



INFLUENCE OF MONOMERS ON THE PROPERTIES OF EPITOPE IMPRINTED SOL-GEL ON SILICA SURFACE FOR HEPARIN ADSORPTION

(Pengaruh Monomer terhadap Sifat-Sifat Sol-Gel Bercetak Epitop pada Permukaan Silika untuk Penyerapan Heparin)

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Abstract

Heparin is a well-known anticoagulant drug commonly utilized in medical practice. Nevertheless, foreign impurities may contaminate heparin extract; thus, molecular imprinting technology is introduced to purify and separate the anticoagulant. In this study, an epitope heparin imprinted polymer was prepared using low molecular weight heparin (LMWH) as a template model in three different functional monomers: (3-aminopropyl) trimethoxysilane (APTMS), (3-mercaptopropyl) trimethoxysilane (MCPTMS), and 3-(2-aminoethylamino) propyltrimethoxysilane (AEPTES). Meanwhile, tetraethoxysilane (TEOS) was used as a cross-linker for the sol-gel process. The prepared epitope heparin imprinted polymer was characterized using Fourier transform infrared spectroscopy (FTIR) and scanning electron microscope (SEM). The AEPTES-MIP demonstrated the best imprinting effect towards heparin (IF = 3.10) due to the high stability and dual functional group created strong hydrogen bonding, in comparison to APTMS-MIP (IF = 2.18) and MCPTMS-MIP (IF = 0.13). Moreover, the epitope heparin imprinted polymer exhibited higher selectivity towards macromolecule heparin than LMWH, indicating the successful identification and selection of macromolecule fragments to determine the biological activities.

Keywords: epitope imprinted, heparin, molecular imprinted polymer, sol-gel process

Abstrak

Heparin adalah ubat anti-gumpalan yang terkenal dan biasa digunakan dalam amalan perubatan. Walau bagaimanapun, ekstrak heparin berisiko tinggi bercampur dengan bahan cemar asing. Oleh itu, teknologi pencetakan molekul diperkenalkan untuk penulenan dan pengasingan heparin. Di sini, polimer bercetak epitop heparin telah dihasilkan dengan menggunakan berat heparin molekul rendah (LMWH) sebagai model templat dalam tiga monomer berfungsi berbeza yang dikaji iaitu (3-aminopropil) trimetoksisilana (APTMS), (3-merkaptopropil) trimetoksisilana (MCPTMS) dan 3-(2-aminoetilamino) propiltrimetoksisilana (AEPTES) manakala, Tetrartoksisilana (TEOS) digunakan sebagai penyambung silang di dalam proses sol-gel. Polimer tercetak epitop heparin yang disediakan telah dianalisis menggunakan spektroskopi inframerah transformasi Fourier (FTIR) dan mikroskop

elektron pengimbasan (SEM). AEAPTES-MIP mempunyai kesan cetakan terbaik terhadap heparin dengan nilai faktor pencetakan tertinggi (IF=3.10) berbanding APTMS-MIP (IF=2.18) dan MCPTMS-MIP (IF=0.13) kerana kestabilannya yang tinggi dan kumpulan dwi berfungsi yang diwujudkan mengukuhkan ikatan hidrogen. Selain itu, polimer tercetak epitope heparin menunjukkan selektiviti tinggi terhadap makromolekul heparin berbanding LMWH. Ini membuktikan bahawa MIP berjaya digunakan sebagai pengenalanpastian dan pemilihan serpihan makromolekul untuk menentukan peranan aktiviti biologinya.

Kata kunci: epitop tercetak, heparin, polimer molekul tercetak, proses sol-gel

Introduction

Heparin is a linear polysaccharide of repeating disaccharide units comprising 1,4-linkage of glucosamine and uronic acid. This drug is a popular anticoagulant for diseases such as deep vein thrombosis (DVT) and pulmonary embolism (PE), extracorporeal (hemodialysis), and surgery [1]. Heparin is derived from animal sources [2], mainly pig intestines and bovine lungs, leading to a high risk of contamination by foreign impurities [3]. Thus, an effective extraction and purification method is essential to produce pure heparin by removing impurities. Heparin extraction and purification are currently performed using capillary electrophoresis, affinity chromatography, and polyacrylamide gel electrophoresis (PAGE). Nevertheless, these techniques demonstrated multiple drawbacks, including time consuming, high maintenance, and regeneration [4, 5]. Therefore, molecular imprinting technology has been proposed as a sustainable heparin extraction and purification alternative.

Molecular imprinting is a technology that produces artificial recognition sites in polymeric matrices complementary to the template in size, shape, and chemical functionality. The functional monomer is polymerized with a template in the presence of a cross-linker. Once the template is removed, cavities are imprinted on the molecular imprinting polymer (MIP). The MIP is produced as a highly selective polymer towards a particular template [6]. Furthermore, MIP manufacturing is simple and cost-effective, with high chemical and physical robustness, and highly resistant in harsh environments, such as drastic changes in temperature, pressure, and pH [7]. Therefore, MIP is extensively applied for separation and purification. For example, MIP can separate small compounds such as enzyme lysozyme from aqueous egg white [8], Congo red dye from water [9] and purify large macromolecular

compounds such as protein [11], DNA acrydite [12] and acetoside from *Cistanche tubulosa* [10].

Several methods are utilized for MIP preparation, including radical and hydro-gel processes. Nonetheless, the sol-gel method is relatively superior due to the simplicity, time efficiency, and hydrophilicity of the protein template [13]. The sol-gel process involves the condensation of a colloidal solution (sol) to form a cross-linked solid (gel) in the presence of solvent and catalyst [14]. Previously, multiple MIPs had been developed using various techniques (bulk, precipitation, and surface imprinting), but these methods share similar weaknesses, such as poor and less selective adsorption performance in recapturing large molecule structures (e.g. protein). Therefore, epitope surface imprinting techniques are introduced due to the low cost, high template utilization efficiency, and high selectivity towards the target molecule, particularly large molecules. This technique involves the imprinting of the epitope template at the material surface. The epitope template functions as a pseudo-template to replace the macromolecule and polymerizes with monomers in the presence of a cross-linker to produce MIP films. Resultantly, highly selective cavities are formed in the MIP film once the epitope template is removed, similar to the normal macromolecule MIP film. Thus, a highly selective MIP is produced to recognize and bind to the targeted macromolecule [15].

In this study, the epitope imprinted polymer for capturing large molecule heparin was developed. The low molecular weight heparin (LMWH) was used as an epitope template to capture the large heparin macromolecule, while tetraethyl orthosilicate (TEOS) acted as the cross-linker for the epitope surface imprinting in the sol-gel process. Three functional monomers, (3-aminopropyl)trimethoxysilane (APTMS), (3-mercaptopropyl)trimethoxysilane

(MCPTMS), and 3-(2-aminoethylamino) propyl]trimethoxysilane (AEAPTMS), were selected to investigate the interaction of heparin adsorption in this study.

Materials and Methods

Materials

The silica-gel (230 – 400 mesh) was purchased from Merck (Germany). The heparin, 98% (for medical purposes), was purchased from AinMedicare (Malaysia). The commercial LMWH, 98%, was purchased from Easybuyer Ltd. (Shanghai, China). The three functional monomers, APTMS, MCPTMS, and AEAPTMS, were purchased from Sigma-Aldrich (Germany). Meanwhile, the cross-linker, TEOS, was purchased from Acros Organics (Germany). Hydrochloric Acid (HCl), 37%, was purchased from Fisher Scientific (Germany), while ethanol, 95%, and methanol, 99.98%, were obtained from HmbG (Germany).

Activation of silica gel

The silica gel was first activated to increase the silanol group and remove metal oxide and nitrogen-containing impurities [16]. Subsequently, the silica gel was dried to remove and reduce the silica gel particle size, thus, increasing the surface area and adsorption capacity [17]. The silica gel (15 g) was then immersed in 120 mL of concentrated 6 M hydrochloric acid (HCl) and refluxed for 10 h with constant stirring. The resulting mixture was filtered, washed thoroughly with deionized water, and centrifuged at 4000 rpm for 10 min. The washing steps were repeated until the mixture became neutral (pH 7.0). Lastly, the silica gel was oven dried at 70 °C overnight.

Preparation of epitope heparin imprinted polymer by sol-gel process

A total of 0.45 g LMWH (0.05 mmol) was first dissolved in 40 mL of distilled water before adding 0.0524 of APTMS (0.1 mmol) and sonicated for 5 min. Subsequently, 0.706 g TEOS (1.8048 mmol) was added into the solution and sonicated for another 5 min. Activated silica-gel (4 g) and 0.4 mL of 0.012 M HCl were added sequentially while stirring at 100 rpm, and the suspension was later polymerized at 40 °C for 24 h.

The imprinted polymer was filtered, washed with 50 mL ethanol, and neutralized with 50 mL of distilled water thrice. Similarly, the non-imprinted polymer (NIP) was prepared using the same protocol but without the template LMWH. The process was repeated using the two remaining monomers: 0.1 mmol MCPTMS and 0.1 mmol AEAPTMS. Once completed, the template LMWH was extracted from the imprinted polymer via sonication for 15 min with a 50 mL mixture of methanol and hydrochloric acid (10% in water) at a ratio of 1:1. Sonication was performed for another 15 minutes with 50 mL of methanol followed by 50 mL of water. The washing steps were repeated thrice. The supernatant from every washing was tested using UV-Vis spectroscopy at 230 nm, and the washing continued until no LMWH was detected. Finally, the MIP was obtained through filtration and dried at 80 °C overnight. A similar protocol was repeated for other imprinted polymers with different monomers and NIP.

Batch rebinding experiment

The MIP and NIP (50 mg) of different monomers were placed in 15 mL centrifuge tubes, followed by adding 5 mL of 0.25 mg/mL heparin or 0.25 mg/mL LMWH solution. Subsequently, the centrifuge tubes were placed horizontally on the shaker for 4 hours at 100 rpm under room temperature. In the adsorption binding test, different concentrations of the prepared mixtures were utilized (0.0625 mg/mL to 1.0 mg/mL). Similarly, the kinetic binding analysis was performed at a constant concentration of heparin of 0.50 mg/mL, except the time measurement was evaluated from 10 min to 240 min. Once the binding has completed, the tubes were centrifuged at 4000 rpm for 10 minutes to remove the imprinted residual from the supernatant. The supernatant was then filtered from the imprinted polymers using a nylon syringe filter (0.45 µm). The LMWH and heparin concentrations in the supernatant were measured by UV-Vis spectrometer at 230 nm or 202 nm, respectively.

Characterization of the Imprinted Polymers

A scanning electron microscope (SEM) was used to study the morphology and texture of the imprinted polymers. The MIP, NIP, and silica gel were coated with platinum for 90 seconds before being observed under

high vacuum conditions at 5 kV and 3 mA current in SEM. The MIP and NIP were examined at 1000 $\times$, 5000 $\times$, and 10,000 $\times$ magnifications. The MIP, NIP, and silica gel were also analyzed using the Perkin Elmer Spectrum 100 Series Fourier-transform infrared (FT-IR) spectroscopy using the KBr pellet method. The polymer (100 mg) and 200 mg of KBr powder were mixed using mortar and pestle and pressed for 2 min at 8 kPa to form transparent pellets. Finally, the pellets were placed in the FT-IR sample holder and observed at wavenumbers between 400 and 4000 cm^{-1}

Results and Discussion

FTIR Characterization of epitope heparin imprinted polymer

The FT-IR spectroscopy was used to prove the presence of amine-functional silane in the developed polymer. Figure 1 illustrates the FT-IR spectra of silica gel, APTMS-MIP, MCPTMS-MIP, AEAPTES-MIP, and APTMS-NIP. Spectra that included silica gel, MIP, and NIP exhibited similar peaks. The strong peaks at 3234.61 cm^{-1} to 3436.78 cm^{-1} corresponded to OH-group vibrations, resulting from the moisture absorbed by the imprinted polymer. Meanwhile, the peaks at 1631.92 cm^{-1} to 1671.75 cm^{-1} indicated the C-C stretching vibration of alkane. The peaks from 1022.38 cm^{-1} to 1100.75 cm^{-1} are associated with the Si-O-Si stretching. The Si-O vibration was identified at the peaks between 747.30 cm^{-1} and 806.36 cm^{-1} .

The developed polymers demonstrated unique FTIR spectra; small peaks were detected at 1410.37 cm^{-1} for APTMS-MIP, 1460.53 cm^{-1} for MCPTMS-MIP, and 1444.48 cm^{-1} for AEAPTES-MIP, representing the N-H peaks of primary amine. In addition, the N-H peaks of secondary amine were identified in AEAPTES-MIP at 1560.87 cm^{-1} . The peak of the S-H thiol group in MCTMS-MIP was observed at 2683.61 cm^{-1} , which slightly deviated from the literature (2600 cm^{-1} – 2550 cm^{-1}). These peaks indicated that the monomers had been imprinted on the silica support, hence, the

successful production of epitope heparin imprinting polymer compared to silica gel.

Previously, Eskandarloo [18] synthesized (3-aminopropyl) triethoxysilane (3-APTES)-functionalized halloysite nanotubes (HNTs) by using heparin as a template to enhance the capacity of MIP binding selectivity. In contrast, the current study utilized LMWH as a template to capture large heparin, thus, known as the epitope imprinted technique. Moreover, the three types of silane groups selected for this study belonged to the trimethoxysilane group, contrary to the triethylxysilane group detected at 2900 cm^{-1} used by - Eskandarloo. The study also claimed that small peaks were detected at 1476 cm^{-1} and 2928 cm^{-1} , represented by the C-N and N-H stretch, which was absent in the presence of a silane coupling agent.

SEM characterization of epitope heparin imprinted polymer

The surface morphology of silica, epitope heparin imprinted polymer, and the non-imprinted polymer was studied using SEM at 5000 $\times$ magnification (see Figure 2). The silica gel [Figure 2(a)] exhibited a highly rough surface compared to other epitope imprinting and non-imprinting heparin [Figure 2 (b) - (e)], owing to the abrasive nature of silica particles. Furthermore, the imprinting polymers, APTMS-MIP [Figure 2 (b)], MCPTMS-MIP [Figure 2 (c)], and AEAPTES-MIP [Figure 2 (d)] demonstrated porous surfaces, representing the cavities resulting from the template removal. These pores can speed up the adsorption process towards the template and improve adsorption kinetic than NIP. Apart from that, this observation indicated that the epitope heparin had been imprinted on the silica gel, hence, the successful preparation of the epitope heparin polymer [19]. Conversely, the non-imprinting polymer in Figure 2 (e) manifested a clear, smooth surface without forming three-dimensional pores due to the non-template polymerization.

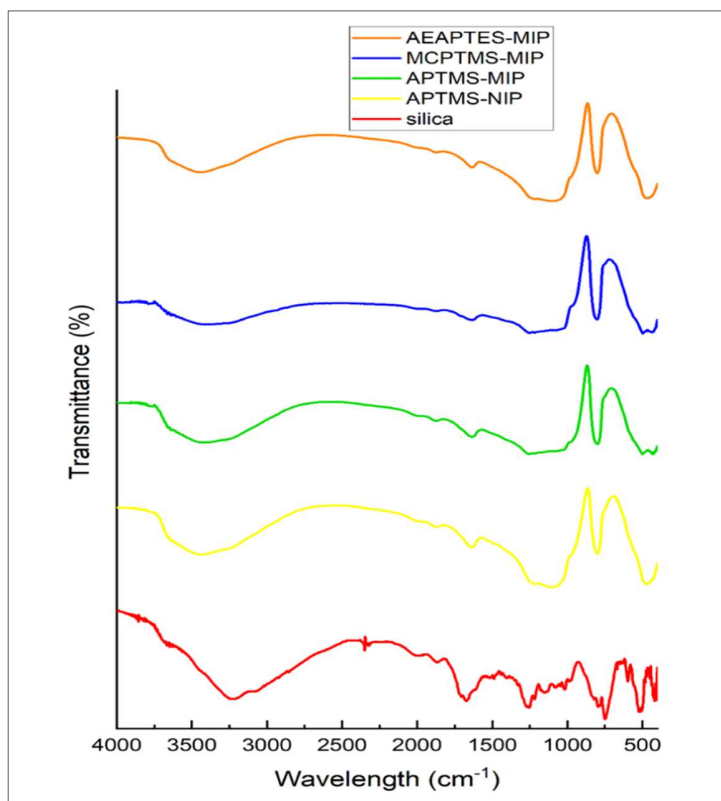


Figure 1. The FT-IR spectra of silica gel (red), APTMS-NIP (yellow), APTMS-MIP (green), MCPTMS-MIP (blue), and AEAPTES-NIP (orange)

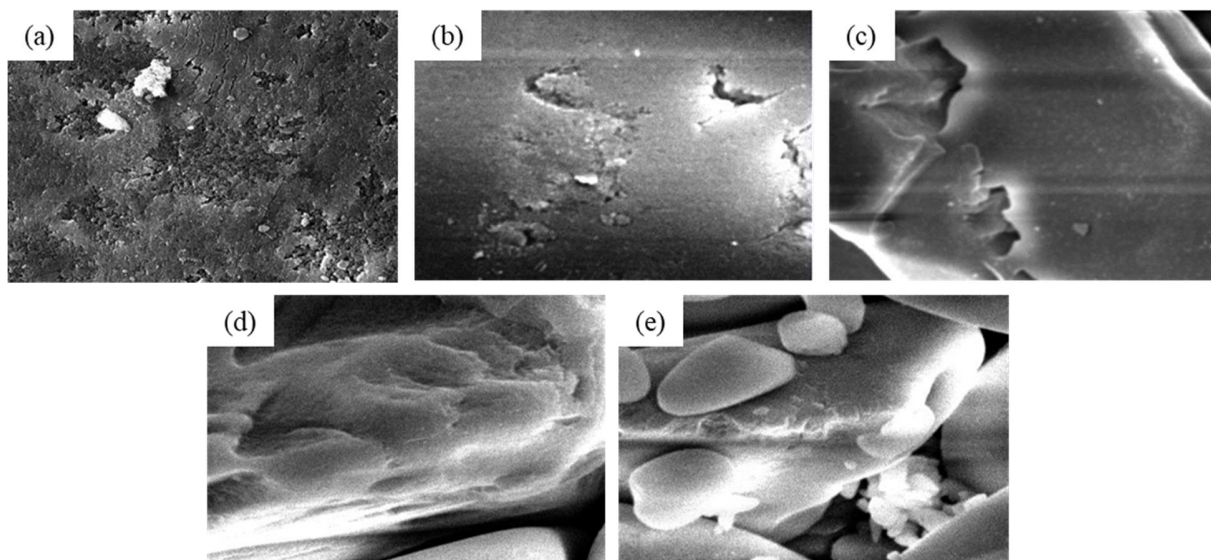


Figure 2. The SEM images of silica (a) APTMS-MIP, (b) MCPTMS-MIP, (c) AEAPTES-MIP, (d) and (e) NIP at 5000x magnification

Gossypol imprinted on silica surfaces via sol-gel surface imprinting [20] exhibited similar findings to the present study. The SEM images of gossypol-MIP illustrated rougher surfaces than gossypol-NIP and silica, indicating a successfully imprinted polymer. In addition, the silica gel surface was distinctly smoother than gossypol-MIP and gossypol-NIP.

Interactions between the functional monomer with heparin and LMWH

The present study tested three monomers, APTMS, MCPTMS, and AEAPTMS, to identify the best functional monomer for the heparin imprinted polymer. The selectivity of epitope heparin imprinted polymer with different monomers was represented as an imprinting factor (IF). The comparison graphs of different monomers are plotted in Figure 3 for heparin rebinding and Figure 4 for LMWH rebinding. Generally, the monomers recorded higher IF values for heparin rebinding than LMWH rebinding, with the highest binding capacity of 23 mg/g for heparin and 9 mg/g for LMWH. The epitope MIP binding of macromolecule heparin was double that of LMWH, indicating higher adsorption towards the former than the latter. Thus, the findings supported epitope MIP utilization to capture macromolecule heparin. Similarly, Gupta et al. [21] reported that epitope-imprinted iron-binding MIP for detecting fbpA protein on *Neisseria meningitidis* bacteria demonstrated high selectivity towards the template fbpA protein (IF = 12.27 ngmL⁻¹). The protein epitope polymer was synthesized using the amino sequence fbpA epitope as the template. This finding suggested that the small epitope molecule could recognize macromolecules. Apart from that, Yoshimi et al. [22] used epitope surface imprinting heparin-imprinted nanoparticles for grafting on unfractionated

heparin (UFH) or LMWH-immobilized glass beads, indicating that the epitope heparin imprinting polymer could capture macromolecule heparin by epitope surface imprinting.

Table 1 shows that AEAPTMS recorded the highest IF values for heparin and LMWH rebinding, followed by APTMS and MCPTMS. Therefore, heparin and LMWH selectivity were the highest in AEAPTMS, followed by APTMS and MCPTMS. This observation could be explained by the presence of double amine groups in AEAPTMS; the primary and secondary amine established stable amine-functionalized surfaces. Resultantly, the interactions between the functional monomer and sulphate group in macromolecule heparin by hydrogen bonding increased in AEAPTMS compared to APTMS with a single amine group. Meanwhile, the sulphhydryl functional group (SH-group) in MCPTMS exhibited low interaction with macromolecule heparin. An earlier study by Zhu et al. [23] reported that AEAPTMS had the highest thickness than other functional silanes, such as 3-aminopropyltriethoxysilane (APTES), APTMS, N-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AEAPTMS), and N-(6-aminohexyl)aminomethyltriethoxysilane (AHAMTES). Moreover, AEAPTMS is a more sterically hindrance of intramolecularly catalyzed hydrolysis, potentially improving the hydrolytic stability. As AEAPTMS has low sensitivity to water, the polymer can generate a more reproducible silane group. Lastly, AEAPTMS possess a unique structure that allows the formation of a cyclic intermediate through the coordination between the secondary amine group and the silicon atom.

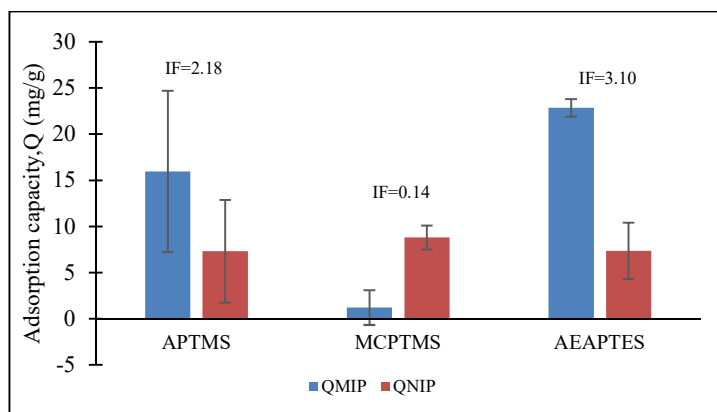


Figure 3. Heparin rebinding of different monomers

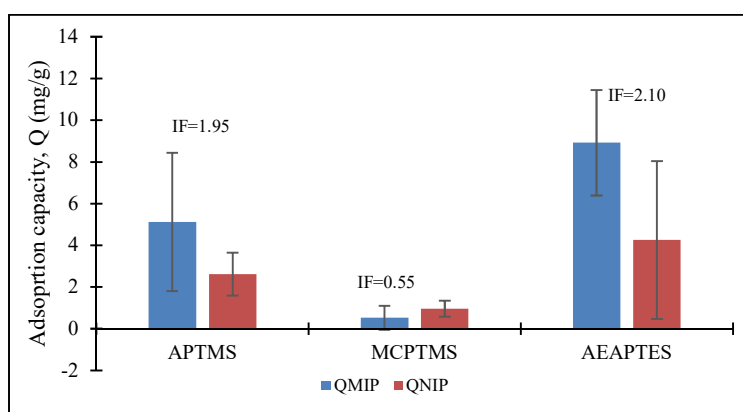


Figure 4. The LMWH rebinding of different monomers

Table 1. Heparin and LMWH rebinding performance of epitope heparin imprinted polymer with different monomers

Sample	Heparin			LMWH		
	Q _{MIP}	Q _{NIP}	IF	Q _{MIP}	Q _{NIP}	IF
APTMS	15.965	7.311	2.18	5.121	2.624	1.95
MCPTMS	1.203	8.789	0.13	0.529	0.96	0.55
AEAPTES	22.843	7.357	3.10	8.919	4.257	2.09

Q_{MIP} = MIP binding capacity, Q_{NIP} = NIP binding capacity, and IF = Imprinting factor

Adsorption and kinetic binding capacity towards heparin

Figure 5 shows the adsorption capacities of AEAPTES-MIP towards different concentrations of heparin (0.062 mg/mL - 1.0 mg/mL). There was a surge in AEAPTES-MIP and AEAPTES-NIP adsorption capacities when the heparin concentration increased from 0.0625 mg/mL to 0.5 mg/mL and remained constant thereafter. As the heparin concentration (target) increased, more heparin was available to adhere to the binding sites, increasing

the heparin adsorption and binding capacities. The polymer became saturated at the equilibrium point (0.5mg/mL) as all the binding sites were occupied by heparin [24]. Based on Figure 5, AEAPTES-NIP adsorption capacity was slightly lower than AEAPTES-MIP. Meanwhile, the highest binding capacities for AEAPTES-MIP and AEAPTES-NIP were 38.19 mg/g and 35.51 mg/g, respectively. This outcome was aligned with a study by Dai et al.[22], which recorded similar adsorption capacities for the structural analogues of

AEAPTES-MIP and AEAPTES-NIP. Conversely, NIP could not produce proper binding sites that are highly complementary to the target due to the absence of a

template, resulting in non-specific adsorption. Therefore, NIP adsorbed less template molecule than MIP, leading to lower adsorption capacities.

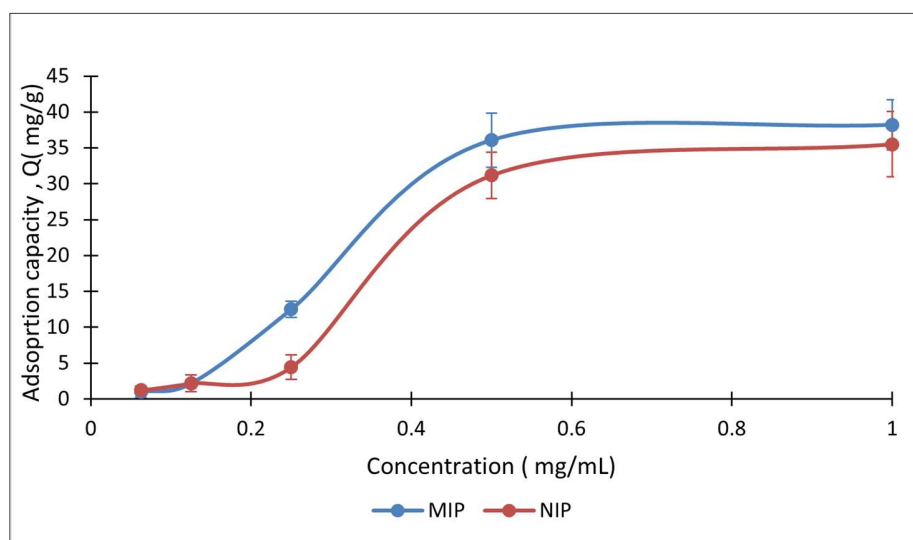


Figure 5. Batch binding test of heparin towards AEAPTES-MIP and AEAPTES-NIP

The AEAPTES-MIP and AEAPTES-NIP adsorption behaviors were determined using Langmuir and Freundlich, isotherm models. The AEAPTES-MIP is best suited to Freundlich isotherm with a linear regression coefficient (R^2) value of 0.8968. This outcome indicated that the heparin adsorption was multilayered and reversible, and the heat adsorption distribution was non-uniform on the AEAPTES-MIP heterogeneous surface. Meanwhile, AEAPTES-NIP followed the Langmuir isotherm model with an R^2 value of 0.9375. This finding suggested the reversible and energetically equivalent heparin adsorption at the homogenous site of the AEAPTES-NIP monolayer surface.

Figure 6 details the kinetic binding of heparin adsorption

at 0.5mg/mL onto AEAPTES-MIP and AEAPTES-NIP between 10 to 240 minutes. The polymers adsorption capacities spiked during the first 10 to 120 minutes and decreased gradually until equilibrium was achieved. The rapid initial heparin adsorption could be explained by the abundance of polymer binding sites exposed to the target, which continued to rise with time. At 60 minutes, the binding sites became saturated, and resistance occurred between heparin and the polymer surface, making it harder to retain heparin in empty binding sites. Consequently, the adsorption rates declined and reached adsorption equilibrium [25]. The AEAPTES-NIP recorded a slightly lower adsorption capacity than AEAPTES-MIP, with the highest binding capacity of 17.1556 mg/g for the former and 18.4478 mg/g the latter.

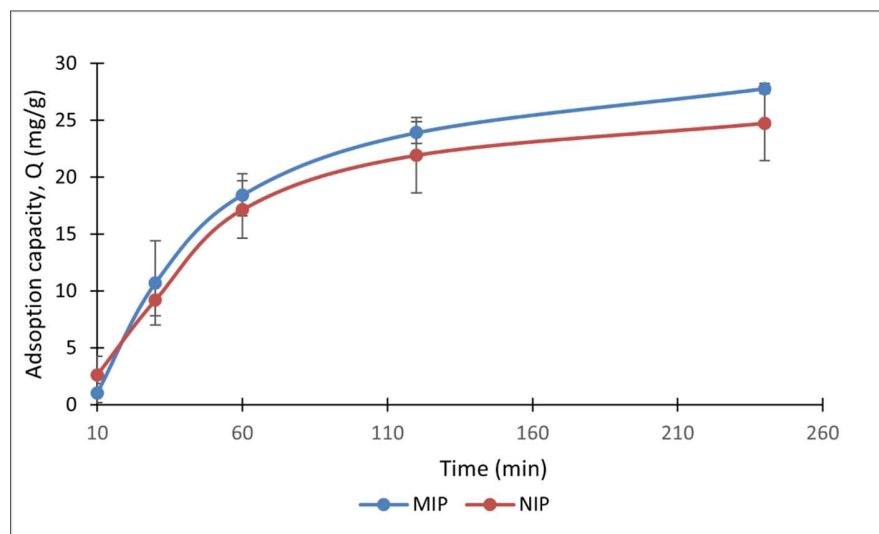


Figure 6. Kinetic binding analysis of heparin on AEAPTES-MIP and AEAPTES-NIP

The AEAPTES-NIP could not bind properly to a highly complementary target without a template, resulting in non-specific adsorption. Consequently, NIP adsorbed less template molecule than MIP, reducing adsorption capacities [26]. Furthermore, AEAPTES-MIP and AEAPTES-NIP were best suited to the pseudo-first-order with R^2 values of 0.9996 and 0.9870, respectively. These results indicated that the rate of change of heparin uptake with time is directly proportional to the difference in concentration of saturation and the amount of heparin uptake with time.

Conclusion

The prepared epitope heparin MIP samples were characterized using FT-IR and SEM in this study. The FT-IR results identified unique functional groups in the epitope heparin polymers: 1) amine group in APTMS-MIP, 2) thiol group in MCPTMS-MIP and 3) secondary amine group in AEAPTES-MIP. Meanwhile, the SEM findings indicated that all MIP exhibited rough surfaces compared to NIP, thus, confirming that the functional monomer and epitope heparin were successfully imprinted on the silica gel. Among the three selected monomers, AEAPTES-MIP demonstrated the best imprinting effect toward heparin (IF = 3.10). This functional monomer exhibited high stability and dual functional groups to create strong hydrogen bonding. Furthermore, the produced epitope heparin was proven

to have high adsorption and specificity towards the macromolecule heparin template due to the higher adsorption toward heparin than LMWH. In conclusion, this study demonstrated that MIP could be synthesized using simple and fast methods, such as the proposed epitope surface imprinting and sol-gel method. These approaches can overcome the limitations of MIP and produce high selectivity heparin imprinted polymers.

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References

1. Haqqani, O. P., Iafrati, M. D. and Freedman, J. E. (2013). Pharmacology of antithrombotic drugs. In vascular medicine: A companion to Braunwald's heart disease: Second Edition. Elsevier Inc, Netherlands: pp.106-107
2. Bolten, S. N., Rinas, U. and Scheper, T. (2018). Heparin: role in protein purification and substitution with animal-component free material. *Applied Microbiology Biotechnology*, 102(20): 8647-8660.

3. Zhu, Y., Zhang, F. and Linhardt, R. J. (2019). Heparin contamination and issues related to raw materials and controls. Springer International Publishing, New York: pp.191-206.
4. Batra, S., Sahi, N., Mikulcik, K., Shockley, H., Turner, C., Laux, Z., Badwaik, V. D., Conte, E. and Rajalingam, D. (2011). Efficient and inexpensive method for purification of heparin binding proteins. *Journal Chromatography B Analytical Technology Biomedicine Life Sciences*, 879(24): 2437-2442.
5. Volpi, N., Maccari, F., Suwan, J. and Linhardt, R. J. (2012). Electrophoresis for the analysis of heparin purity and quality. *Electrophoresis*, 33(11): 1531-1537.
6. Ertürk, G. and Mattiasson, B. (2017). Molecular imprinting techniques used for the preparation of biosensors. *Sensors (Switzerland)*, 17(2): 1-17.
7. Vasapollo, G., Sole, R. Del, Mergola, L., Lazzoi, M. R., Scardino, A., Scorrano, S. and Mele, G. (2011). Molecularly imprinted polymers: Present and future prospective. *International Journal of Molecular Sciences*, 12(9): 5908-5945.
8. Odabaşı, M., Say, R. and Denizli, A. (2007). Molecular imprinted particles for lysozyme purification. *Materials Science and Engineering C*, 27(1): 90-99.
9. Shafqat, S. R., Bhawani, S. A., Bakhtiar, S. and Ibrahim, M. N. M. (2020). Synthesis of molecularly imprinted polymer for removal of Congo red. *BMC Chemistry*, 14(1): 1-15.
10. Zhao, X., Pei, W., Guo, R. and Li, X. (2020). Selective adsorption and purification of the acteoside in *cistanche tubulosa* by molecularly imprinted polymers. *Frontiers in Chemistry*, 7(January): 1-13.
11. Zhang, C., Wang, Y., Zhou, Y., Guo, J. and Liu, Y. (2014). Silica-based surface molecular imprinting for recognition and separation of lysozymes. *Analytical Methods*, 6(21): 8584-8591.
12. Dong, Z., Tan, J., Pinelo, M., Zhang, H., Wan, Y. and Luo, J. (2022). Engineering mussel-inspired coating on membranes for green enzyme immobilization and hyperstable reuse. *Industrial & Engineering Chemistry Research*, 61(15): 5042-5053.
13. Chen, L., Wang, X., Lu, W., Wu, X. and Li, J. (2016). Molecular imprinting: Perspectives and applications. *Chemistry Society Review*, 45(8): 2137-2211.
14. Esposito, S. (2019). Traditional sol-gel chemistry as a powerful tool for the preparation of supported metal and metal oxide catalysts. *Material (Basel)*, 12(4): 1-25.
15. Yang, K., Li, S., Liu, L., Chen, Y., Zhou, W., Pei, J., Liang, Z., Zhang, L. and Zhang, Y. (2019). Epitope imprinting technology: Progress, applications, and perspectives toward artificial antibodies. *Advanced Materials*, 31(50): 1902048.
16. Budiman, H., Sri H. K. F. and Setiawan, A. H. (2009). Preparation of silica modified with 2-mercaptoimidazole and its sorption properties of chromium(III). *E-Journal of Chemistry*, 6(1): 141-150.
17. Shi, L., Liang, L., Wang, F., Ma, J. and Sun, J. (2014). Polycondensation of guanidine hydrochloride into a graphitic carbon nitride semiconductor with a large surface area as a visible light photocatalyst. *Catalysis Science and Technology*, 4(9): 3235-3243.
18. Eskandarloo, H., Arshadi, M., Enayati, M. and Abbaspourrad, A. (2018). Highly efficient recovery of heparin using a green and low-cost quaternary ammonium functionalized halloysite nanotube. *ACS Sustainable Chemistry and Engineering*, 6(11): 15349-15360.
19. Wang, L., Zhi, K., Zhang, Y., Liu, Y., Zhang, L., Yasin, A. and Lin, Q. (2019). Molecularly imprinted polymers for gossypol via sol-gel, bulk, and surface layer imprinting-A comparative study. *Polymers (Basel)*, 11(4):1-18.
20. Zhi, K., Wang, L., Zhang, Y., Jiang, Y., Zhang, L. and Yasin, A. (2018). Influence of size and shape of silica supports on the sol-gel surface molecularly imprinted polymers for selective adsorption of gossypol. *Materials*, 11(5): 1-16.
21. Gupta, N., Singh, R. S., Shah, K., Prasad, R. and Singh, M. (2018). Epitope imprinting of iron binding protein of *Neisseria meningitidis* bacteria through multiple monomers imprinting approach. *Journal of Molecular Recognition*, 31(7): 1-9.

22. Yoshimi, Y., Oino, D., Ohira, H., Muguruma, H., Moczko, E. and Piletsky, S. A. (2019). Size of heparin-imprinted nanoparticles reflects the matched interactions with the target molecule. *Sensors (Switzerland)*, 19(10): 1-11.
23. Zhu, M., Lerum, M. Z., & Chen, W. (2012). How to prepare reproducible, homogeneous, and hydrolytically stable aminosilane-derived layers on silica. *Langmuir*, 28(1): 416-423.
24. Baydemir, G., & Denizli, A. (2015). Heparin removal from human plasma using molecular imprinted cryogels. *Artificial Cells, Nanomedicine and Biotechnology*, 43(6): 403-412.
25. Öter, Ç. and Zorer, Ö. S. (2021). Molecularly imprinted polymer synthesis and selective solid phase extraction applications for the detection of ziram, a dithiocarbamate fungicide. *Chemical Engineering Journal Advances*, 7(4): 100118.
26. Dai, C., Zhang, J., Zhang, Y., Zhou, X. and Liu, S. (2013). Application of molecularly imprinted polymers to selective removal of clofibric acid from water. *PloS One*, 8(10): 78167.