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Research Article

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Hemoglobin Purification Using His-Tag Affinity Chromatography With the Assistance of Ni Ions

His-Tag Afinite Kromatografisi Kullanarak Hemoglobinin Ni iyonları Yardımıyla Saflaştırılması

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ABSTRACT

Nickel ions were were utilized in this study due to forming of strong complexes with histidine and its ability to interact with histidine-containing proteins and for this goal, an affinity adsorbent of poly(2-hydroxyethyl methacrylate) (p(HEMA) was developed to purify hemoglobin with the assistance of Ni ions. Initially, cryogel membranes of p(HEMA) were prepared and their surfaces were modified with the dopamine monomer to create polydopamine (PDA) modified p(HEMA) cryogel PDA(HEMA) membranes. After the modification, the nickel (Ni) ions were immobilized on PDA(HEMA) for Hb purification in the aqueous solution. According to experimental findings, at pH 5, the adsorbent successfully adsorbed 23.5 mg/g of Hb. The Langmuir adsorption isotherm model was found to accurately describe this adsorption process, as evidenced by the high R2 and q max values. Furthermore, it was observed that the adsorption capacity of the prepared adsorbent remained consistent without significant decreases, even after multiple uses.

Key Words

Hemoglobin, adsorption, affinity chromatography, his-tag affinity chromatography, cryogel.

ÖZ

Bu çalışmada histidin ile güçlü kompleksler oluşturması ve histidin içeren proteinlerle etkileşime girme yeteneğinden ötürrü nikel iyonları kullanılmış ve bu amaçla, Ni iyonlarının yardımıyla hemoglobini saflaştırmak için poli(2-hidroksietil metakrilat) (p(HEMA) aesaslı bir afinite adsorbenti geliştirilmiştir. İlk olarak, p(HEMA) kriyojel membranlar sentezlenmiş ve yüzeyleri dopamin monomeri kullanılarak polidopamin (PDA) kriyojeller PDA(HEMA) hazırlanmıştır. Modifikasyon sonrasında, nikel (Ni) iyonları PDA(HEMA) üzerine sulu çözeltiden hemoglobin (Hb) saflaştırılması için immobilize edilmiştir. Deney sonuçlarına göre pH 5 de, adsorbent tarafından 23.5 mg/g Hb'ni adsorpladığı görülmüştür. Langmuir adsorpsiyon isoterm modeli yüksek R2 değeri ve q maks değerlerine doğrultusunda bu adsorpsiyon işlemi için uygun olarak bulunmuştur. Ayrıca, birçok kez tekrar kullanım sonunda, hazırlanmış olan adsorbentin adsorpsiyon kapasitesinde önemli kayıpların olmadığı görülmüştür.

Anahtar Kelimeler

Hemoglobin, adsorpsiyon, afinite kromatografi, his-tag afinite kromatografi, kriyojel.

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INTRODUCTION

Hemoglobin is an extraordinary molecular machine composed up of four subunits and each have a ring-like heme group. This exceptional protein binds oxygen reversibly and carries it to the tissues in order to produce the energy required for the entire metabolic process [1,2]. The level of hemoglobin in the blood provides a vital information about whether adequate oxygen is present at the tissues. Apart from this unique function in the body, the high level of hemoglobin in the blood is one of the early warnings of diseases such as polycythemia and advanced lung diseases, so the availability of sensitive and fast methods for its determination is one of the hot topics that do not fall on the scientific agenda [3]. From these perspectives, isolation, and sensing of hemoglobin are of great importance due to its main functions mentioned above.

The histidine content of hemoglobin is quite high and using histidine-specific ligands such as metal ions have paved the way for the his-tag affinity chromatography in the recent years. One of the reasons why histidine comes to the fore is that it is widely used among depletion methods by selecting it as a labeled protein due to its high affinity for transition metals.

In research where high selectivity and high purity are sought, materials with strong affinity such as histidine-metal play a leading role especially in purification techniques. Various adsorption and depletion studies of hemoglobin have been carried out with metal chelates formed as a result of strong interaction between metal ions such as Cu^{2+}, Ni^{2+}, Zn^{2+} or Co^{2+} and proteins e.g., hemoglobin containing sequential histidine. It effectively binds to nickel histidine-containing proteins but may also show non-specific binding. Moreover, if substantial purification quantities are needed, nickel ions might be favored over other transition metals. [4,5]. In addition to benefiting from the metal-histidine interaction by labeling the histidine on the surface of the materials, purification can also be done with different surface modification approaches like used of polydopamine (PDA) due to its non-toxicity and surface functional. PDA, containing catechol and amine groups, is one of the most widely used methods for surface functionalization and compatibilization in the last decade. As a bioinspired synthetic polymer, PDA can be easily obtained by the self-assembly and oxidative polymerization of dopamine under slightly basic conditions**.** [6–9]

Recently, cryogels with the superior key features such as macropore structures, fast-swelling kinetics, and robustness have employed in affinity chromatography applications including protein purification studies [10,11]. Many studies using the unique adaptive nature of cryogels in protein purification have taken their place as a promising approach in biomedical applications [12–15]. In one of the special studies on the use of cryogels in protein purification, poly(2-hydroxyethyl methacrylate) p(HEMA)-based microbeads were synthesized, and immunoglobulin G (IgG) purification was aimed. It has been shown that the adsorption capacities increase drastically after PDA coating on bioinspired magnetic microbeads by oxidative polymerization [16].

In this research, we introduced a novel metal-chelate adsorbent for purifying hemoglobin in the aqueous solution. Initially, cryogel membranes based on p(HEMA) were synthesized utilizing 2-hydroxyethyl methacrylate (HEMA) as the functional monomer. Then, the dopamine was polymerized under basic conditions on adsorbent surfaces to introduce the catechol groups for Ni ions attachments. Finally, the hemoglobin adsorption performance of the metal-chelate adsorbent was examined in the aqueous solution with the adjustment some adsorption parameters.

MATERIALS and METHODS

Materials

HEMA, dopamine hydrochloride, and hemoglobin (bovine blood) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals were of reagent grade and during the whole experimental studies, all water purified with using Barnstead (Dubuque, IA, USA) ROpure LP® reverse osmosis unit system.

p(HEMA) based cryogel synthesis

A basic monomer, HEMA, was polymerized with ammonium persulfate (APS) and N,N,N',N'-tetramethyl ethylenediamine (TEMED) ion pairs under semi-frozen conditions, during the synthesis, deionized water (DW) and N,N'-Methylenebis(acrylamide) (MBAAm) were

used as pore formers and crosslinkers, respectively. The preparation procedure involved dissolving of HEMA (1.3 mL) in DW (3.7 mL) and MBAAm (0.283 g) in a separate solution (10 mL DW). After mixed of two solutions, the cyrogel was fabricated via free-radical polymerization at -16°C for 24 hours. Then, the cryogel was washed and cut into circular discs (1.5mm thick), which were stored in containing 0.02% sodium azide a phosphate buffer at 4°C.

Surface Modification of p(HEMA) cryogel with PDA

The surface modification of PDA was conducted in the basic conditions at room temperature (RT) using an orbital rotator. Before the surface modification, the plain, p(HEMA), cryogels were washed with DW and then pH of the surface modification media was adjusted at pH 8.5 Tris-HCI buffer solution after that, the plain cryogels to be modified were added into the medium to swell for 30 min at RT. After the addition of dopamine into solution containing the adsorbents, the solution was stirred for nearly 60 min at RT for the polymerization of dopamine until the surface color of the cryogels turned into brownish. Finally, the surface modified PDA(HEMA) cryogels were collected and were washed with DW.

Ni ions interactions with the PDA(HEMA) cryogel membranes

The metal-chelate interactions of the Ni ions and the PDA modified surface were carried out with the previous work [17]. For that purpose, the modified cryogels that were treated with 100 mM ($NiSO_4$) solution were stirred for 2h at RT and after that, the adsorbents were washed with DW to remove the unbounded metal ions from the modified surface.

Characterization Studies

The surface morphologies of both cryogels were investigated with a scanning electron microscopy (SEM) and before recording their images, the dried cryogels were layered on a stamp and their surfaces were coated with a gold.

The swelling test was applied to understand the swelling degree (SD) of both cryogels and before the calculation of the SD, the dried cryogels were weighted (W_{dry}) and the dried cryogel was put into the DW for 2h. After that, to remove the water from the cryogel surface, cryogels were wiped out via a filter paper and the cryogel (W_{wet}) was weighted again. Finally, SD of the cryogel was calculated with the following equation.

$$SD\ (\%) = [(W_{wet}-W_{dry})/W_{dry}] \times 100 \qquad \text{(Eq 1)}$$

The degree of macroporosity in cryogels was determined by measuring the weights of both swollen and squeezed cryogels. The weight of swollen cryogels ($m_{swollen\ gel}$) was measured after they had been submerged in water. To determine the weight of squeezed cryogels ($m_{squeezed\ gel}$), excess water within the cryogel structure was removed, and their weights were subsequently measured [18]. The macroporosity degree of the cryogels was calculated using Equation (2)

$$\text{Macroporosity degree (\%)}: (m_{swollen\ gel}-m_{squeezed\ gel})/m_{swollen\ gel} \times 100 \qquad \text{Eq (2)}$$

Adsorption studies

Hemoglobin adsorption was conducted in an aqueous solution, systematically varying parameters and utilizing a batch system with an orbital rotator at RT. Before the experimental studies, one of the membranes was chosen and the pH, concentration effects on hemoglobin adsorption were determined with the UV vis spectrum (Shimadzu, Model 1601, Tokyo, Japan). After the adsorption studies, %5 (w/v) SDS solution was interacted to adsorbent for 5 min to remove adsorbed of hemoglobin**.**

RESULTS and DISCUSSION

The surface morphologies and the inner parts of both cryogels were investigated with a SEM and their images were depicted in Figure 1. As given in Figure 1, the p(HEMA) cryogel (A and B) and PDA(HEMA) (C and D) have macroporous structures due to the cryotropic gelation process, but, the PDA cryogel surface was quite different and the macropores of modified cryogel was more distinctive as compared to plain cryogel. Probably, the surface morphology differences of PDA(HEMA) may be dependent on the polymerization of dopamine monomer.

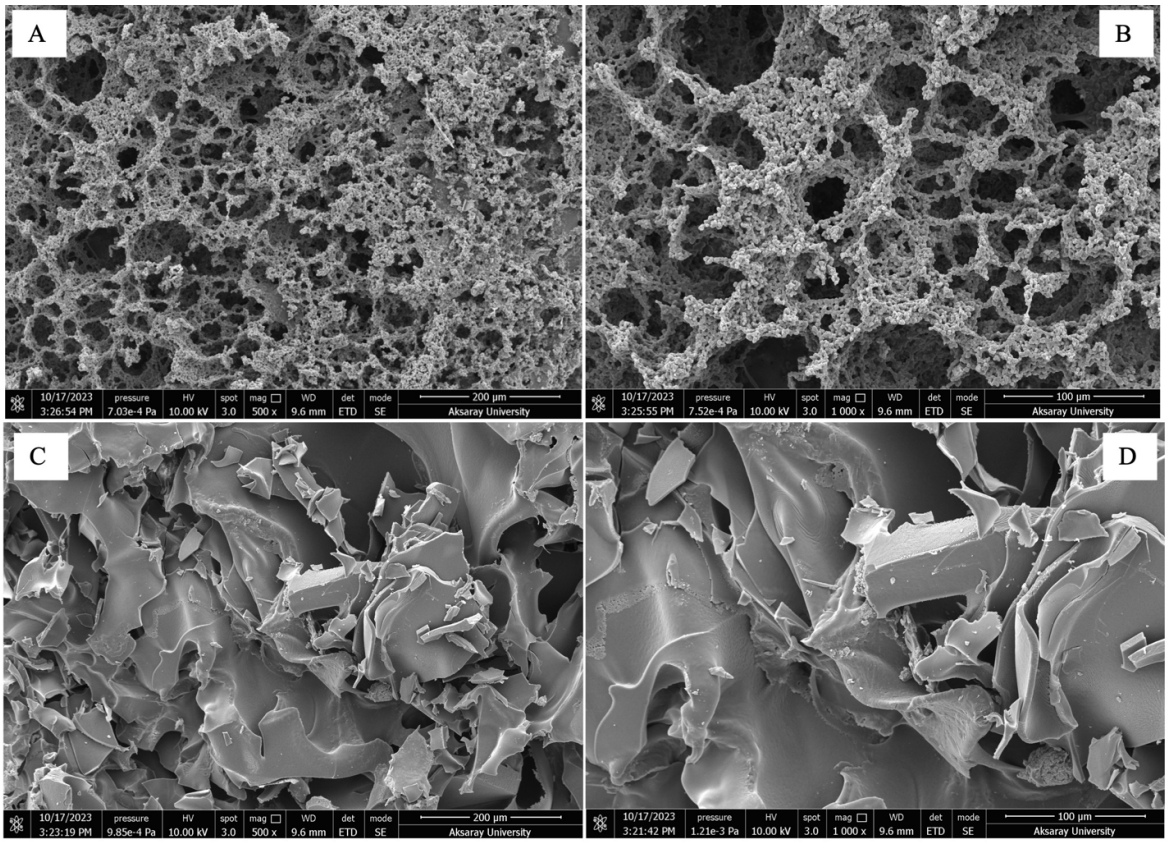


Figure 1. A, B represents the SEM images of p(HEMA) and C, D represents the SEM images of PDA(HEMA) cryogels.

EDX analysis results of of p(HEMA) and PDA(HEMA) cryogels were given in Figure 2 and as depicted in Figure 2, the amount of Ni ions found in PDA(HEMA) was calculated as (0.34%) and this result indicates successful interaction between Ni ions and the catechol groups of PDA.

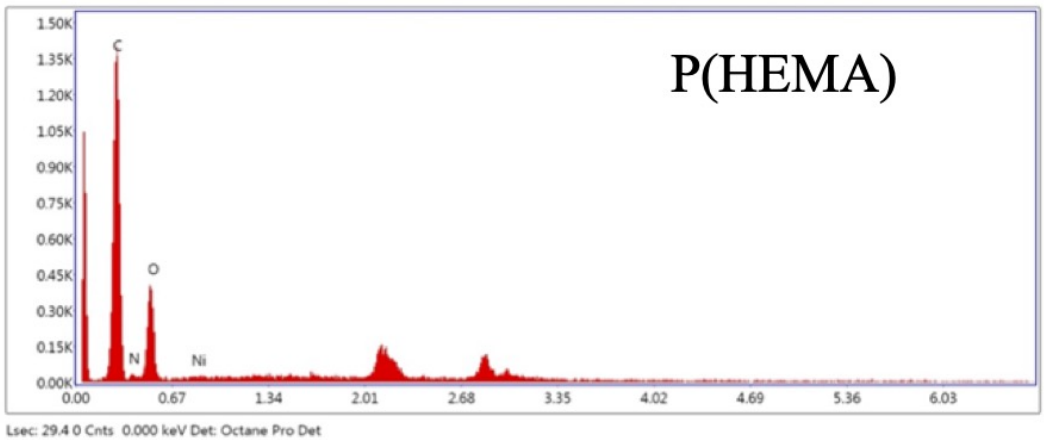
The swelling degrees (SDs) of the cryogels were investigated via swelling test for 2h in DW and the SDs of p(HEMA) and PDA(HEMA) were calculated as 283.81% and 534.69%, respectively. On the other hand, the macroporosity of p(HEMA) and PDA(HEMA) were found as 73.95% and 84.24%, respectively. The macroropores of PDA(HEMA) were larger than p(HEMA) and allow to penetration of water into the polymer chain leading to increasing of swelling ratio of modified cryogel; furthermore, the introducing of new hydrophilic catechol groups could enhance the swelling of PDA(HEMA).

During the adsorption studies, the adsorption studies was carried out via an orbital rotator for 10 rpm at RT for 2 h. Before the investigation of pH effect on hemoglobin adsorption, the modified cryogel membrane was equilibrated with the buffer solutions and then, 0.1 mg/mL hemoglobin solution was interacted with the modified cryogel for 2h. Following that, the cryogel that was treated with the desorption solution for 5 min to remove the bounded hemoglobin from the adsorbent. The adsorbed amount of hemoglobin was measured at 406 nm and was calculated with Eq 3.

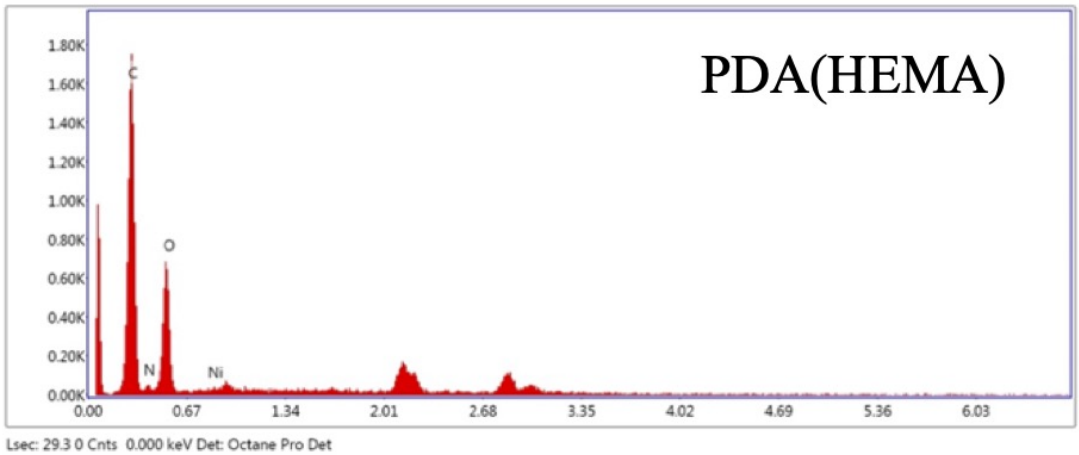
$$q = [(C_i - C_f)] \times V / M \qquad \text{Eq (3)}$$

Herein, C_i and C_f are the initial and the final concentrations (mg/mL) of hemoglobin, respectively, and V, the volume of the adsorption media (mL) and M, the weight of the polymer (g).

The pH effect of hemoglobin adsorption was examined in pH 4-7 values and the results of pH effect were depicted in Figure 3. The adsorbed amount of hemoglobin was calculated as 23.5 mg/g at pH 5 and after the increased of pH, the adsorbed amount of hemoglobin was gradually decreased at pH 7. The hemoglobin imidazole group



Element	Weight %	Atomic %	Net Int.	Error %
C K	64.83	70.63	248.84	4.62
N K	5.16	4.83	3.6	31
O K	30.01	24.55	70.21	10.96
NiL	0	0	0	99.99



Element	Weight %	Atomic %	Net Int.	Error %
C K	56.15	62.56	298.39	4.86
N K	8.12	7.76	9.38	21.14
O K	35.39	29.6	125.49	10.13
NiL	0.34	0.08	0.99	52.13

Figure 2. The EDX results of p(HEMA) and PDA(HEMA) cryogels

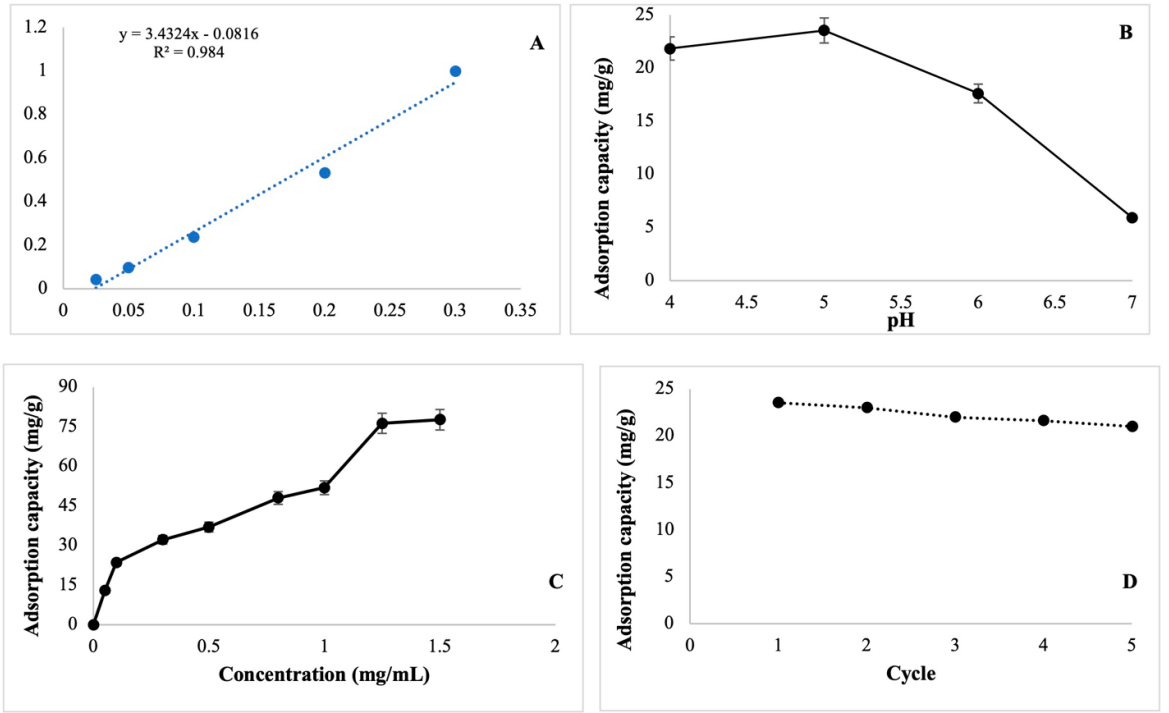


Figure 3. The calibration curve (A), the pH effect (B) on hemoglobin adsorption (hemoglobin concentration; 0.1 mg/mL, orbital rotator rate; 10 rpm, RT), the concentration effect (C) on hemoglobin adsorption (pH 5, orbital rotator rate; 10 rpm, RT) and the reusability study (D) of hemoglobin adsorption (pH 5, hemoglobin concentration; 0.1 mg/mL, orbital rotator rate; 10 rpm, and RT).

Table 1. Figure-of-merit (FOM) calculation of the sensor at different glycerol concentrations (FWHM represents full width at half maximum of the curves).

	Langmuir	Freundlich
R2	0.9627	0.9552
Kl(L/mg)	0.03	
qmax (mg/g)	64.5	58.55
1/n		0.47

Table 1. Figure-of-merit (FOM) calculation of the sensor at different glycerol concentrations (FWHM represents full width at half maximum of the curves).

Adsorbent Material	Functional Group/Chelating Agent	Adsorption Capacity (mg/g)	Reference
Vinyl-coated magnetic Fe_3O_4 particles	Deep eutectic solvent (DES)	164.20	[22]
Carbon-based nanospheres	Amino asit and copper ion	673.0	[23]
Fe_3O_4@SiO_2 core-shell particle	Zinc	571.3	[24]
Imprinted EGDMA microspheres	N-methacryloylamino folic acid-Nd(III)	361	[25]
Mesoporous silicate aggregates	Copper	4150	[26]
Imprinted bacterial cellulose nanofibers	N-methacryloyl–(L)-histidinemethylester	281.94	[27]
Silk fibroin/gelatin composite hydrogel	-	724.0	[28]
Magnetic Fe_3O_4 composite	Copper (II)-DTPA (Diethylene triamine pentacetate acid)	911.3	[29]
Composite imprinted macroporous hydrogels	Acrylamide and methacrylic acid	3.6	[30]
Magnetic mesoporous rare-earth silicate microspheres	Rrare-earth (Er, Tm, Yb)	304.4	[31]
polyoxometalate (POM)-based hybrid P8W48-APTS	Aminopropyltriethoxysilane	355.0	[32]
SiO_2@Fe_3O_4 hollow spheres	Cu2+, Ni2+, Mg2+	4416.8	[33]
TiO_2-based monodisperse porous microspheres	Iminodiacetic acid-3-glycidoxypropyltrimethoxysilane	137	[34]
CNTs/Fe_3O_4 composite	Cu-Silicate	302.3	[35]
p(HEMA) cryogel	Polydopamine/Ni ions	77.69	present study

pka is about 6 that is nearly close to pH 5, which is the maximum pH value of this study. Furthermore, the coordination of histidine has occurred via three coordination sites via with its amino, carboxyl, the imidazole groups and the pKa values of amino, carboxyl and the imidazole groups of hemoglobin were reported 6.1, 1.9 and 9.1, respectively [19–21]. In the acidic conditions, negatively charged groups of hemoglobin could enhance bind Ni^{+2} ions via electrostatic interactions; so, we fixed pH 5 for the further adsorption studies.

The concentration effect of hemoglobin adsorption was carried out with various hemoglobin concentrations, and the experimental findings were illustrated in Figure 3. As given in Figure 3, the adsorbed of hemoglobin was sharply increased especially in the interval of 0.05-0.1 mg/mL concentrations; however, above the 1.5 mg/mL concentration, the recognition sides of Ni^{+2} and histidine residues probably reached saturation and caused to equilibrate the adsorption of hemoglobin as well.

The suitability of hemoglobin adsorption was investigated by applying the Langmuir and the Freundlich adsorption models. The Langmuir model, the adsorption has occurred with a homogenous and a monolayer surface with the equal binding sites whereas, the Freundlich adsorption refers to define the adsorption occurring a heterogenous surface.

As depicted in Table 1, hemoglobin experimental results were in accordance with the Langmuir adsorption isotherm thanks to the R^2 value and the similarity of q_{max} values of the experimental result and the Langmuir model; thus, hemoglobin adsorption occurred on homogenous surfaces with the equal binding sites.

Reusability studies

Reusability studies are one of the key parameters for adsorption studies and reflect the stability and adsorption capability of the prepared adsorbent. So, in this work, the stability and adsorption capability of PDA(HEMA) adsorbent for hemoglobin adsorption was investigated with reusability studies. For that aim, one of the PDA(HEMA) adsorbent cryogel was selected and tested 5 repetitive adsorption-desorption-regeneration cycles and the results of this parameter was illustrated in Figure 3D. As depicted in Figure 3D, the PDA(HEMA) adsorbent preserved not only in terms of stability but also adsorption capacity as well.

In Table 2, the comparison results and their adsorption capacities of several adsorbents were given.

Conclusion

Hemoglobin is a vital protein in living organisms, playing a crucial role in various essential functions. Given its significance, the method employed for the isolation or purification of this protein becomes highly critical. In this work, we aimed to purify hemoglobin from the aqueous solution using his-tag affinity chromatography due to the histidine residues of hemoglobin and for that purpose, we first prepared p(HEMA) based cryogel membrane and afterwards, the adsorbent surface was modified with dopamine monomer to generate the catechol groups on their surfaces. Following that, the Ni ions were immobilized PDA surface to interact with the histidine residues of hemoglobin during the purification process by several adsorption parameters.

The optimum pH of hemoglobin purification using PDA was observed at pH 5 value and the adsorbent was adsorbed 77.69 mg of hemoglobin that was comparable with the other adsorbents. The adsorption of hemoglobin suited well the Langmuir adsorption model and hemoglobin adsorption occurred in homogenous layers; furthermore, the prepared adsorbent protected the adsorption performance after the reusability studies, in conclusion, the potential alternative use of the designed cryogel discs was supported and presented through further studies.

References

1. A. Maton, J. Hopkins, C.W. McLaughlin, S. Johnson, M.Q. Warner, D. LaHart, J.D. Wright, Human biology and health, Prentice Hall, 1993.
2. A.N. Al-Shura, Hemoglobin, Advanced Hematology in Integrated Cardiovascular Chinese Medicine, Elsevier, 2020.
3. D. Kumar, Clinical Molecular Medicine, Elsevier, 2020.
4. J.H. Ryu, P.B. Messersmith, H. Lee, Polydopamine Surface Chemistry: A Decade of Discovery, ACS Appl. Mater. Interfaces 10 (2018) 7523–7540.
5. H. Lee, S.M. Dellatore, W.M. Miller, P.B. Messersmith, Mussel-Inspired Surface Chemistry for Multifunctional Coatings, Science 318 (2007) 426–430.
6. H. You, X. Zhang, D. Zhu, C. Yang, P. Chammingkwan, T. Taniike, Advantages of polydopamine coating in the design of ZIF-8-filled thin-film nanocomposite (TFN) membranes for desalination, Colloids Surf. A: Physicochem. Eng. Asp., 629 (2021) 127492.
7. M. Zhang, X. Zhang, X. He, L. Chen, Y. Zhang, A self-assembled polydopamine film on the surface of magnetic nanoparticles for specific capture of protein, Nanoscale 4 (2012) 3141.
8. S. El Yakhlifi, V. Ball, Polydopamine as a stable and functional nanomaterial, Collids Surf. B: Biointerfaces., 186 (2020) 110719.
9. G. Fredi, F. Simon, D. Sychev, I. Melnyk, A. Janke, C. Scheffler, C. Zimmerer, Bioinspired polydopamine coating as an adhesion enhancer between paraffin microcapsules and an epoxy matrix, ACS Omega 5 (2020) 19639–19653.
10. Y. Saylan and A. Denizli, Supermoacroporous composite cryogels in biomedical applications, GELS 5 (2) (2019) 1-20.
11. R.F. Lane, A.T. Hubbard, Differential Double Pulse Voltammetry at Chemically Modified Platinum Electrodes for in vivo Determination of Catecholamines, Anal. Chem., 48 (1976) 1287–1293.
12. I. Perçin, N. Idil, A. Denizli, Molecularly imprinted poly(N-isopropylacrylamide) thermosensitive based cryogel for immunoglobulin G purification, Process Biochem., 80 (2019) 181–189.
13. M. Bakhshpour, A.A. Topcu, N. Bereli, H. Alkan, A. Denizli, Poly(Hydroxyethyl methacrylate) immunoaffinity cryogel column for the purification of human immunoglobulin M, Gels 6 (2020) 1-13.
14. I. Perçin, G. Sener, A.H. Demirçelik, N. Bereli, A. Denizli, Comparison of Two Different Reactive Dye Immobilized Poly(Hydroxyethyl Methacrylate) Cryogel Discs for Purification of Lysozyme, Appl. Biochem. Biotechnol., 175 (2015) 2795–2805.
15. N. Avcibaşi, M. Uygun, M.E. Çorman, S. Akgöl, A. Denizli, Application of supermacroporous monolithic hydrophobic cryogel in capturing of albumin, Appl. Biochem. Biotechnol., 162 (2010) 2232–2243.
16. F. Yilmaz, K. Kose, M.M. Sari, G. Demirel, L. Uzun, A. Denizli, Bioinspired surface modification of poly(2-hydroxyethyl methacrylate) based microbeads via oxidative polymerization of dopamine, Colloids Surf. B: Biointerfaces., 109 (2013) 176–182.
17. J. Yang, K. Ni, D. Wei, Y. Ren, One-step purification and immobilization of his-tagged protein via Ni^{2+}-functionalized Fe_3O_4@polydopamine magnetic nanoparticles, Biotechnol. Bioprocess Eng., 20 (2015) 901–907.
18. G. Bayrak, I. Perçin, A. Kılıç Süloğlu, A. Denizli, Amino acid functionalized macroporous gelatin cryogels: Characterization and effects on cell proliferation, Process Biochem., 110 (2021) 100–109.
19. R.J. Sundberg, Interactions of histidine and other imidazole derivatives with transition metal ions in chemical and biological systems, Chem. Rev., 74 (1974) 471–517.
20. R.N. V. Krishna Deepak, R. Sankararamakrishnan, N–H..N Hydrogen Bonds Involving Histidine Imidazole Nitrogen Atoms: A New Structural Role for Histidine Residues in Proteins, Biochemistry 55 (2016) 3774–3783.
21. G. Zheng, M. Schaefer, M. Karplus, Hemoglobin Bohr Effects: Atomic Origin of the Histidine Residue Contributions, Biochemistry 52 (2013) 8539–8555.
22. W. Xu, Y. Wang, X. Wei, J. Chen, P. Xu, R. Ni, J. Meng, Y. Zhou, Fabrication of magnetic polymers based on deep eutectic solvent for separation of bovine hemoglobin via molecular imprinting technology, Anal. Chim. Acta 1048 (2019) 1–11.
23. X. Chen, J. Chai, X. Yang, F. Chai, M. Tian, Amino acid-immobilized copper ion-modified carbon-based adsorbent for selective adsorption of bovine hemoglobin, J. Chromatogr. A 1680 (2022) 463440.
24. X. Zhang, J. Tan, X. Xu, F. Shi, G. Li, Y. Yang, A coordination polymer based magnetic adsorbent material for hemoglobin isolation from human whole blood, highly selective and recoverable, J. Solid State Chem., 253 (2017) 219–226.
25. G. Canpolat, Molecularly imprinted polymer-based microspheres for selective extraction of hemoglobin from blood serum, Process Biochem., 129 (2023) 86–93.
26. W. Hu, L. Liu, Y. Fan, M. Huang, Facile synthesis of mesoporous copper silicate aggregates for highly selective enrichment of hemoglobin, Microchem. J., 167 (2021) 106256.
27. M. Bakhshpour, E. Tamahkar, M. Andaç, A. Denizli, Surface imprinted bacterial cellulose nanofibers for hemoglobin purification, Colloids Surf. B: Biointerfaces 158 (2017) 453–459.
28. J.P. Fan, Z.T. Lai, D.Y. Mao, C.F. Xie, H.P. Chen, H.L. Peng, Preparation of a silk fibroin/gelatin composite hydrogel for high-selectively adsorbing bovine hemoglobin, Colloids Surfaces A Physicochem. Eng. Asp., 660 (2023) 130869.
29. J. Wang, H. Guan, Q. Liang, M. Ding, Construction of copper (II) affinity- DTPA functionalized magnetic composite for efficient adsorption and specific separation of bovine hemoglobin from bovine serum, Compos. Part B Eng., 198 (2020) 108248.
30. S. Hajizadeh, K. Kettisen, M. Gram, L. Bülow, L. Ye, Composite imprinted macroporous hydrogels for haemoglobin purification from cell homogenate, J. Chromatogr. A 1534 (2018) 22–31.
31. J. Wang, S. Tan, Q. Liang, H. Guan, Q. Han, M. Ding, Selective separation of bovine hemoglobin using magnetic mesoporous rare-earth silicate microspheres, Talanta 204 (2019) 792–801.
32. D.D. Zhang, L.L. Hu, Q. Chen, X.W. Chen, J.H. Wang, Selective adsorption of hemoglobin with polyoxometalate-derived hybrid by solidification of super-lacunary phosphotungstate polyoxoanions, Talanta 159 (2016) 23–28.
33. Y. Zhang, M. Zhang, J. Yang, L. Ding, J. Zheng, J. Xu, Facile synthesis of sea urchin-like magnetic copper silicate hollow spheres for efficient removal of hemoglobin in human blood, J. Alloys Compd., 695 (2017) 3256–3266.

34. Ç. Kip, R.B. Tosun, S. Alpaslan, İ. Koçer, E. Çelik, A. Tuncel, Ni(II)-decorated porous titania microspheres as a stationary phase for column chromatography applications: Highly selective purification of hemoglobin from human blood, Talanta 200 (2019) 100–106.

35. M. Zhang, Y. Wang, Y. Zhang, L. Ding, J. Zheng, J. Xu, Preparation of magnetic carbon nanotubes with hierarchical copper silicate nanostructure for efficient adsorption and removal of hemoglobin, Appl. Surf. Sci., 375 (2016) 154–161.