponge surface morphology and the increase in the amount of FITC-BSA at low pH of the desorption solution.

4. Discussion

The focus of the present study was to understand the protein adsorption and desorption mechanisms of the Gel sponge by GlcNAc crosslinking. The reason for adding CTS was to increase the interaction between $-NH_2$ in Gel or CTS and $-CHO$ in GlcNAc. The results revealed a slight difference between Gel and Gel/CTS sponges, which could be attributed to the amount of only 0.04 wt%
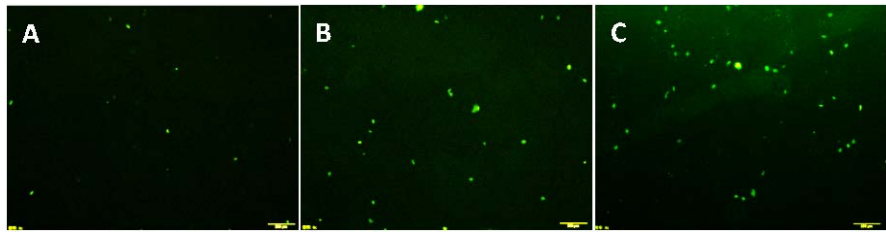
Figure 10. Fluorescein microscope images after desorption at different pH of Gel/CTS sponges: (A) pH 7.4, (B) pH 4.0, and (C) pH 2.0: Scale bar is 200 μm.

CTS. Therefore, this may affect the physical properties of sponges such as the tensile or compression property [21] but only slightly affect protein adsorption and desorption, which are controlled by hydrophobic interactions, hydrogen bonding, and electrostatic interactions, particularly by the hydrophilicity and surface charge of materials [22] [23].

5. Conclusion

The Gel/CTS composite sponge was prepared using GlcNAc as a cross-linker into the form of a sponge by freeze-drying. The present study focused on FITC-BSA adsorption and desorption on the sponge as a protein model. FITC-BSA adsorption on the sponge increased with FITC-BSA concentration in the solution, until equilibrium was reached at around 30 mg/g. Furthermore, the Langmuir isotherm model fit with the experiment data with $R^2 \geq 0.99$. The thermodynamic parameter was calculated, and the results indicated a spontaneous exothermic adsorption reaction. The adsorption reaction effectively lowered the temperature for the Gel sponge and at every test temperature for the Gel/CTS sponge. The desorption behavior evaluated for different concentrations and pH values of the FITC-BSA solution showed that a high adsorbed amount of FITC-BSA on the sponge resulted in a high desorbed amount, up to 55% from 3.5 mg/mL adsorbed concentration (around 1.5 mg from 3 mg adsorbed amount) in sponge. Sponges released FITC-BSA at pH 7.4 more rapidly than at low pH. The association may be influenced by the net protein charge and interaction between sponge and FITC-BSA. However, Gel and Gel/CTS sponges show the same overall trend. Also, it was suggested that the crosslinking method with GlcNAc is an extremely useful to prepare the gelatin sponges. As the method can apply for other biopolymers such as proteins and polysaccharides, it will be useful tool for the preparation of various biocompatible and non-toxic biomaterials.

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