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Synthesis of Membrane Structures Containing Nanocomposites for Immunoglobulin G (IgG) Adsorption

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ABSTRACT: The discovery of markers of many diseases, such as cancer, inflammation, diabetes, cardiovascular diseases and other autoimmune diseases, can be made through the characterization of serum protein or peptide. Proteomics, the comprehensive study of proteins on a large scale, is poised to significantly enhance our comprehension of gene functionality in the post-genomic age. It entails the quantitative and physical delineation of all proteins within a specific cell line, tissue, bodily fluids, or organism. Our study is aimed to adsorb antibodies from serum with GO-HEMA nanocomposite materials. In this context, GO-HEMA membranes were synthesized via the photopolymerization method, and subsequently, it was observed through scanning electron microscopy (SEM) that the particles exhibited a cylindrical structure. Fourier Transform Infrared Spectroscopy (FTIR) was employed to characterize the inclusion of graphene oxide into the HEMA membrane structure. During IgG binding to GO-HEMA membranes, pH, concentration, and time parameters were examined. As a result, the optimum binding conditions were determined to be pH 5.0, a concentration of 0.5 mg/mL, and an adsorption time of 30 minutes. This study is suitable for highly selective IgG adsorption.

Keywords: *proteomics, antibody removal, composite materials, membrane systems, graphene*

1 INTRODUCTION

Nanotechnology's advancements impact diverse fields such as physics, chemistry, materials science, medicine, and biotechnology. Biosensing, utilizing nanotech, relies on advanced materials and nanostructures. Serum and plasma are vital for disease marker detection, reflecting abnormal conditions in organs or tissues through the serum proteome [1]. Proteomic techniques have emerged to identify marker proteins in serum, crucial for early disease detection, monitoring, and treatment. Recently, various commercial products have been created to eliminate albumin and immunoglobulins from plasma, aiding in protein analysis [2].

Since its discovery, graphene has sparked considerable excitement. Furthermore, graphene oxide (GO) shows advantageous properties for biosensing due to its excellent abilities in biocompatibility, solubility, and selectivity [3]. Among these nanocarbons, the low-cost GO and reduced graphene oxide (rGO) have received more attention due to their large electrochemically

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active surface area, oxygenic functional groups in complexing with metal ions/metal nanoparticles (NPs) (redox probes), and immobilization of active antibodies, and good electrical conductivity [4]. Materials derived from GO are widely applied in electronics, membrane engineering, and medicine due to their remarkable electrical, thermal, mechanical, and optical properties. The hydrophilic nature of GO, along with its easy modification using various molecules through abundant oxygen functionalities, highlights its significant potential in drug delivery and biosensing applications [5]. As an illustration, tumor-targeting agents or GO-based materials functionalized with phospholipid polyethylene glycol (PEG) derivatives are effective anticancer agents [6].

Proteomics entails the quantitative and physical mapping of the entire complement of proteins within a specific cell line, tissue, bodily fluids, or organism [7]. The prevailing experimental methodologies in proteomics predominantly encompass two-dimensional protein electrophoresis (2-DE) for protein separation and mass spectrometry for subsequent protein identification [8]. The potential identification of disease markers across various conditions such as inflammation, diabetes, infection, cancer, cardiovascular diseases, Alzheimer's, and autoimmune diseases has spurred interest in proteomic studies. Consequently, there is increasing focus on comprehensively characterizing individual serum proteins and peptides, leading to numerous efforts to broadly understand the protein composition of human serum [9]. The serum harbors a plethora of over one million distinct proteins and peptides, characterized by a broad dynamic concentration range (i.e., 60-80 mg/mL^{-1}), presenting numerous opportunities and potentially advantageous attributes for proteomic scrutiny [10]. Nonetheless, the substantial protein content in serum poses significant challenges for proteomic analysis due to the remarkably dynamic concentration range of serum proteins, spanning from highly abundant proteins (tens of mg/mL to ~2 mg/mL), such as albumin, immunoglobulins (IgG and IgA), to antitrypsin [11].

Transferrin and haptoglobin have the capability to bind to proteins of exceedingly low abundance, such as the vasoconstrictor peptide endothelin -1. An indispensable factor exacerbating the analytical challenge of characterizing the serum proteome is the fact that approximately 90% of the protein content is constituted by merely 10 proteins [12,13]. Within the remaining 10%, a mere 12 proteins encompass 90% of the residual total. Consequently, only 1% of the complete serum protein content consists of proteins deemed to be in low abundance, thus sparking substantial interest in proteomic inquiries focused on identifying potential biomarkers [14]. The

primary challenge in serum proteome analysis is reducing sample complexity to minimize interference on biomarkers. Blood, serum, and plasma proteomic analysis is hindered by highly abundant proteins like albumin, immunoglobulins (IgG and IgA), antitrypsin, haptoglobin, and transferrin, masking proteins present in lower concentrations [15,16]. The removal of these proteins, which constitute approximately 90% of the total protein, increases the relative concentration of other proteins in small quantities and facilitates their detection [17]. Due to its extensive protein composition, plasma plays a pivotal role in disease diagnosis [18]. Early detection depends on spotting proteins that change with biological processes or diseases, vital for treatment response and detecting cancer recurrence. Yet, plasma analysis is complex due to varied protein concentrations [19]. Yet another challenge encountered in serum protein analysis pertains to the depletion of elevated concentrations of IgG [20]. The elimination of IgG is commonly achieved by immobilizing protein A or protein G onto affinity resins, which possess a binding affinity for the Fc region of IgG. However, specific antibodies can also be employed for this purpose [21,22].

Affinity chromatography is a method based on the specific and reversible adsorption of the target biomolecule onto ligands immobilized on an insoluble support matrix [23]. Biological interactions between ligands and target molecules involve electrostatic, hydrophobic, Van der Waals, and/or hydrogen bonds. Target molecule selection can be achieved by using a competitive ligand or adjusting the pH, ionic strength, or polarity (mobile phase) of the medium [24]. Moreover, the removal of IgG from human plasma is employed in the management of immune disorders. Depletion of plasma proteins can be done using different strategies, but the ultimate goal is to separate high amounts of proteins from low amounts of proteins [25]. The identification of cost-effective and highly efficient methods for the depletion of abundant proteins from blood, serum, and recently plasma has emerged as one of the foremost concerns and holds significant importance [2]. Currently, various removal technologies are available for the removal of albumin and antibodies (IgG) from serum, including ultracentrifugal filtration, dye affinity, immunoaffinity, immobilized metal affinity chromatography (IMAC), and suppression methods [2, 26].

Removal of IgG is usually achieved by protein A/G affinity adsorbents that are attached to the Fc region of IgG, but specific antibodies may also be used. Monoclonal antibodies targeting protein A/G and IgG are obtainable from commercial vendors [27]. Nonetheless, IgG is found in serum at concentrations ranging from 8 to 16 mg mL^{-1},

necessitating substantial quantities of antibodies and/or protein A/G for quantitative removal [28]. The high specificity of bioligands provides excellent selectivity [29]. Despite their high selectivity, adsorbents with protein A/G or antibodies have drawbacks: (i) Cost is often prohibitive. (ii) Immobilizing them in the correct orientation is challenging. (iii) Ligands may leak from the stationary phase, which is unacceptable in clinical practice where high removal of IgG from serum is necessary [30]. Among the materials and methods used for removal, it is seen in the literature that composite systems using nano-membrane systems together are not included [31]. Natural surface properties of nanomaterials (e.g., high surface/volume ratio) combined with membrane system dynamics: easy preparation, high selectivity and stability in harsh conditions, nanomaterials are expected to surpass conventional methods [32]. The main aim of the proteomic analysis of serum and plasma is to obtain the most reliable information for diagnosis and treatment [33].

The aim of this particular analysis is to remove antibodies present in blood, serum, and plasma and to develop new generation nanomembrane composite systems. In pursuit of this objective, an innovative and biocompatible GO-HEMA-based membrane composite system has been developed for the removal of IgG.

2 MATERIAL AND METHOD

2.1 Materials

Graphene oxide (GO), IgG, 2-Hydroxyethyl methacrylate (HEMA), Ethylene glycol dimethacrylate (EGDMA), as an initiator 2,2-Dimethoxy-2-phenylacetophenone (DMPA), Graphene powder were taken from Sigma Aldrich. All the other chemicals utilized in this study, such as Tetrahydrofuran (THF), Sodium hydride (NaH), vaseline, parafilm, Ethanol (C_2H_6O), Potassium permanganate ($KMnO_4$), Hydrogen peroxide (H_2O_2), Sulfuric acid (H_2SO_4), Phosphoric acid (H_3PO_4), Hydrochloric acid (HCl), Nitrogen gas, Potassium persulfate (KPS), Polyvinylalcohol (PVA) are of analytical purity.

FTIR spectra of monomers and polymers were obtained using the FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). Scanning electron microscope (SEM, Philips XL-30S FEG) and Zeta potential measuring device (Malvern) were used. Support was received from Ege University in the implementation of other devices and experiments. Centrifuges (Centrion Scientific Benchtop Centrifuges) were used for washing and settling processes. Precision balance (KERN&Sohn GmbH), pH meter (ISTEK, NeoMet), magnetic stirrer (Dragon lab, MX-F), sonicator for homogeneous mixing of solutions (Lab Companion, UC-10), UV lamp, oven

(Memmert) was used.

2.2 Nanographene Oxide Synthesis

Graphene oxide will be prepared from graphene powder by the improved Hummers method [34]. In short, 2 g of graphite powder, 12 g of potassium permanganate, and 261 mL of acid (235 mL H_2SO_4 + 26 mL H_3PO_4) will be mixed at 50°C for 12 hours in a balloon reactor. This mixture will then be poured onto the frozen mixture containing 260 mL of pure water and 2 mL of 30% H_2O_2. Then the resulting final solution will be centrifuged for 4 hours at 4000 rpm. The precipitated substance will be removed and washed first with 10% HCl solution and then with ethanol and dried overnight at 80°C.

2.3 Synthesis of Nano-Membrane Composite Structures

The polymerization mixture (10 mL) will be prepared by adding and dissolving 2500 µL HEMA and 100 µL EGDMA (as cross-linking agent), 100 mg graphene oxide and DMPA (as initiator). The mixture will be incubated in the bath of sonic for 5 minutes. And it will be cleaned with nitrogen for about 5 minutes and degassed.

The mixture will then be poured into cylindrical glass molds (diameter 10 mm) and the synthesis of composite structures will be carried out by UV-initiated photopolymerization. For this purpose, the mixture shall be exposed to UV light with a wavelength of 1 $mWcm^{-1}$ UV light 365 nm.

After polymerization, the synthesized nano-membrane composite structures will be cut into circular parts using a perforator. It will be washed with distilled water several times to remove reacting monomers, initiators, and other debris and left for orbital mixing for 24 hours. After the washing process, the nano-membrane composite structures will be dried overnight at 37°C [35].

2.4 Optimization of Adsorption Conditions of IgG

Optimization studies were conducted to achieve high levels of IgG binding. For this purpose, graphene oxide (GO) nanoparticle synthesis was performed using the Hummers method [34]. The synthesis of the GO-HEMA nanocomposite structure was carried out

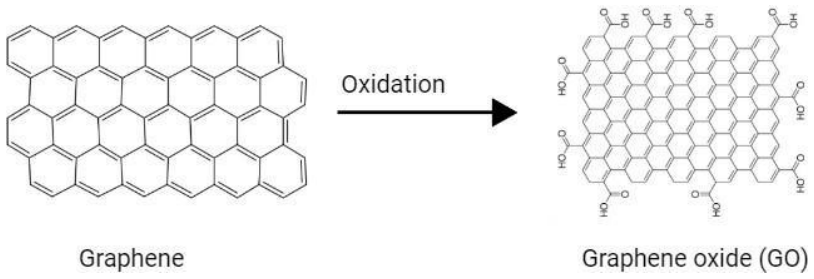

Figure 1. From Graphene oxidation to Graphene Oxide.

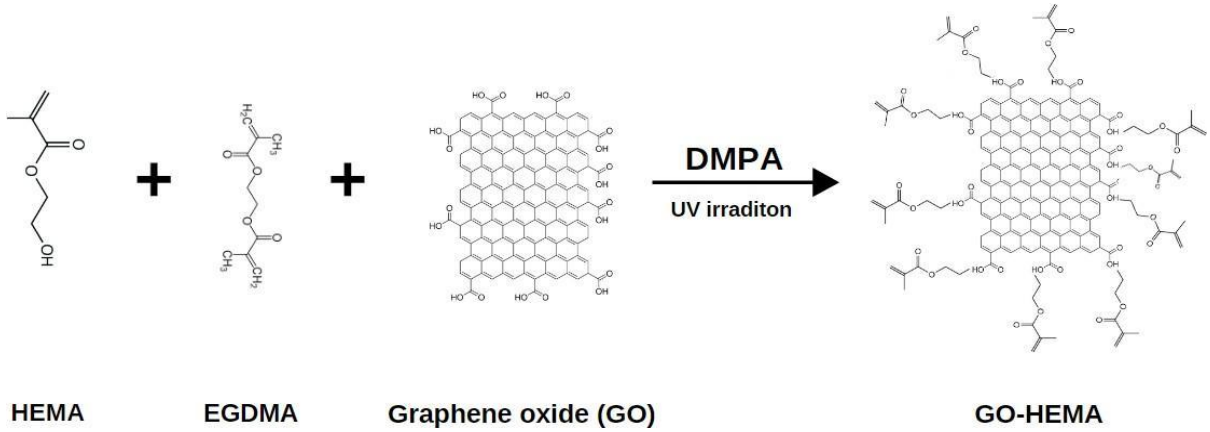

Figure 2. EGDMA with 2-hydroxyethyl methacrylate (HEMA) has formed a membrane structure under UV and is related to the functionalization of GO with 2-hydroxyethyl methacrylate (HEMA).

using the photopolymerization technique. The effect of pH (5.0, 6.0, 7.0, 8.0), time (30 min., 60 min., 90 min., 120 min.), and initial IgG concentration (0.05, 0.1, 0.25, 0.5, 0.75, 1 mg/mL) on adsorption was examined for the GO-HEMA nanocomposite. All experiments were conducted at 20°C [36].

3 RESULT

3.1 FTIR Analysis

The GO-HEMA membrane FTIR spectrum is shown in Figure 5.

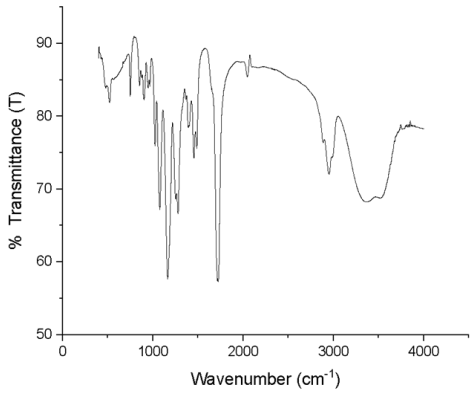

Figure 3. FTIR spectrum of membrane structures containing graphene oxide.

Figure 3 depicts the FTIR spectra of the GO/HEMA nanocomposite. The strong peak at 3441 cm^{-1} corresponds to the OH functional group; the peak at 1735 cm^{-1} is attributed to the C=O group, which is functionalized to produce the carboxylic group (COOH); and the peaks at 1156 cm^{-1}, 1459 cm^{-1}, and 1405 cm^{-1} are associated with the alcoholic C-OH and the C=C aromatic bond on the GO surface. Peaks of HEMA are observed at 2923 cm^{-1}, 2852 cm^{-1}, and 1365 cm^{-1}, corresponding to the bending vibrations of the C-H bonds of methylene and methyl groups. The peaks at 900 cm^{-1}, 945 cm^{-1}, 850 cm^{-1}, and 748 cm^{-1} are related to bending vibrations outside the aromatic plane or bending vibrations outside the CH surface. Additionally, the tensile vibrations of the -OH groups of GO-HEMA membranes in the range of 3300-3700 cm^{-1} indicate the incorporation of graphene oxide into the HEMA membrane structure.

3.2 SEM Analysis

Scanning electron microscope images showed the morphological structure of HEMA Membrane, GO-HEMA Membrane

nanopolymers in Figure 4.

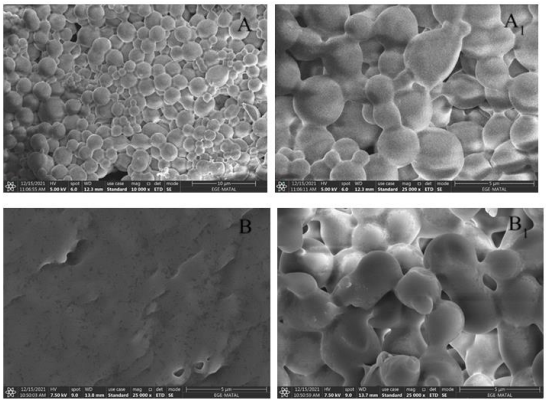

Figure 4. SEM images of HEMA-EGDMA membrane nanopolymer. (A, A_1: HEMA Membrane; B, B_1: GO-HEMA Membrane).

It is shown from its horizontal and vertical sections that the particles have a cylindrical structure. It can be said that these structures can be grafted with GO and turned into nanocomposites.

3.3 IgG Adsorption/Desorption to GO HEMA Membrane

3.3.1 Investigating the Effect of Concentration and Time on IgG Adsorption

IgG adsorption experiments were performed on the synthesized GO-HEMA membrane systems. IgG adsorption is highly influenced by initial IgG concentration, incubation period, ambient pH, temperature, and ionic strength. Therefore, the initial concentration of IgG was changed from 0.1 mg/mL to 2.0 mg/mL; The impact of incubation duration on IgG adsorption was investigated across various adsorption periods (30-120 minutes).

In typical IgG adsorption experiments, HEMA membranes (diameter: 0.75 cm) were mixed with a total of 1.0 mL of IgG solution at different concentrations under an orbital mixing at 400 rpm and at room temperature. Upon reaching the equilibrium time for optimal IgG adsorption, the HEMA membranes were recovered from the solutions via uncomplicated physical filtration. The adsorbed amount of IgG (Q) was quantified spectrophotometrically at 280 nm, deriving from the discrepancy between the initial and residual IgG concentrations in the adsorption solution. The Q values were determined utilizing the equation presented herein (Equation 1).

$$Q = \frac{(Ci - Cf) x V}{weight\ of\ membrane\ (g)} \quad \text{(Equation 1)}$$

In this context, within the adsorption solution (mg/mL), Q represents the quantity of IgG adsorbed into the HEMA membrane systems (mg/g), where Ci symbolizes the initial IgG concentration, Cf denotes the final IgG concentration, and V indicates the total volume of the adsorption solution (mL), while m represents the mass of the membrane. Since the HEMA membrane has a cylindrical structure, it is calculated with the cylindrical volume formula.

As shown in Figure 5, graphene oxide HEMA membrane structures are determined at best in 0.5 mg/mL concentration at 30 minutes. In the graph of Figure 3, IgG at 0.5 mg/mL

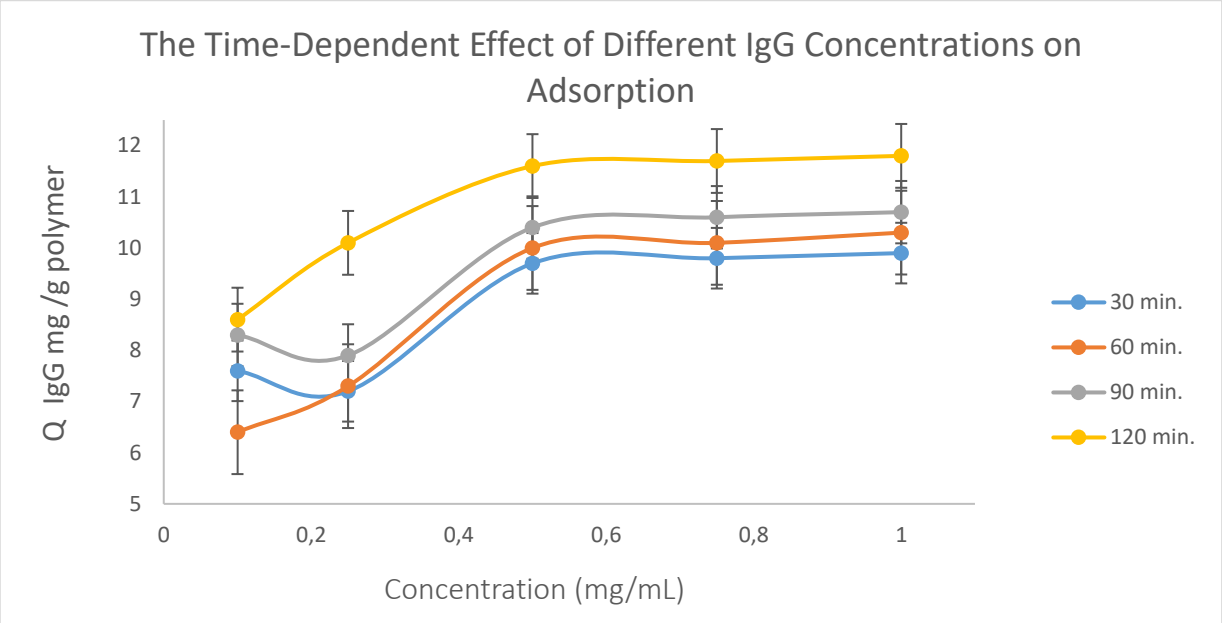

Figure 5. The Q equation was calculated by taking the absorbance measurements of the amount of IgG adsorbed to the GO-HEMA membrane structures and the standard graph was drawn.

concentration was bound to (GO-HEMA) membrane through secondary interactions, such as hydrogen bonding, hydrophobic interactions, etc.

For the calculation of the amount of adsorbed substance, the mass of the GO-HEMA membrane structures was calculated from the volume of the cylinder. At 30 and 45 minutes, the values showed similar results. In trials at 60 and 120 minutes, it was observed that IgG was more absorbed into the GO-HEMA membrane surface. It can be said that IgG can be bound to the nanocomposite materials synthesized with the obtained data.

3.3.2 Investigating the effect of pH on IgG adsorption

The pH-dependent impact on IgG binding to GO-HEMA membranes was explored through a systematic investigation of pH values ranging from 5.0 to 8.0 To this end, buffered solutions were meticulously prepared, comprising a 0.1 M acetate buffer for pH 5.0 and 0.1 M PBS for pH 6.0, pH 7.0, and pH 8.0, with rigorous pH level verification. Subsequently, 0.5 mg/mL IgG binding assays were meticulously conducted under these varied pH conditions.

The optimum pH value for IgG binding was determined as 5. After this value, decreases in bonding may be seen due to the deterioration of the IgG structure or the decrease in the area to be bound on the membrane surface.

Based on the data acquired from the optimization studies, optimum binding was determined as pH 5.0, 0.5 mg/mL concentration, and 30 min adsorption time. Figure 5 and Figure 6 demonstrate that a

protein

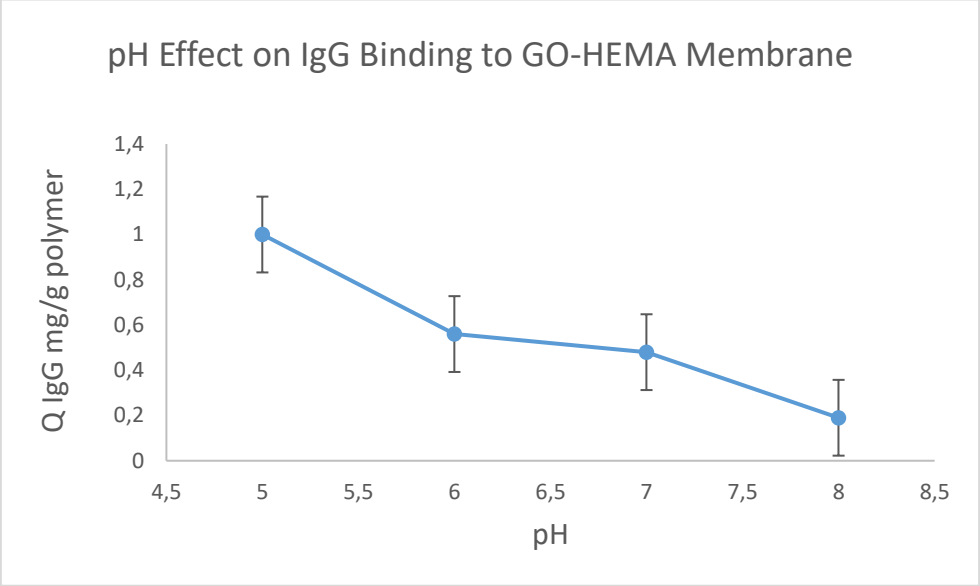

Figure 6. pH effect on IgG binding to GO-HEMA membrane (Room temperature, 30 min).

concentration of 0.5 mg/mL IgG is adequate for binding to GO-HEMA membrane structures.

4 DISCUSSION

Previous studies assert that plasma harbors a multitude of proteins, thus rendering it pivotal in disease diagnosis. They further elaborate that the removal of these proteins, constituting approximately 90% of the total protein content, amplifies the relative concentration of other proteins present in minor quantities, thereby facilitating their detection. Because the characterization of thousands of individual serum proteins/peptides can lead to the discovery of markers of many diseases, such as inflammation, infection, diabetes, cancer, cardiovascular disease, Alzheimer's, and other autoimmune diseases, human serum is attracting increasing interest in its proteomics studies with many attempts to characterize its components broadly. Treatment methods and drug systems for these diseases on a large scale are quite numerous. With the advancement of technology, the production of composite nanomaterials and their use in diseases are becoming widespread. Based on the most basic, it would be more accurate to separate proteins from blood, serum and plasma and use them in diagnosis and treatment. In our own study, we aimed to use inexpensive and highly efficient techniques for the removal of proteins and preferred nanocomposite materials. In addition to this, results show that the developed sorbent system has a great potential for use for the removal of IgG from human blood in proteomic studies.

In a study conducted by Demir et al. in 2017, Concanavalin A-poly (2-hydroxyethyl methacrylate-ethylene dimethacrylate) hydrogel membranes were employed for IgG adsorption, yielding a recorded adsorption

capacity of 3.52 mg/g [37]. Additionally, in a study by Bayramoğlu and Arıca in 2009, poly(HEMA/EGDMA) microspheres were utilized, revealing optimal IgG adsorption between pH 5.0 and 6.0 [38]. In our study, using GO-HEMA composite membrane at pH 5.0, an adsorption capacity of 11.8 mg/g was observed, demonstrating sufficient adsorption. The preference for the synthesized polymeric material in proteomic studies may stem from its cost-effectiveness and adequate adsorption capability.

In conclusion, a cost-effective and easily synthesizable nanocomposite polymeric structure demonstrating high specificity to IgG was synthesized for IgG detection. The use of non-toxic nanomaterials synthesized via photopolymerization technique and optimized polymer is anticipated to provide a precise detection and analysis method at the molecular level. This suggests the potential to offer an innovative diagnostic method in the biomedical field, providing high sensitivity and accuracy in detecting structural alterations in IgG.

5 ACKNOWLEDGEMENTS

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