BSA Adsorption and Antibiofilm Properties of (N-Cyclohexylacrylamide-co-Acrylamide/AMPSNa) Hydrogels

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Authors’ contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

The hydrogels poly (N-cyclohexylacrylamide-co-acrylamide/2-acrylamido-2-methyl-1-propanesodiumsulfonate) i.e.poly(NCA-co-AM/AMPSNa) (HG41,HG42,HG43&HG44) were synthesised via free-radical copolymerization of NCA and (AM) in a fixed proportion (50:50), but varying the ionic monomer-AMPSNa (0.1g,0.3g,0.5 d 0.7g) in a medium of mixture of water and methanol at 60°C in an oil bath, Potassium persulfate (KPS) was used as an initiator, while N,N'-methylene-bisacrylamide (MBA) was used as a cross-linker. FT-IR spectral data, SEM, XRD, and TGA techniques were used to characterise the synthesised hydrogel (HG43). On changing pH(3.0, 5.0, 7.0 & 9.0), the amount of Bovine Serum Albumin (BSA) adsorption efficiency by these hydrogels was evaluated. At pH 5.0, which is near to BSA’s isoelectric point (4.7), the maximum adsorption was found. BSA adsorption increased as the amount of AMPSNa increased. SEM and XRD were used to examine the BSA-adsorbed hydrogel (HG43). The antibiofilm abilities of the hydrogel (HG43) by Microtiter plate Assay(MTP), Fluorescence microscopy and SEMI against Staphylococcus aureus and Pseudomonas aeruginosa displayed outstanding efficacy.

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1. INTRODUCTION

Hydrogels having a three-dimensional network structure have drawn a huge interest because of their high water content, low friction coefficient, and good biocompatibility [1-2]. Hydrogels have a wide range of applications as biological materials, including bio-adhesives [3], tissue engineering [4], and drug delivery carriers [5]. In addition, some hydrogels have effective antimicrobial characteristics [6-7]. Biomolecules can be immobilised by very simple chemistries on hydrogels, which have a large number of polar reactive sites [8]. Poly (NCA-co-AM/AMPSNa) hydrogels were prepared using a free-radical crosslinking copolymerization of NCA and AM in fixed amounts but changing amounts of AMPSNa co-monomer in methanol/water medium at 60°C using KPS as the initiator and MBA as cross linker. The adsorption of BSA on the obtained hydrogels was tested at various pH levels. SEM, TGA, and XRD analyses all supported BSA adsorption. Antibiofilm tests were also done.

2. EXPERIMENTAL

2.1 Materials

The reaction of Acrylonitrile and cyclohexanol yielded N-cyclohexylacrylamide (NCA). As received, Acrylamide (am), 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), potassium persulphate (KPS) initiator, N,N'-methylenebisacrylamide (MBA) cross linker, and Bovine Serum Albumin (BSA) were utilised. The Britton-Robinson (BR) buffer solution was made using standard protocol with acetic acid, phosphoric acid, and boric acid. The above solution was neutralised using a standardised NaOH solution in order to prepare varied pH solutions. By neutralising 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) with sodium hydroxide, 2-acrylamido-2-methyl-1-propane sodium sulfonate (AMPSNa) was created.

2.2 Preparation of Hydrogels

Hydrogels were made via free-radical crosslinking copolymerization [9]. NCA (0.50g), AM (0.50g), and AMPSNa (0.10,0.30,0.50&0.70g) were added to the reaction vessel, followed by KPS (0.005g) and MBA (0.03g) in a mixture of methanol/water. After a 15-minute nitrogen purge, they were heated to 60°C in a thermostatic water bath for one day of polymerization. After the process, the recovered hydrogels (HG41-HG44) were dried to a constant weight in a vacuum oven at 50°C.

2.3 Adsorption of BSA on to Hydrogels

1 g dry hydrogel was dissolved in 20 mL BR buffer (various pH levels) with 4.0 mg/mL BSA and kept at 30°C till equilibrium is attained. A fraction of the BSA solution was taken out and the concentration was measured at 279 nm using a UV spectrophotometer. The amount of BSA (qe) adsorbed by the hydrogel was calculated using the equation below.

\[ qe \text{ (mg/g)} = (C_i - C_e) \frac{V}{m} \]

Where \( qe \) is the amount of BSA adsorbed in mg/g, \( C_i \) is the concentration present initially, \( C_e \) is the concentration of the solution after the attainment of equilibrium, \( V \) is the volume of the solution taken, and \( m \) is the mass of the dried hydrogel.

2.4 Antibiofilm Activity of the Hydrogel (HG43)

2.4.1 Microtiter plate assay (MTP) for biofilm inhibition

To investigate the efficacy of produced hydrogel in interrupting biofilm development, the procedure given by Christensen et al. [10] i.e. MTP assay using 96 well-flat bottom polystyrene titer plates was followed. Before being treated with 10µL of pathogenic bacterial culture overnight, multiple wells were filled with 180µL of BHI broth. The test solution (HG43) was added to the prepared stock solution at doubling concentrations of 1, 2, 4, 8, 16, 32, 64, 128 and 256 µg/mL, respectively, and incubated at 37°C for 24 hours with the control (without the test sample). After incubation, the contents of the wells were removed and washed with 0.2 mL of phosphate buffer saline (pH 7.2) to remove bacteria. Sessile bacteria adhesion was fixed with sodium acetate (2%) and stained with crystal violet (0.1 percent, w/v). The deionized water wash was used to remove the excess discoloration, which was then let to dry. Optical density was evaluated using a microtiter plate.
reader (Thermo) at 600 nm after dried plates were cleaned with 95 percent ethanol. Using the formulas, the percentage of biofilm inhibition was estimated.

\[
\% \text{ Biofilm inhibition} = \left( \frac{\text{Control OD} - \text{test OD}}{\text{Control OD}} \right) \times 100
\]

2.4.2 ETBr/AO staining by fluorescence microscope

In a 24 well culture plate, 5x10^6 cells/mL of Staphylococcus aureus/Pseudomonas aeruginosa cells were plated on coverslips and treated with 50 μg/mL of hydrogel (HG43) in nutritional broth. For 24 hours, the plate was incubated at 37°C in a bacteriological incubator. After the wells had been incubated, 50μL of 1 mg/mL Ethidium bromide and Acridine orange were added and gently mixed together. Finally, the plate was centrifuged for 2 minutes at 800 rpm before being analysed within an hour, with at least 100 cells examined under a fluorescence microscope with a fluorescent filter.

3. RESULTS AND DISCUSSION

3.1 IR Spectral Characterization

The existence of peaks in the polymeric hydrogel chain that correspond to the functional groups of monomeric units was shown by FT-IR analysis of the hydrogels (Fig. 1). Around 3430 cm⁻¹, a prominent peak corresponding to NH of AMPSNa as well as NH stretching of acrylamide was found. Another wide peak at 2929 cm⁻¹ agreed with hydrogel backbone's C-H stretching. Peaks were also seen at 1636 cm⁻¹, which corresponded to the C=O of the NCA unit, and 1448 cm⁻¹, which corresponded to the S=O of the NCA unit (Sym).

3.2 The pH Effect on Protein Adsorption

BSA adsorption was highest in poly (NCA-AM/AMPSNa) hydrogels when pH was 5.0. (Table 1 and Fig. 2) [11]. More acidic and alkaline pH values yielded lower adsorption capacities. The amount of adsorbed BSA decreases when the charge on protein molecules. Variations exist, as do conformational changes. BSA adsorption may be caused via interactions between the negatively charged sulphonate groups of poly(NCA-AM/AMPSNa) hydrogels and the positively charged lysyl or arginyl residues of BSA, as well as the conformational state of BSA molecules at this pH. At pH 5.0, the macromolecules have a compact and folded structure. The adsorption increased with the increase in amount of AMPSNa.

3.2.1 Morphology of the Surface

The plain hydrogels reveal smooth and regular topology while the adsorption caused rougher surface because of the adsorbed protein molecules on the surface. (Fig. 3). The porous structure disappeared after BSA adsorption which confirmed the adsorption of BSA.

![Scheme 1. Preparation of poly (NCA-co-AM/AMPSNa) hydrogel](image-url)
Fig. 1. FT-IR spectrum-HG43 hydrogel

Fig. 2. The pH Effect on Protein Adsorption

Table 1. The pH effect on protein adsorption

| pH | Protein adsorption |
|----|-------------------|
|    | 0.1   | 0.3   | 0.5   | 0.7   |
| 3  | 73.6   | 79.0  | 83.0  | 93.6  |
| 5  | 108.4  | 120.4 | 124.4 | 129.6 |
| 7  | 71.5   | 75.0  | 82.0  | 90.2  |
| 9  | 65.4   | 71.2  | 77.8  | 80.4  |
3.2.2 XRD studies
The studies revealed that the hydrogel (HG43) does not exhibit any sharp peaks in XRD pattern and the broad peaks showed its amorphous nature. The broad peak becomes sharpened for BSA adsorption at 2θ, which supplemented BSA adsorption. (Fig. 4).

3.3 Antibiofilm Effect of Hydrogels

3.3.1 Microtiter plate method
For varying quantities of Staphylococcus aureus (Gram positive bacteria) and Pseudomonas aeruginosa (Gram negative bacteria), the optical density (OD) was assessed using a microtiter plate reader (Thermo) at 600 nm (Figs. 5a and 5b). Using the formula, the inhibitory concentration was calculated. (Table.2 & Fig.6.). The biofilm inhibition is calculated by the formula:

\[ \% \text{ Biofilm inhibition} = \left( \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \right) \times 100 \]

Using the Graphad prism Software, the inhibitory concentration IC_{50} for S. aureus and P. aeruginosa was determined to be 27.93 µg/mL and 31.48 µg/mL, respectively. The hydrogel is more efficient against S. aureus than P. aeruginosa, according to the results. The hydrogel poly(NCA-co-AM/AMPSNa) is anionic. The ionisation of carboxyl and phosphate gives the bacterial cell surface a negative charge. As a result, when negatively charged surfaces and bacteria cells come close together, electrostatic repulsion occurs, which explains why bacterial adherence is reduced [12]. The structure of attached bacteria has been found to be altered as a result of significant electrostatic effects, and this ability has been proved.

Fig. 3. SEM images of a) Hydrogel (HG43), b) BSA adsorbed hydrogel.

Fig. 4. XRD of a) Hydrogel(HG43)b) BSA adsorbed Hydrogel
Fig. 5. a) Crystal violet Stain and OD against *S.aureus*; b) Crystal violet Stain and OD against *P.aeruginosa*

Fig. 6. Percentage of inhibition by poly(NCA-co-AM/AMPSNa) hydrogel (HG43) against a) *S.aureus* b) *P.aeruginosa*
Table 2. Percentage inhibitory concentration

| Conc. of Hydrogel(HG43) (µg/mL) | % of inhibition |  |
|----------------------------------|-----------------|---|
|                                  | S. aureus       | P. aeruginosa |
| 1                                | 45.52           | 90.12         |
| 2                                | 37.47           | 84.3          |
| 4                                | 27.84           | 74.03         |
| 8                                | 20.44           | 51.43         |
| 16                               | 19.20           | 19.20         |
| 32                               | 9.30            | 15.95         |
| 64                               | 8.13            | 11.51         |
| 128                              | 2.96            | 9.58          |
| 256                              | 0.2             | 7.04          |
| 0                                | 0               | 0             |

![Fluorescent microscopic images](image)

Fig. 7. Fluorescent microscopic images of a) S. aureus and hydrogel treated S. aureus; b) P. aeruginosa and hydrogel treated P. aeruginosa
3.3.2 Fluorescent microscopy

Fluorescence microscopy was used to analyse bacterial cells treated with poly(NCA-co-AM/AMPSNa) hydrogels. (Fig.7) Green fluorescence with well-organized nuclei looked to be normal living cells. With AO nuclear staining, early apoptotic cells appeared as a crescent-shaped or granular yellow-green. The ETBr staining of late apoptotic cells was intense and asymmetrically localised orange nuclear staining. At the periphery of necrotic cells, there was an uneven, orange-red fluorescence with no chromatin fragmentation. Hydrogel-treated cells with IC_{50} values showed dead apoptotic bodies that could be seen under fluorescence microscopy.

3.3.3 Scanning electron microscope

*S. aureus* and *P. aeruginosa* biofilms were examined for anti-biofilm activity, and biofilm development was seen using SEM at 200 X and 10 times magnification. (Fig.8.) In the presence of bacterial liquid culture medium, biofilms formed a monolayer. Microbial colonies were reduced and eliminated when biofilms were treated with HG43, and fewer individual cells were adhered to the glass slides.

4. CONCLUSION

Free radical copolymerization in methanol at 60 °C has been used to prepare various compositions of poly (NCA-co-AM/AMPSNa) hydrogels in this study. FT-IR, SEM, and XRD were used to characterise the hydrogels. At room temperature and pH 5.0, the maximum BSA adsorption of 129.6 mg was attained. In vitro antibiofilm performance of the hydrogels against *Staphylococcus aureus* and *Pseudomonas aeruginosa* exhibited promising inhibitory efficacy.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by
the producing company rather than the personal efforts of the authors.

**CONSENT**

It is not applicable.

**ETHICAL APPROVAL**

We conducted our research after obtaining proper approval.

**COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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