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Research Article

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Lectin Affinity Based Recognition Nanomaterial for Glucose

Glukoz için Lektin Afinite Temelli Tanıyıcı Nanomalzeme

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ABSTRACT

Glucose is an important biomolecule because it is an important source of energy for cells and is used as an intermediate/metabolic agent. Selective recognition of glucose is important for diagnosing many metabolic diseases. In this study, lectin ligand (Con A) attached p(GMA) nanopolymer was synthesized and characterized. The best interaction of p(GMA)-ConA nanopolymer and glucose was determined to be 10 mM glucose concentration at pH = 8.0. In the selectivity assay, p(GMA)-Con A was found to be 2-fold selective for glucose than galactose. Lectin affinity based nanopolymeric system that is selective, with high surface area, low cost and highly biocompatible with high adsorption capacity has been developed for recognition of glucose.

Key Words

Nanotechnology, glucose, lectin affinity chromatography, concanavalin A.

ÖZ

Glukoz hücreler için önemli bir enerji kaynağıdır ve bir ara / metabolik ajan olarak kullanılır. Glukozun seçici olarak tanınması birçok metabolik hastalığın teşhisi için önemlidir. Bu çalışmada, p(GMA) nanopolimerine lektin ligandı (Con A) bağlanarak p(GMA)-ConA sentezlendi ve karakterize edildi. p(GMA)-ConA nanopolimeri ve glikozun en iyi etkileşiminin 10 mM glukoz konsantrasyonu ve pH = 8.0 koşullarında olduğu belirlenmiştir. Seçicilik analizinde p(GMA)-Con A'nın, glukoz için galaktozdan 2 kat seçici olduğu bulunmuştur. Glukozun tanınması için seçici, yüksek yüzey alanına sahip, düşük maliyetli ve yüksek adsorpsiyon kapasitesiyle yüksek oranda biyouyumlu olan lektin afinite esaslı nanopolimer sistem geliştirilmiştir.

Anahtar Kelimeler

Nanoteknoloji, glukoz, lektin afinite kromatografisi, concanavalin A.

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INTRODUCTION

Glucose is a primary source of energy for cell metabolism. Control of glucose level is important for regulation of signaling protein-involved pathways, and to manage chronic diseases [1]. Glucose sensors have been constructed based on a variety of signal transduction mechanisms, including electrochemistry, colorimetry, fluorometry, and gravimetry [2].

Biomolecules have been separated using several techniques based on the interaction of specific molecules. Among these molecules of lectin-carbohydrate interactions are widely studied, from basic to applied natural and clinical sciences [3]. Lectins are used not only as molecular unifying to immobilize proteins but also used as the recognition element of biosensors, because of binding carbohydrate chains with high selectivity [2].Concanavalin A (Con A) from Canavalia ensiformis, a type of bean is a protein that is extracted. Con A is a protein with a total molecular weight of about 104 kDa, composed of 4 similar subunits at neutral and alkaline pHs. Concanavalin A (Con A) is a typical lectin protein, a tetramer at physiological pH (7.4) comprised of four identical monomeric subunits (MW ~ 25 kDa). Concanavalin A (Con A) is also a metalloprotein containing Mn^{2+} and Ca^{2+} [16]. Concanavalin A (Con A) has been extensively used in the isolation, fractioning and structural characterization of glycoproteins and other important glycoconjugates carrier glucose and/or mannose residues [3-5]. Each monomer contains an independent carbohydrate binding site, for glucose and mannose. It has been found that ConA has the ability to reversibly bind to glucose with an affinity of ~400 M−1.The affinity allows capability for ConA to monitorize glucose concentrations in physiological conditions [6-8].

Glycidyl methacrylate (GMA) with a double bond and a highly reactive oxirane (epoxy) ring group is a versatile monomer, widely used as one of the most effective support materials for polymer chemistry, materials science and biochemistry. GMA is low cost, highly reactive and suitable for a wide variety of polymerization reactions. In addition, the oxirane ring in GMA can be modified with nucleophiles, such as the thiols, amines, diphenyl phosphoryl chloride, sodium azide and others [9-13].
The ring-opening reaction of the epoxy groups of GMA with various secondary amines can provide to supply bifunctional polymers containing tertiary amine and hydroxyl groups. These modified-PGMA polymers have weak basic groups as commonly seen in tertiary amine methacrylate polymers such as poly[2-(diethylamino) ethyl methacrylate] (PDEA), poly[(2-N-morpholino) ethyl methacrylate] (PMEMA), poly[2-(dimethylamino)ethyl methacrylate] (PDMA),and poly[2-(diisopropylamino) ethyl methacrylate] (DPA). Furthermore they exhibit very close solution actions to tertiary amine methacrylate type polymers PGMA polymer and its derivative functional polymers containing morpholine, 1-methylpiperazine and diethyl amine have been prepared by post-polymerization modification method [12].

In this study, Lectin ligand (Con A) attached p(GMA)-Con A nanopolymer were prepared and characterized by different techniques such as scanning electron microscope (SEM), Atomic Force Microscopy (AFM), Fourier transform infrared spectroscopy (FTIR), elemental analysis, zeta-size analysis. The Con A attached poly (glycidyl methacrylate) [p(GMA)-Con A] were used for glucose detection. For glucose binding to the p(GMA)-Con A nanopolymer, a selectivity assay was made with galactose that is the glucose-4-epimer. Glucose was determined by DNS method. Optimization of glucose adsorption on to p(GMA)-Con A nanopolymer was investigated at different conditions. In this context, we aimed to develop a nanomaterial that can be used for determination of glucose from diagnosis chronic diseases such as diabetes. This nanopolymer provides basis for the application of glucose detection.

MATERIALS and METHODS

Materials and Devices

Ethylene glycol dimethacrylate (EGDMA), Glycidyl methacrylate (GMA), Potassium persulfate (KPS), Polyvinyl alcohol (PVA) (Mw 146.000-186.000), Concanavalin A (Canavalia ensiformis Type V), Glucose were used for the synthesis of nanopolymers and they were purchased from Sigma Aldrich.

The laboratory devices were used in the experiments: Scanning Electron Microscope (SEM- Quanta 250 S FEG, IYTEMAM), Attenuated Total Reflectance- Fourier-Transform Infrared Spectroscopy (FTIR-ATR) (Thermo FTIR, Hacettepe University), ZETA Dimensional Analysis (Malvern Zeta-Sizer), Atomic Force Microscopy (AFM) (BRUKER Dimension Edge with ScanAsyst, Ege MATAL).

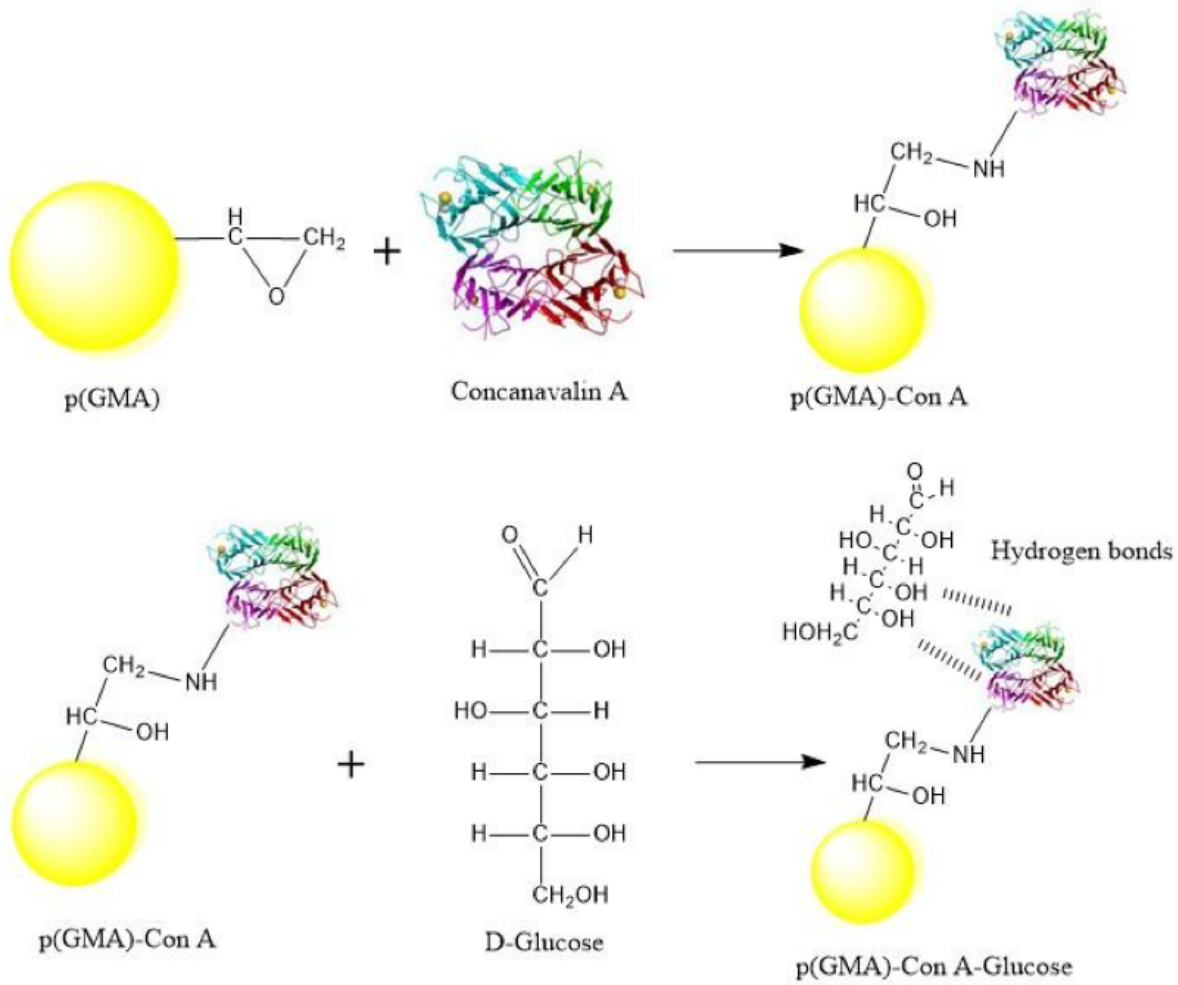

Figure 1. Molecular structure of Con A and Glucose adsorption of p(GMA) nanopolymers.

Methods

Synthesis of lectin attached p(GMA)-Con A nanopolymers

0.275 grams of PVA with high molecular mass (Mw 146.000-186.000) used as a stabilizer for the formation of the spherical shape, was dissolved by heating in 25 ml of distilled water. Then, 0.3 ml of ethylene glycol dimethacrylate (EGDMA), which was used as the crosslinker with 0.6 ml of glycidylmethylmethacrylate (GMA), which is the main monomer, were mixed. This mixture was taken into a laboratory-type reactor (single neck, 100 ml volume) and GMA, EGDMA was added. Finally, 19,8 mg of KPS, dissolved in 45 ml of distilled water, was added to the reactor by shaking. p(GMA) nanopolymers were polymerized by non-surfactant emulsion polymerization method with the mixing rate of 65 rpm in a water bath with a polymerization shaker heater was carried out at 70°C for 4 hours.

The polymer was precipitated by centrifugation 14500 rpm for 30 minutes and after separation of the supernatant, the eluents were removed without further reaction by washing with 70 mL of water, ethanol, and d-water, respectively. The nanopolymer mixed in the ultrasonic bath for 45 minutes was then stored at + 4°C for use in optimization and characterization assays [14]. Calibration curve was prepared with the Con A concentration between 5 and 60 µg/mL was prepared. For binding Con A to p(GMA) nanopolymers, 0.1 M phosphate buffer was used at pH = 8.0 where the thiol groups reacted with the epoxy groups. Optimization of ConA concentration to the p(GMA) nanopolymers were tested with 5; 10; 20; 30; 45; 60 µg/mL concentrations of ConA. 50 µL p(GMA), 300 µL Con A, 150 µL pH = 8.0, 0.1 M phosphate buffer were added to the eppendorf. Eppendorf tubes were placed in an angled mixer and stirred for 1 hour. They were centrifuged for 20 minutes

at 14500 rpm. The lectins remained in the supernatant which did not bind to p(GMA). The absorbance values were measured at 280 nm at the spectrophotometer by taking the upper phase. Con A initial concentration (Ci) and unbound Con A amount were determined by using standard prepared Con A standard graphic. The amount of Con A bound in gram nanopolymer is in mg: The Q values were calculated with following equation (Eq. (1)). It also should be noticed that all adsorption curves are averages of at least triplicated experiments.

$$Q\left(\frac{mg}{g}\right) = \frac{(Ci-Cf)x\,V}{m_{pol}\,x\,10^{-3}} \qquad (1)$$

Q is the Con A and Glucose adsorption capacity of p(GMA) nanopolymers (mg/g), Ci is the initial concentrations of Con A and Glucose in the solution (mg/mL), Cf is the final concentration of Con A and Glucose in the solution (mg/mL), V is the volume of the the aqueous phase (mL), mpol is the mass of nanopolymer (mg).

Experimental studies have shown that Con A specifically binds D-mannose and D-glucose (Figure 1). In a study, it was determined that the amino acids Asp 81, Gly 99, Asn 125, Ala 30, Phe 123 and Glu 31 were involved in the binding region of Con A. It has been found that these amino acids form a network of hydrogen atoms with the oxygen atoms in the hydroxyl groups bound to the 3rd, 4th, 5th, and 6th carbon groups of mannose and glucose sugars [15,16].

Characterization of lectin attached p(GMA)-Con A nanopolymers

For the characterization of synthesized and functionalized nanopolymers, SEM (Scanning Electron Microscopy), FT-IR (Fourier Transform Infrared Spectrophotometer), Zeta Size and Potential Analysis, Atomic Force Microscopy (AFM), volume-mass graph and surface area calculations were performed.

The following equation (Eq. (2)) was used to determine the surface area of synthesized nanopolymers, giving the number of particles in the 1 mL suspension [17].

$$N = 6 \text{ x } 10^{10} \text{ x } S / \pi \text{ x } \rho s \text{ x } d^3 \qquad (2)$$

where N is the number of nanopolymers in the 1 mL suspension; S, % solids; d, diameter (µm); ρs is the polymer density (g/mL). The amount of mg nanopolymer in the mL suspension was theoretically determined using the standard mass-volume plot of the nanopolymers. The specific surface area of the synthesized nanopolymers was calculated in m^2/g using the equilibrium surface area equation.

Surface area of nanopolymer= $4 \text{ x } \pi \text{ x } r^2$ (π, 3,14; r, nanopolymer radius (m))

Optimization of Glucose binding studies to p(GMA)-Con A nanopolymers

3,5-Dinitro salicylic acid (DNS) method was used to determine glucose amount. To obtain the DNS reagent, 1 g of DNS was stirred by heating in 20 mL of 2N NaOH. 30 g of sodium potassium tartrate was added slowly and finally 50 mL of water was added. In order to make a standart graphic, 0,5 mL of concentrations of 1, 2, 4, 6, 8, 10 mM glucose samples and 0.5 mL of DNS reagent was added and mixed with vortex. The mixed tubes were placed in a beaker and boiled in the heating plate for 10 minutes to ensure the reaction was carried out. 3 mL each water was added to the tubes taken from the heater. The absorbance values were read at 546 nm wavelength in spectrophotometer [18].

In order to optimize glucose concentration, 300 µL of glucose at a concentration of 1; 2; 4; 6; 8; 10 mM was added p(GMA)-Con A nanopolymer respectively. Eppendorf tubes were placed into angle mixer for 1 hour. It was centrifuged for 20 minutes at 14500 rpm. In the supernatant, the amount of unbound glucose was measured spectrophotometrically at 546 nm by the DNS method.

To investigate the pH effect of glucose binding to p(GMA)-Con A nanopolymer were investigated with 10 mM pH= 5-9 buffers. 400 µL of buffers, 50µL p(GMA)-Con A nanopolymer were added to 50µL 10 mM glucose and stirred for 1 hour at room temperature in an angled mixer. The samples were centrifuged at 14500 rpm for 20 minutes.

For the temperature assay, 10 mM glucose solution in pH = 8 0.1 M phosphate buffer was used. Temperature experiments were carried out at 4°C, room temperature (23°C), 37°C and 40°C.

Selectivity studies

For glucose binding to the p(GMA)-Con A nanopolymer, a selectivity assay was made with galactose that is the

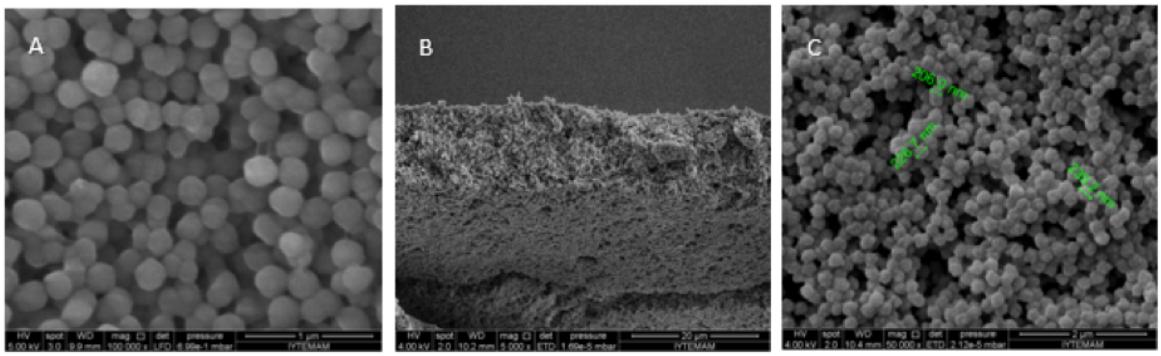

Figure 2. A) SEM images of p(GMA) (100000x), B) p(GMA)-Con A (5000x) C) and p(GMA)-Con A (50000x).

glucose-4-epimer. For the selectivity assay, p(GMA)-Con A nanopolymer was used to determine the optimum conditions for pH=8.0, 0.1 M phosphate buffer, 40°C temperature and 10mM concentration of glucose and glucose 4-epimer galactose solutions were used.

RESULTS

Characterization of p(GMA)-ConA nanopolymers

SEM Analysis

The morphological structure of p(GMA) and p(GMA)-ConA nanopolymers were determined by SEM analysis. As shown in Figure 2, p(GMA) and p(GMA)-ConA nanopolymers have spherical shapes with averagely 223.9 nm sizes and it can be said that binding of ConA to p(GMA) nanopolymers did not change the sizes of nanopolymers as visibly.

As shown in Figure 3, the surface morphology of the p(GMA) and p(GMA)-Con A nanopolymers was also confirmed by AFM analysis. When the AFM results of p(GMA) and p(GMA)-Con A nanopolymers were examined, average roughness (Ra) of the p(GMA) nanopolymer was found as 0.0347 µm, root mean square roughness (Rq) was obtained as 0.0566 µm; average

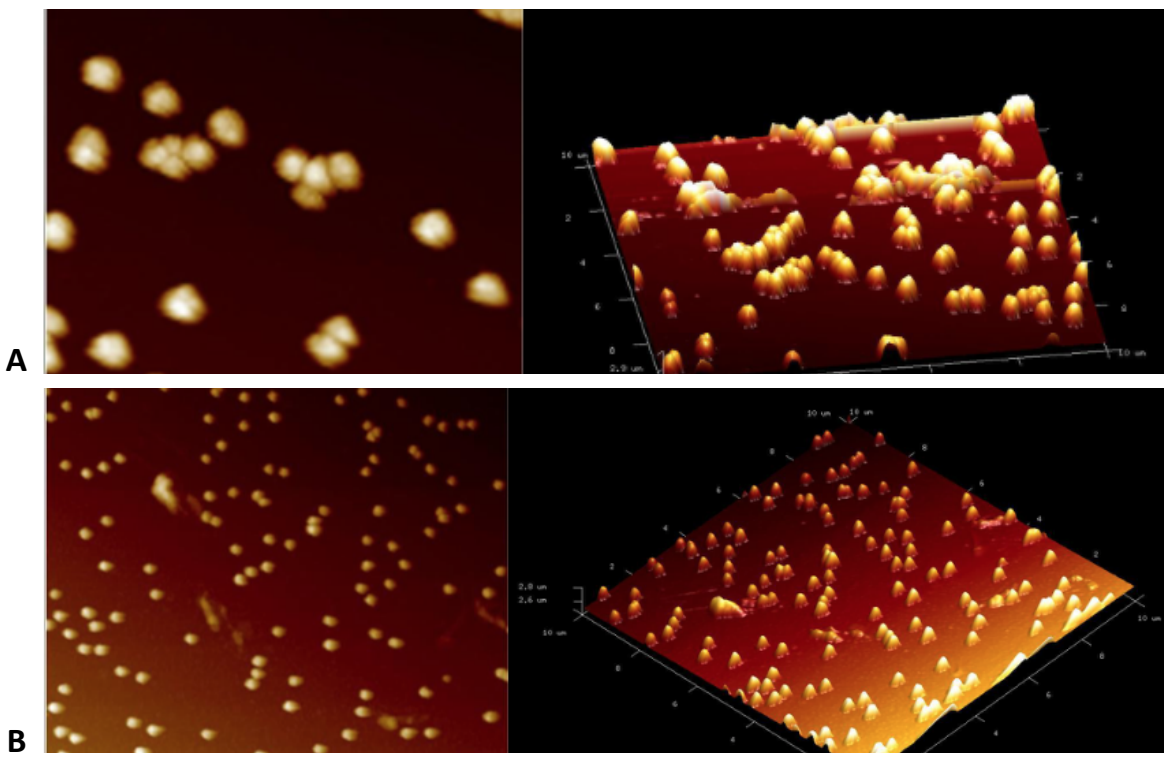

Figure 3. AFM Analysis of A) p(GMA) and B) p(GMA)-Con A Nanopolymers (2D and 3D).

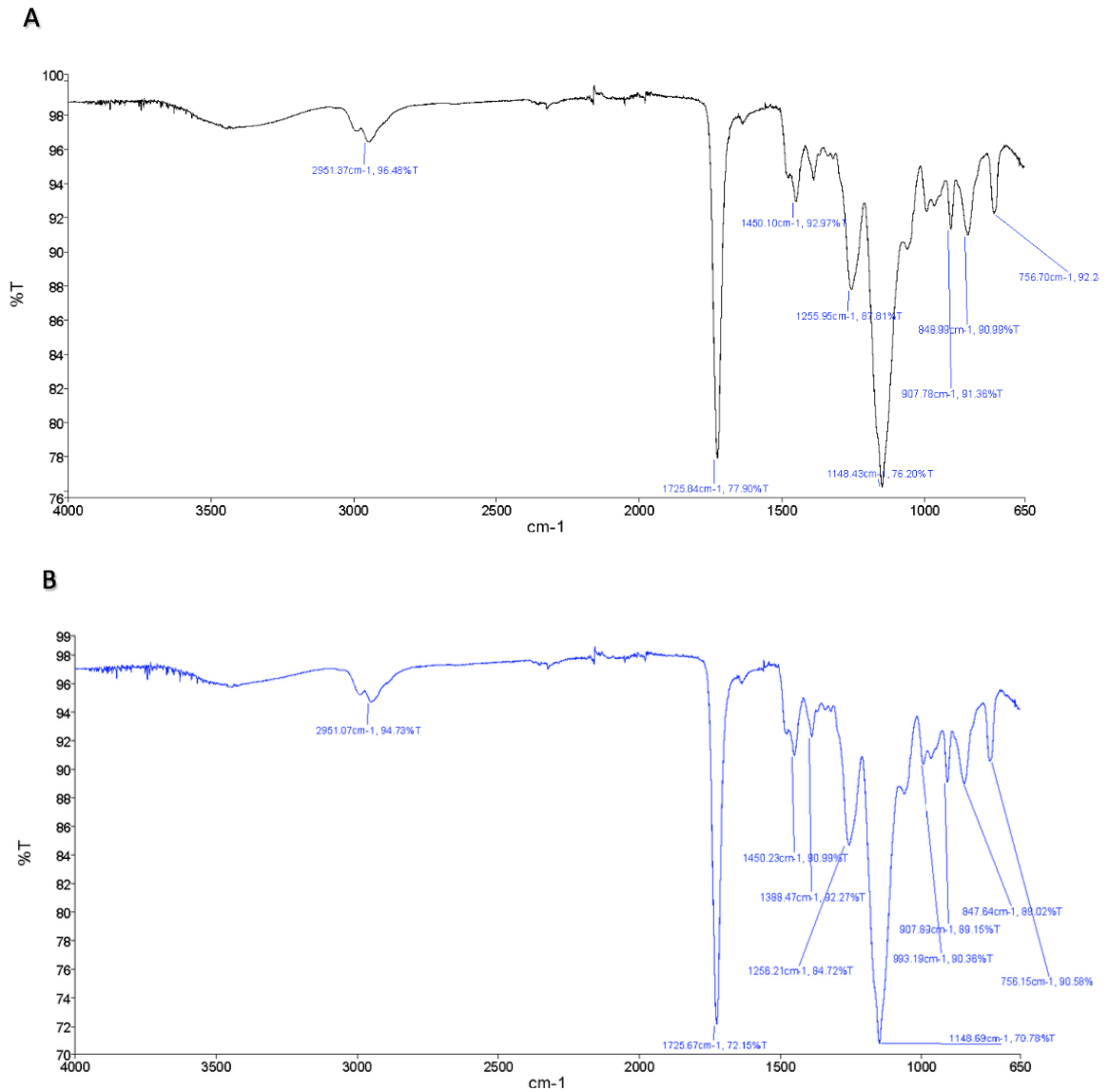

Figure 4. FTIR Analysis of A) p(GMA) and B) p(GMA)-Con A nanopolymers.

roughness (Ra) of the p(GMA)-Con A nanopolymer was found as 0.0153 µm, root mean square roughness (Rq) was obtained as 0.0261 µm. Ra is the arithmetic average of the absolute values of the height of the surface profile. The root mean square of roughness (Rq) is a function that takes the square of the measures [19]. Ra value increases, surface roughness increases [20]. In this case, Con A binding to p(GMA) nanopolymer reduced surface roughness. Rq value is more sensitive to high hills and valleys depending on the square of the amplitude according to Ra value [19]. The decrease in Rq value with Con A binding also supports the change in morphological structure.

As seen in the FTIR spectrum in Figure 4, the presence of epoxy groups with the 908 cm^{-1} peak characteristics showing the presence of oxirane groups in the structure has been proven. Asymmetric ring bending can be mentioned for the peak region 993-907 cm^{-1}. The peaks 847-756 cm^{-1} indicate C-H bending. The vibration at 1725 cm^{-1} can be correlated with C=O (ester carbonyl) stress and the vibration at 1148 cm^{-1} can be attributed to C-O stress representing the ester configuration of p(GMA) nanopolymer. It refers to the C-O-C binding state at 1069 cm^{-1}. It shows symmetrical ring stretching of peak epoxies at 1256 cm^{-1}. The peak at 1450 cm^{-1} shows the

CH2 bending state. The peaks in the range 2951- 2980 cm^{-1} are due to the presence of C-H tensile vibrations. In the light of this results, we conclude that lectin molecules (Con A) attached onto the p(GMA) nanopolymers covalently.

Despite of the SEM analysis, in zeta size measurement is performed by utilizing the brownian motion, which is the ability of the particles in dilute suspension to determine the particle size [21]. According to the Zeta-Size analysis of p(GMA)-Con A nanopolymers in Figure 5, the average particle size was 241±2.1 nm; Polydispersity value (PDI) was found to be 0.065. This 3 repetitive zeta size results support to SEM analysis.

According to the Zeta-Size analysis of p(GMA)-Con A nanopolymers in Figure 6, the average particle potential was found to be -31.2 mV. Particles with zeta potentials more negative than -30 mV are normally considered stable [22].

Surface Area Calculations

Using the prepared volume-mass graph, the amount of g polymer per unit volume (mL) was calculated as 0.0084 g nanopolymer/mL with using Eq (1). The diameters of the p(GMA)-Con A nanopolymers were determined to be 223.9 nm, with a specific surface area of 971.5 m^2 / g.

Ligand Density Studies

Lectin affinity method has recently been carried out for the glycoprotein recognition and purification. Lectins are carbohydrate-binding proteins. Con A is the most notable and commonly used lectin, and also a biospecific ligand, because of the specific interactions via carbohydrate residues. Con A loading is the amount of Con A molecules as ligand per unit volume of the p(GMA) nanopolymers. Figure 1 show the binding stucture of Con A to the p(GMA) nanopolymers. Con A was covalently binding onto the p(GMA) nanopolymers through coupling reaction between the free amine groups of Con A and the epoxy groups of the nanopolymers [23].

In the experiment, it was aimed to obtain p(GMA)-ConA nanopolymer by utilizing the reaction of –SH groups between the epoxy groups in GMA structure in pH = 7.5-8.5. Figure 7 indicates the effect of Con A concentration to the loading. The amount of Con A loading increased

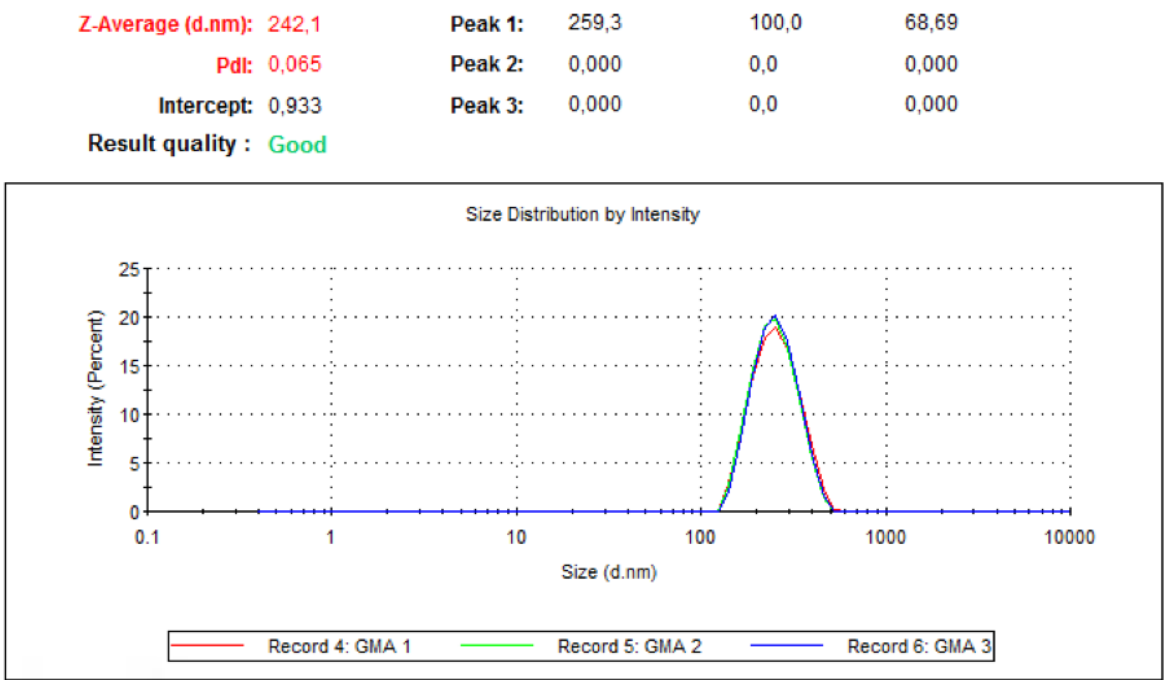

Figure 5. Zeta Size Analysis of p(GMA)-Con A nanopolymers.

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -30,9	Peak 1:	-30,9	100,0	11,5
Zeta Deviation (mV): 11,5	Peak 2:	0,00	0,0	0,00
Conductivity (mS/cm): 0,0132	Peak 3:	0,00	0,0	0,00

Result quality : Good

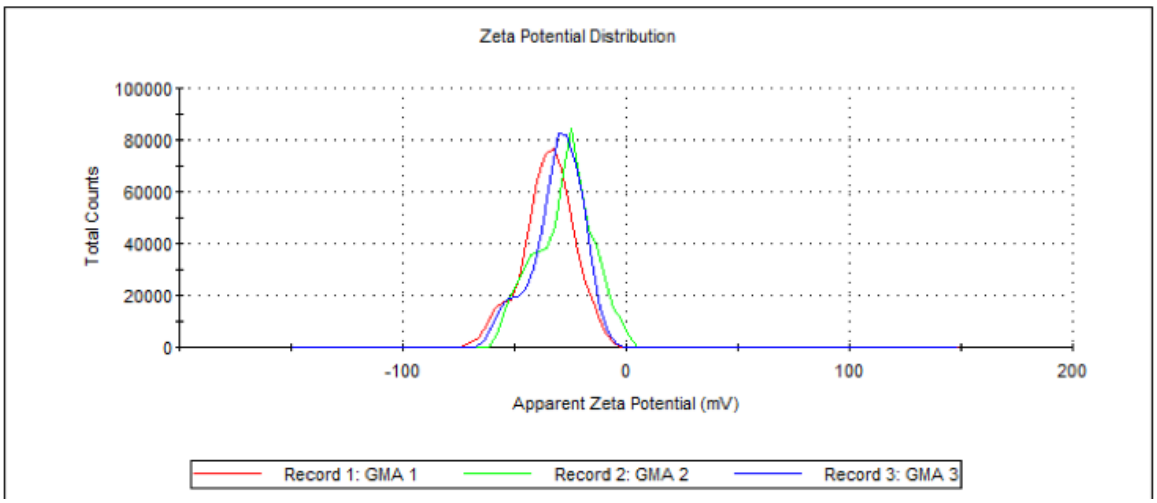

Figure 6. Zeta Potential Analysis of p(GMA)-Con A nanopolymers.

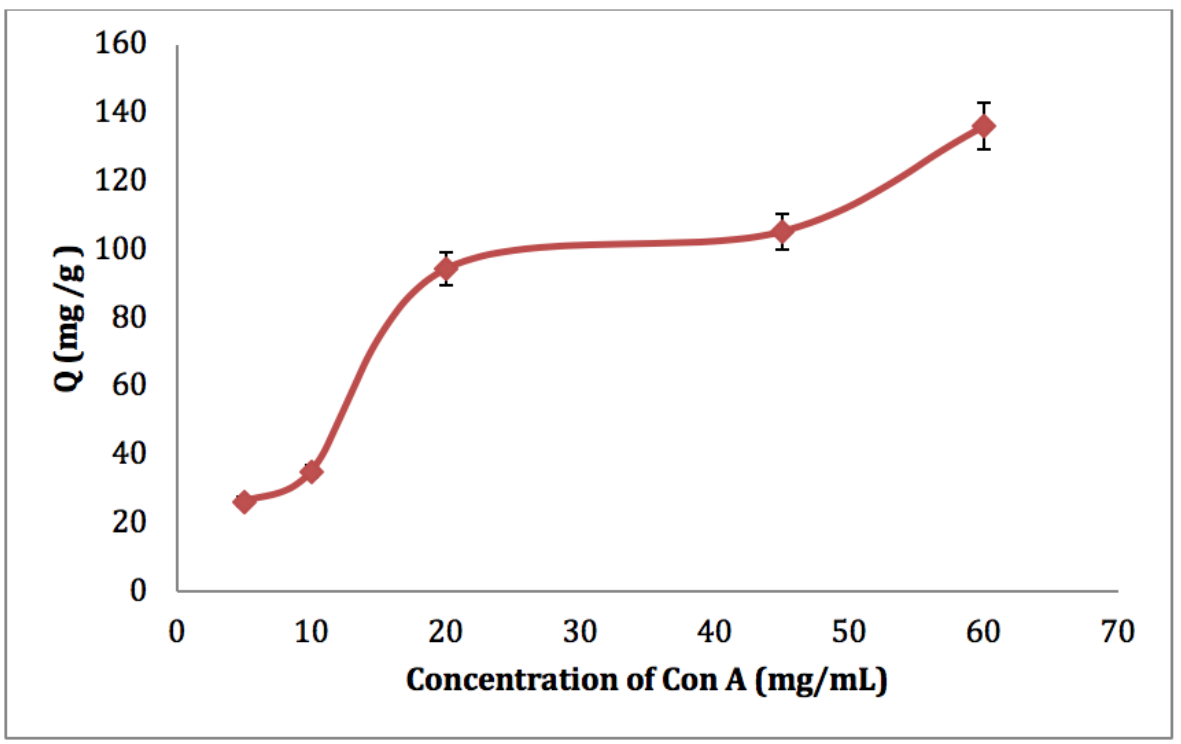

Figure 7. Con A loading capacity onto the p(GMA) nanopolymer (0.1 M pH= 8 buffer, T= 25°C).

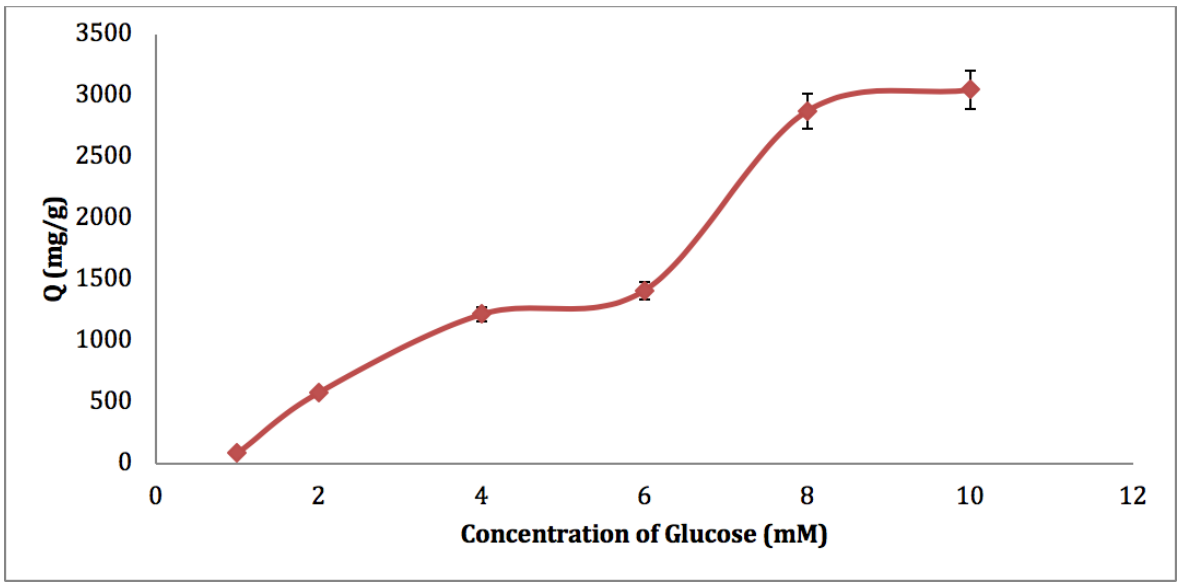

Figure 8. Glucose concentration effect on glucose binding to p(GMA)-ConA nanopolymer (0.1 M pH=8.0 buffer, t=1 hour, T=23 oC).

with increasing amount of Con A. The maximum concentration of ConA that binded to p(GMA) nanopolymer is determined as Q=136.3 mg/g at a concentration of 60 µg /mL ConA.

Optimization of Glucose binding studies to p(GMA)-Con A nanopolymer

Concentration Effect

Figure 8 shows the glucose concentration effect onto the p(GMA)-ConA nanopolymer. It can be understood from the figure that the amount of sugar binding firstly increased with increasing sugar concentrations and then reached the highest value. This rising incline of the graphic indicated the high affinity between glucose and p(GMA)-Con A nanopolymers. Maximum glucose binding to p(GMA)-Con A nanopolymer was determined as 3049.2 mg/g at a concentration of 10 mM glucose. At this point, the p(GMA)-Con A nanopolymer reached saturation.

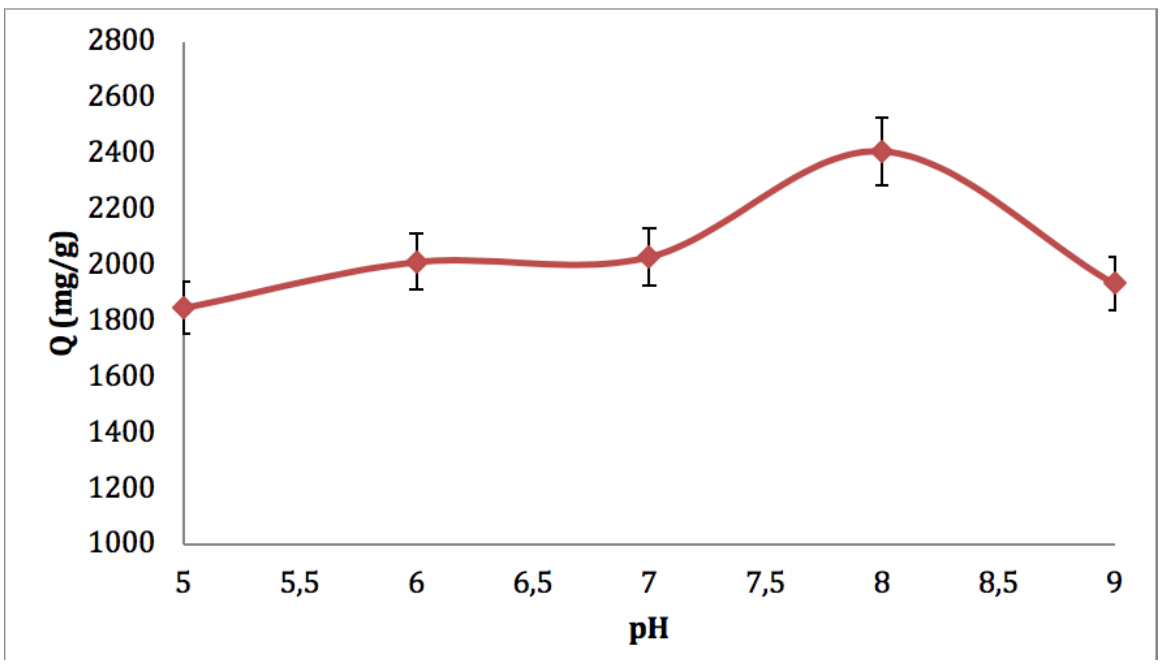

Figure 9. pH effect on glucose binding to p(GMA)-ConA nanopolymer (C_i= 10 mM, t= 1 hour, T=23°C).

Figure 9 shows the pH effect to the glucose binding. The pH of the medium generally effects lectin and carbohydrate interactions. Because, lectins are proteins and total charge, solubility, chemical stability and spatial regulation, which is a function of the primary, secondary, tertiary and quaternary structure of proteins, strongly depend on it. In the experiments, the effect of different pH values could not be clearly observed, but the point of pH = 8.0, maximum binding was observed as 2409 mg/g.

Temperature Effect

The effect of the temperature on glucose binding was investigated with a temperature range of 4°C- 40°C. Figure 10 shows that adsorbed amount of the glucose have been firstly stable and increased at 40°C. In the experiments, the maximum temperature for glucose binding was determined as 40°C with the Q value of 2405.41 mg /g. Due to the increase in the kinetic energy of the molecules with increasing temperature, the molecules were interpreted as a higher rate of interaction with each others. So adsorption of glucose on the p(GMA)-Con A was increasing but, after 40°C, it is expected that adsorbed amount of the glucose will decrease because of the structure of the carbohydrate molecules were also corrupted at high temperatures

Selectivity of p(GMA)-Con A nanopolymers for Glucose

In order to determine the sugar group specificity of the p(GMA)-Con A, optimum conditions (pH = 8.0; 0.1 M phosphate buffer, 40°C temperature, Ci = 10 mM) of the glucose and 4-epimer galactose p(GMA)-ConA was investigated. Then, it was determined that nanopolymer was showed 2-fold selectivity to glucose due to using of ConA lectin (Figure 11). It can be assumed that ConA can recognise all sugar groups with different selectivity. Then this nanopolymer can be used for recognition of sugar groups. Galactose, glucose and mannose were isomers differ only in the axial/ equatorial configuration of their hydroxyl groups and they are the three most common hexoses present in mammalian physiology [24].

DISCUSSION

Within the scope of the study, lectin affinity chromatography based nanopolymeric system that is selective, low cost and highly biocompatible with high adsorption capacity has been developed for detection of glucose. As a result of the experiments, optimum glucose recognition conditions were determined for Con A which is one of the well-known lectin.

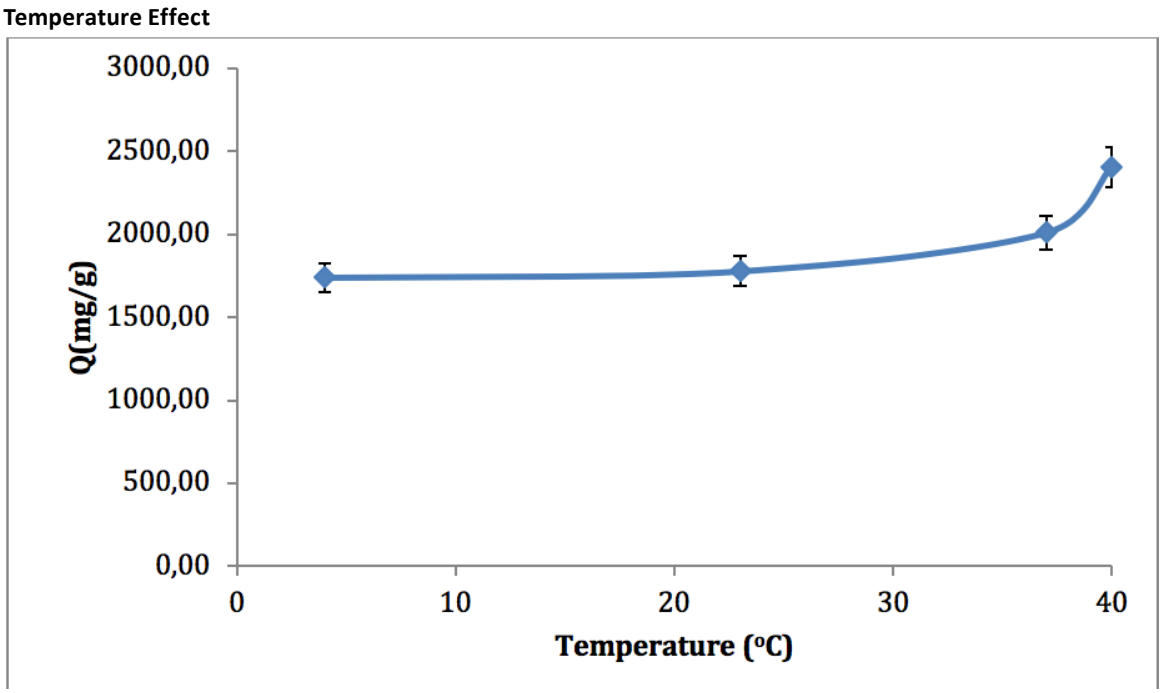

Figure 10. Temperature effect on glucose binding to p(GMA)-ConA nanopolymer (C_i= 10 mM, pH= 8.0, 0.1 M phosphate buffer, t= 1 hour).

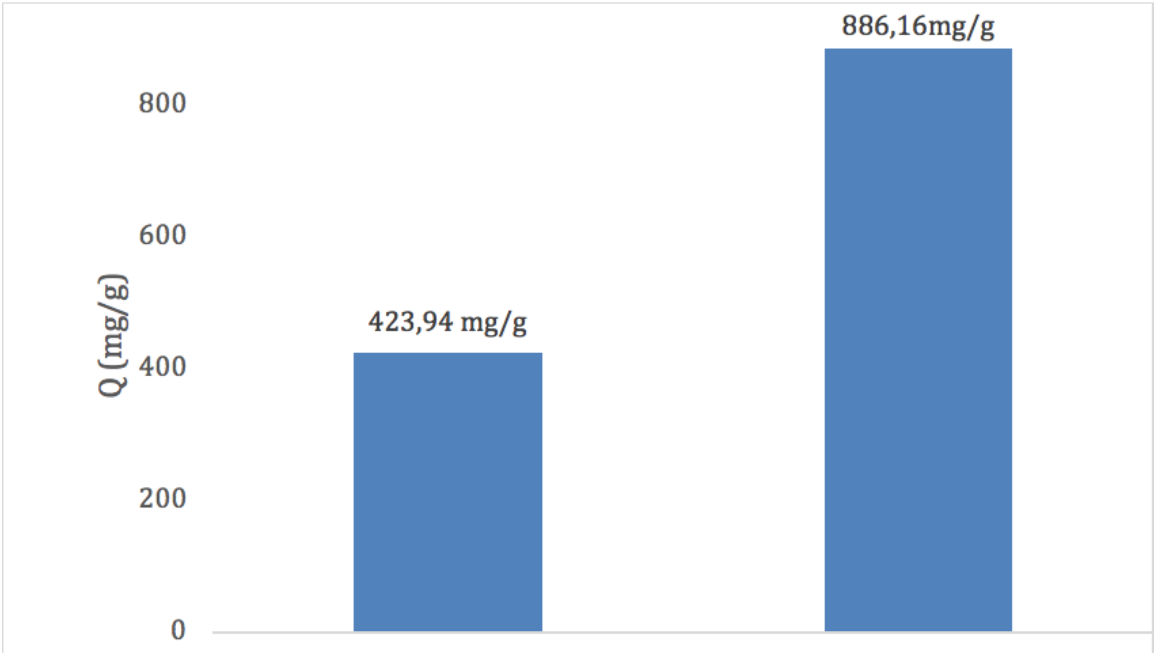

Figure 11. Selectivity assay between glucose and galactose (0.1 M pH= 8.0 phosphate buffer, C_i= 10 mM, T=40°C).

Results from our groups have already demonstrated lectin attached affinity cryogels for amyloglucosidase adsorption [5], mannose based polymeric nanoparticles for lectin separation [24] and inulinase immobilized lectin affinity cryogels for the high-fructose syrup [25].

Here, we demonstrated that specifically identify glucose by taking advantage of the specific interaction between the large surface area of nanopolymers and Con A-glucose with high yield. The best interaction of p(GMA)-ConA nanopolymer and glucose was determined to be 10mM glucose concentration, pH = 8.0, 0.1M phosphate buffer and 40°C. In the selectivity assay, p(GMA)-Con A was found to be 2-fold selective for glucose than galactose, the 4-epimer. It was found from the results of this study that these newly synthesized p(GMA)-Con A have good capability for the specific recognition of the glucose analyzed. Based on the advantages of the good glucose-recognition properties, these nanopolymers can potentially be used in biosensor applications for the specific recognition of the glucose.

In parallel to our results, Antonyuk et al. developed specific polysaccharide-immobilized monodisperse poly(glycidyl methacrylate) core–silica shell microspheres and they achieved to immobilize 50mg of polysaccharide (yeast mannan and gum arabic) per 1g of the matrix, which is 10 times higher than the capacity of epoxy-activated Sepharose 6B (agarose gel matrix). This study is important in order to show interaction and affinity between polysaccharides and lectins [26]. Another study, Locke et al. reported that PEGylated ConA were potential improving of a ConA-based long term continuous glucose monitoring device for in vivo applications [6].

In conclusion, these findings support the hypothesis that these nanopolymers may be used for the recognition of the glucose from biological fluids. According to the results of this study, p(GMA)-ConA based lectin affinity system for glucose recognition represents promising route to the large scale applications process. Further studies are expected to pave the way for various recognition studies for different diagnosis devices.

Conflict of Interest

The authors declare that they have no conflict of interest.

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