Antimicrobial pH-sensitive protein carrier based on modified xanthan gum

Mahmoud H. Abu Elella, Magdy W. Sabaa, Demiana H. Hanna, Marwa M. Abdel-Aziz, Riham R. Mohamed

* Chemistry Department, Faculty of Science, Cairo University, Giza, 12613, Egypt
* Regional Center for Mycology and Biotechnology, Al-Azhar University, Al-Azhar University, 11651, Egypt

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ABSTRACT

Proteins have poor absorption efficiency through the gastrointestinal (GI) tract due to their physical and chemical instabilities and short half-life. The aim of the present study was to encapsulate bovine serum albumin (BSA) protein in an efficient potentially biodegradable pH-sensitive carrier based on crosslinked xanthan gum-g-poly (N-vinyl imidazole) for delivery in the intestines. The structure of crosslinked grafts was characterized with different analysis techniques: FTIR, XRD, TGA and FE-SEM/EDX. BSA uptake and release from loaded grafts were performed in two different pH media, acidic (pH 1.2) and slightly alkaline (pH 7.4) solutions, imitating the simulated gastric and intestinal pH, respectively, within various time intervals at body temperature (37 °C). The results showed that the swelling rate and the accumulative BSA release % were faster in pH 7.4 medium than in pH 1.2 medium. The BSA release results revealed that BSA release mechanism in both media followed non-Fickian release. The structural integrity of released BSA was elucidated with SDS-PAGE analysis and the obtained data confirmed that BSA was released without any deformation in its structure. Cytotoxicity of crosslinked grafts was studied against normal cell line by neutral red uptake assay test and the obtained results showed that the prepared grafted carriers possesses good biocompatibility and considered as safe carriers for protein delivery. In addition, investigation of their antimicrobial activities against Aspergillus niger and Staphylococcus aureus confirmed that they can be used as antimicrobial protein carriers to transfer the proteins through GI tract.

1. Introduction

Protein is one of the main constituents of human body as it plays both dynamic and diverse roles such as catalyzing reactions, building cellular structures and controlling cell fates. Protein has been used since 1922 as therapeutic agent when insulin was used for diabetes treatment as an injection. After that, FDA approved it as therapeutic human insulin that led to discovery of many numbers of proteins. Recently, proteins are widely used as therapeutic agents in treatment of various diseases: metabolic disorders, autoimmune and cancer diseases [1,2]. However, they have some drawbacks as physical and chemical instabilities, short half-life and poor absorption efficiency through the gastrointestinal (GI) tract. Thus, protein drugs are traditionally administered by parenteral route rather than oral route [2-6]. Consequently, many scientific research groups worldwide were concerned to prepare smart protein drug carriers to solve the previous mentioned drawbacks, to control the protein release and also, to improve its therapeutic efficacy [2,7].

Nowadays, pH-sensitive hydrogels have attracted attention to design proteins oral delivery because they can be able to change their characteristics when exposing to external different pH media. Different synthetic and natural polymers with acidic or basic pendant groups have been used to prepare pH-sensitive hydrogels to be suitable carriers for the desired proteins controlled release [3].

Polysaccharides are natural polymers that have been widely used in biomedical and industrial applications for their excellent properties such as easy viability, amenability to modification, easy processing, excellent biocompatibility, biodegradability, non-toxicity, water solubility and bioactivity [8-12].

Some hydrogels based on polysaccharides are used to effectively deliver the pharmaceutical drugs that can be incorporated into their chains with physical interactions [13].

Xanthan gum (XG) is a high molecular weight natural anionic polysaccharide. XG is secreted by Xanthomonas campestris (Gram-negative bacterium). The primary structure of XG composed of pentasaccharide repeating unit including β-glucose, α-mannose and α-glucuronic acid with molar ratio 2:2:1. The main chain of XG consists of a linear β-(1→4)-α-glucose that is similar to cellulose backbone. The side
chain is composed of terminal β-D-mannose that is linked via β-(1-4) to α-glucuronic acid which is linked via α- (1→2) to α-mannose (Fig. 1) [8,14–18].

XG is biocompatible, thermally stable, non-toxic, biodegradable, stable over acidic and alkaline conditions and has intrinsic ability as immunological agent [8,19,20]. Thus it is widely used in food, oil, pharmaceuticals, cosmetics, textile, petrochemical and agriculture industries as suspending, stabilizing and thickening agent [18,21–24]. In addition, XG is used in drug delivery system as a gelling agent because of its stability and water solubility [25]. Moreover, XG can conjugate with biocompatible polysaccharide as chitosan through electrostatic attractions and can be copolymerized with synthetic polymer as poly(acrylic acid) through free radical polymerization technique in presence of crosslinking agent as glutaraldehyde to form stable physical and chemical crosslinked hydrogels, respectively [18,26].

N-vinyl imidazole is polymerized to water-soluble synthetic polymer known as poly (N-vinyl imidazole) (PVI) via free radical polymerization mechanism [27,29,31–39]. PVI contains imidazole ring that is a main component in primary bio-macromolecules such as proteins, nucleic acids, certain vitamins and hormones [32]. Moreover, PVI has also excellent properties as thermal stability, biocompatibility, antimicrobial activity, biodegradability, pH sensitivity, so it has wide applications in antimicrobial and antitumor activity, gene delivery, wastewater treatment and pharmaceutical fields [27,29,31–39].

Bovine serum albumin (BSA) is a water-soluble natural albumin protein. It is the globular protein with distinct isoelectric point (pI) 4.8. Thus, BSA is carrying negative charge at physiological pH (pH 7.4). Moreover, BSA is one of the most common protein plasma that is widely used in many applications such as protein/drug and antigen delivery and pharmaceutical industry because it has good features: biocompatibility, antimicrobial activity and unusual ligand-binding [40–44].

Moreover, microbial infection is considered as one of the most serious problems in our daily life, because it causes various diseases such as pneumonia, nausea, abdominal pain, vomiting, diarrhea, pyogenic liver abscess, hemorrhagic colitis, meningitis and hemolytic uremic syndrome. Microbial infection is resulted in pathogenic microorganisms including Staphylococcus aureus and Aspergillus niger (A. niger) [45–48]. Furthermore, antimicrobial agents with low molecular weight have many disadvantages: short-term antimicrobial activity and environmental toxicity. Consequently, academic researchers have been interested in some polymers offering better antibacterial activities to reduce the low molecular weight antimicrobial agents drawbacks by minimizing their toxicity and improving the efficiency, selectivity, and lifetime of the agents controlling microbial infection [48].

The present work aims to use crosslinked xanthan gum graft as an efficient antimicrobial pH-sensitive protein carrier to the intestine avoiding protein degradation in stomach. BSA was used as a model to study protein loading and release. Cytotoxicity effect of the synthesized graft was examined against normal cell line. In addition, different parameters were affecting loading, encapsulation efficiency and in-vitro release of BSA. Moreover, kinetic studies of in-vitro BSA release from loaded grafts were performed. Moreover, the structure integrity of released BSA from the grafts was confirmed with SDS-PAGE technique. Moreover, antimicrobial activities of crosslinked XG-g-PVI were evaluated against Aspergillus niger and Staphylococcus aureus.

2. Experimental

2.1. Chemicals

Xanthan gum (Average molecular weight = 1.7 X 10^6 gmol⁻¹), was purchased from Alpha-Chemika, India. Bovine Serum Albumin (BSA), N-vinyl imidazole (NVI), N, N'-Methylene bisacrylamide (MBA), potassium persulfate (PPS), Coomassie Brilliant Blue G-250 and acetone were purchased from Sigma-Aldrich, Germany. Phosphate buffered saline (PBS) was purchased from Loba chemi Pvt. Ltd., Mumbai-India. Hydrochloric acid and benzene were purchased from Merck-Germany. Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Lonza (Basel, Switzerland). Aspergillus niger (A. niger, ATCC 16888) and Staphylococcus aureus (S. aureus, ATCC 12600) were provided by the regional center for mycology and biotechnology at Al-Azhar University-Egypt.

3. Xanthan gum-based crosslinked grafts synthesis

Crosslinked xanthan gum grafts (XG-g-PVI/MBA) were synthesized via free radical polymerization mechanism as follows; XG (1.0 g), PPS (45 m M) as initiator, N-vinyl imidazole monomer, (0.25–1.0 g) and N,N'-methylene bisacrylamide, MBA, (2.5–10%, %w/w of XG) as crosslinking agent while a bubbling stream of N₂ gas was purged in the reaction [49]. Crosslinked XG grafts were synthesized with various graft yields (GY) % in presence of 5% MBA: 0% (P1), 20% (P2), 63% (P3) and 95% (P4). Also, GY (95%) was synthesized in presence of
different MBA concentrations: 2.5% (P5), 5% (P4), 7.5% (P6) and 10% (P7).

4. Kinetic studies of crosslinked xanthan gum-based grafts swellability

Swellability of crosslinked XG grafts in both phosphate buffered saline (PBS), pH 7.4 and pH 1.2 was kinetically studied and compared with the swellability of crosslinked XG (P1) for various time intervals (15–420 min) at 37 °C (body temperature).

Definite amounts of graft (0.05 g) (Wf) were soaked in 25 mL PBS at various time intervals 15–420 min till reach equilibrium. Then swollen grafts were weighed after wiping off excess water on their surface with filter paper (Wp). Swelling uptake % was calculated using the following equation [50,51].

\[
\text{Swelling uptake\%} = \frac{(W_p - W_f)}{W_i} \times 100 \quad (1)
\]

Where Wf and W_i are the swollen and dry graft weights, respectively.

5. BSA loading/in-vitro release from XG crosslinked grafts

BSA loading into XG crosslinked grafts was studied via swelling method. Briefly, definite weight of XG grafts (Wf) was soaked in 30 mL of BSA solution (1mg/1mL) (W2) under continuous shaking for 24 h at room temperature (30 °C). Then grafts were centrifuged at 2000 rpm. Then the amount of loaded BSA was determined according to Bradford method using Coomassie brilliant blue G250 protein assay [52]. Unloaded BSA hydrogels were used as blank. Drug loading (DL) % of BSA and encapsulation efficiency (EE) were evaluated according to equations (2) and (3) [50].

\[
\text{Drug (BSA) loading\% (DL)} = \frac{(W_2 - W_3)}{W_1} \times 100 \quad (2)
\]

\[
\text{BSA encapsulation efficiency\% (EE)} = \frac{(W_2 - W_3)}{W_1} \times 100 \quad (3)
\]

Where W_1, W_2 and W_3 are the weights of dry XG (non-loaded), total amount of BSA and free BSA in supernatant, respectively. The % DL and %EE within XG-g-PVI/MA and XG/MA were affected with different variables: Graft yield% (0–95%), MBA concentration (2.5–10%), graft concentration (5–15 mgmL\(^{-1}\)) and loaded BSA concentration (1–3 mgmL\(^{-1}\)) that are illustrated in Table 1.

6. In-vitro release of BSA from XG-based graft

In-vitro release studies of BSA from loaded grafts were performed in two different release media: 0.1 N HCl (pH 1.2) and PBS (pH 7.4). In brief, the prepared BSA loaded grafts were dispersed in conical flask containing 30 mL of the release medium under constant incubator (100 rpm) at 37 °C for different time intervals (12, 24, 48, 72 and 120 h). After incubation time intervals, the BSA loaded samples were centrifuged at 2000 rpm for 5 min, then 5 mL of supernatant solution was collected from the release medium to determine the BSA concentration.

Meanwhile, the equal amount (5 mL) of fresh release medium was replenished to preserve constant the original volume (30 mL). The BSA concentration in supernatant solution was evaluated using Coomassie brilliant blue assay. Different formulation variables: % graft yield (0–95%), MBA concentration (2.5–10%), graft concentration (5–15 mgmL\(^{-1}\)) and loaded BSA concentration (1–3 mgmL\(^{-1}\)) were affecting the extent of BSA release in two pH media. The unloaded BSA sample was taken as a control. The percentage of in-vitro BSA release was determined using the following equation (4) [50,53].

\[
\text{Accumulative BSA release\%} = \left( \frac{C_f}{C_i} \right) \times 100 \quad (4)
\]

Where C_f and C_i are the weight of BSA in both the supernatant and BSA initial weight, respectively. All experiments of BSA loading and release were done in triplicates for each sample and the mean values were taken.

7. Kinetic studies of in-vitro BSA release

In-vitro BSA release from XG/MA/BSA (P1) and XG-g-PVI/MA/BSA (P2–P11) matrix was determined kinetically with the diffusion exponent (n) which can be calculated according to Koresmeyer model as represented in equation (5) [43,54,55].

\[
M_t/M_\infty = K t^n \quad (5)
\]

Where M_t and M_\infty are the weights of BSA released at a given interval time (t) and at infinite time (120 h), respectively. k is a constant and n is the diffusion exponent.

The mechanism of in-vitro BSA release from both XG/MA/BSA and XG-g-PVI/MA/BSA depends on n values: diffusion controlled (Fickian) release (n = 0.45–0.5), diffusion and erosion controlled (non-Fickian) release (n = 0.5–0.89), Case II transport (n = 0.89–1.0), Super case II transport (n > 1.0).

8. Cytotoxicity (cell compatibility) assay

Cell compatibility of XG-g-PVI/MA was tested against Human Lung normal cell line (WI38) by neutral red uptake assay test (NRU) [56]. We collected exponentially the growing cells by 0.25% Trypsin-EDTA, then we counted the cell suspension by hemocytometer then we incubated the 96 well plates at 37 °C for 48 h. Then, the unwanted cells were incubated for 24 h before treatment with the tested prepared samples to permit attachment of cells to the wall of the plate. Different concentrations of XG-g-PVI/MA hydrogels were prepared (300, 200, 100 and 50 μg mL\(^{-1}\)) by dissolving and diluting it by Dulbecco’s modified Eagle’s medium (DMEM).

Next, we dispensed 200 μl of the treated medium into 4 replicates for each concentration, other wells were filled with untreated cells only (as a negative control) and other wells were filled with media containing Doxorubicin HCl (6 μg/mL\(^{-1}\)) as a positive control. After that, we incubated the 96 well plates at 37 °C for 48 h. Then, we added 100 μl of neutral red solution (50 mg/mL\(^{-1}\)) and then incubated at 1800 rpm for 10 min to remove any precipitated dye crystals. The dye medium was discarded, the microplate was washed twice with 150 μl PBS after incubation at 37 °C for 3 h, to remove the unabsorbed neutral red dye contained in the wells. The well plates were then read using
Inverted Microscope Leica (DMI3000B).

We measured the absorbance of acidified ethanol solution containing extracted neutral red dye with microplate reader (BioTek, ELX808) at 540 nm to determine the optical density (OD), then cell viability was calculated according to the following equation (6):

\[
\text{Cell viability} = \frac{1 - (\text{OD}_t / \text{OD}_c)}{100}
\]

Where OD\(_t\) is the mean optical density of wells treated with polymeric sample and OD\(_c\) is the mean optical density of untreated cells.

9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis studies

The structural integrity of released BSA was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [40]. Released BSA from XG graft was lyophilized and reconstituted in PBS. The molecular weight reference marker, native BSA and released BSA from loaded graft were mixed with sample dilution buffer [1.5 M Tris/HCl pH 6.8, 8.8% (w/v) SDS, 40% (v/v) glycerol and 0.01% bromophenol blue] after that, they were boiled for 2 min prior to electrophoresis.

Finally, the previously prepared samples were separated by SDS-PAGE in a 1.5 mm thick, 4% stacking gel and 12% resolving gel. The electrophoresis was performed by a Protean II vertical electrophoresis cell (Bio-Rad) at 200 V. The bands were visualized by Coomassie blue staining.

10. Antimicrobial activity

Antimicrobial activity of all tested samples was evaluated against Staphylococcus aureus (S. aureus, ATCC 12600) as a pathogenic bacterium and Aspergillus niger (A. niger, ATCC 16888) as a pathogenic fungus with agar well diffusion method using nutrient agar medium for S. aureus and sabouraud dextrose agar medium for A. niger, in presence of amoxicillin and amphotericin B as reference drugs for S. aureus and A. niger, respectively [18,57].

The diameter of wells was 6 mm and 5 mg mL\(^{-1}\) of tested samples and reference drug using water as control solvent was added to them. The plates of S. aureus were incubated at 37 °C for 24 h. While, the incubation of A. niger plates was 48 h at 25 °C.

After incubation time, antimicrobial activity was calculated by measuring the inhibition zones diameter against microbial growth for all tested samples and was compared with reference drugs. The inhibition zones diameters for antimicrobial activity were expressed in millimeters (mm). The experiment was done in triplicates; the average inhibition zone diameter was calculated and shown as mean plus standard deviation (± SD).

11. Instrumentation

Fourier Transform Infrared (FTIR) Spectroscopy, Jasco FTIR 4100 spectrometer (Japan), was used to elucidate the chemical composition of XG, PVI, XG/MBA and XG-g-PVI/MBA in solid state using KBr pellets within wavenumber range of 4000–600 cm\(^{-1}\) at 25 °C.

Field Emission Scanning Electron Microscope (FE-SEM), Quanta 250 FEG, FEI Company, Netherlands with a magnification ×1000 and accelerating voltage of 30 KV, was used to examine the microstructure of tested samples. The small part of tested samples was placed on a carbon tape on a stub and coated with Au.

X-Ray Diffractometry (XRD) (Phillips Xpert MPD Pro) was used to investigate the crystalline structure of tested samples using Ni-filter and CuK\(\alpha\) radiation source at an accelerating voltage/ current (50 KV/40 mA). The relative intensity was recorded in the scattering range 20 from 4° to 60° at a scanning rate 2° min\(^{-1}\).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was investigated by Vertical Polyacrylamide Gel Electrophoresis Apparatus (a Protean II vertical electrophoresis cell (Bio-Rad) at 200 V to investigate the structure integrity of released BSA from loaded grafts.

Inverted Microscope (Leica DMI3000B) was used to examine the cellular morphology of the treated Human Lung normal cells with the tested XG-g-PVI/MBA.

Microplate reader (BioTek, ELX808) was used to measure the optical density (OD) of acidified ethanol solution containing extracted neutral red dye.

12. Results and discussion

12.1. Loading and encapsulation efficiency of BSA

The protein (BSA) incorporation within XG-g-PVI/MBA matrices were subsequently investigated using different variables such as: different graft yields %, different MBA concentrations, loaded BSA concentrations and graft concentrations which affect %DL and %EE and the data was illustrated in Table 2.

The effect of % GY, keeping all other parameters constant; [MBA] = 5% (w/w), [BSA] = 1 mg mL\(^{-1}\) and [graft] = 10 mg mL\(^{-1}\), showed that grafted samples P2 (20%), P3 (63%) and P4 (95%) have higher % DL and %EE values than ungrafted XG (P1).

In addition, the values of %DL and %EE increased with increasing the grafting % due to the electrostatic interactions between the nitrogen atom in PVI imidazole ring and the carboxylic acid (-COOH) of BSA. Similar results were reported in literature [58]. Also, the increase in grafted PVI % increased the number of pores and irregular cavities (shown through FE-SEM images) that tended to absorb consequently more BSA within the crosslinked grafted chains. So, P4 (%GY = 95%) had the highest %DL (9.7%) and %EE (96.6%) values.

The effect of MBA concentration on BSA loading was studied using different MBA concentrations (2.5% (P5), 5% (P4), 7.5% (P6) and 10% (P7)), while keeping all other parameters constant; %GY = 95%, [BSA] = 1 mg mL\(^{-1}\) and [graft] = 10 mg mL\(^{-1}\), and the data exhibited that the increase in MBA concentration from 2.5% to 10% (w/w) led to a decrease in both %DL and %EE values due to the increase in rigidity and compactness of the matrix which led to less intermolecular distances between crosslinked graft chains. These results were similar to uploading diclofenac sodium within carboxymethyl locust bean gum/carboxymethyl cellulose beads [59]. So, P5 (%GY = 95% and [MBA] = 2.5%) had the highest %DL (9.8%) and %EE (98.3%) values.

The effect of BSA concentration on uploading BSA was studied using different BSA concentrations (1 mg mL\(^{-1}\) (P5), 2 mg mL\(^{-1}\) (P8) and 3 mg mL\(^{-1}\) (P9), while keeping %GY = 95%, [MBA] = 2.5% (w/w) and [graft] = 10 mg mL\(^{-1}\), the data showed that % DL and % EE increased with increasing BSA concentration to reach a maximum value of 49.2% and 99.9% for %DL and %EE, respectively.

The highest BSA concentration (3 mg mL\(^{-1}\) (P9) led to an increase in the electrostatic interactions between both BSA and PVI imidazole...
moiety. Consequently, the amount of loaded BSA within XG-g-PVI/MBA increased. The obtained data is similar to some results previously reported in literature [58,60]. Furthermore, the effect of graft concentration on uploading BSA was studied in different graft concentrations (5 mg mL$^{-1}$ (P10), 10 mg mL$^{-1}$ (P5) and 15 mg mL$^{-1}$ (P11), while keeping %GY = 95%, [MBA] = 2.5% (w/w) and [BSA] = 1 mg mL$^{-1}$, the data showed that % EE increased with increasing the graft concentration that led to an increase in the electrostatic interactions between BSA and XG grafts. While, %DL decreased as it is inversely proportional to XG-g-PVI/MBA concentration (Equation (2)).

12.2. Characterization of BSA loaded crosslinked XG graft

The chemical structure of crosslinked XG-g-PVI BSA loaded were elucidated with different analysis tools: FTIR, XRD, FE-SEM and EDX compared with BSA and native XG and PVI.

12.3. FTIR spectroscopy

The characteristic functional groups in XG, PVI, unloaded grafts P5, BSA and BSA loaded grafts P5 were proven with FTIR spectroscopy and the results were shown in Fig. 2.

XG FTIR spectrum showed different absorption peaks at 3431, 2922, 1730, 1639, 1420 and 1065 cm$^{-1}$ referring to stretching vibrations of –OH groups, aliphatic –CH groups, –C=O of acetate groups that overlapped with asymmetric stretching vibrations of carboxylate groups (at 1639 cm$^{-1}$), symmetric carboxylate groups and glycosidic ether (C–O–C) bond, respectively. While, PVI spectrum illustrated various absorption peaks at 3109, 2922, 1639, 1498 and 912, 1415, 1083, 1282, 1229, 662, 822 and 743 cm$^{-1}$ corresponded to stretching vibrations of olefinic (=C–H) groups in imidazole ring, aliphatic (–C–H) hydrocarbon groups in backbone, alkene (C=C) groups in imidazole moiety, C=N and C–C of imidazole ring, in-plane bending vibration of aliphatic C–H groups, –CN groups of imidazole ring and out-of-plane bending vibration of -CH groups in imidazole ring, respectively [30,61–63]. Moreover, FTIR spectrum of unloaded grafted XG (P5) showed broader absorption peak at 3431 cm$^{-1}$ than that found in native XG but it had less intensity because of both consumption of some of –OH groups in grafting process and the intermolecular hydrogen bonding interaction among P5 chains. Moreover, it showed the absorption peaks at 2930, 1730, 1642 and 1433 cm$^{-1}$, referring to the stretching vibration of SP$^2$ =CH groups of imidazole moiety, aliphatic –C–H groups in PVI backbone, –C=O of acetate groups in XG, asymmetric and symmetric carboxylate groups in native XG, respectively. In addition the absorption peaks appeared at 1288, 612, 882 and 695 cm$^{-1}$ for C–N bond of imidazole ring and out-of-plane bending vibration of aliphatic –C–H groups in PVI, respectively. On the other hand, Fig. 2b showed the FTIR spectra of BSA and BSA loaded P5. FTIR spectrum of BSA exhibited the characteristic absorption peaks for protein such as a broad absorption peak at 3417 cm$^{-1}$ corresponding to stretching vibration of carboxylic (–OH) groups and amino groups. In addition, absorption peak appeared at 2952 cm$^{-1}$ referring to stretching vibration of aliphatic –C–H groups. Moreover, three absorption peaks appeared at 1654, 1525 and 1246 cm$^{-1}$ assigned for stretching vibration of carbonyl groups (amide I), secondary amine groups (amide II) and amide III, respectively [62]. The characteristic peaks of BSA were similar to other FTIR spectrum of BSA that previously reported in literature [52,64,65]. While, the FTIR spectrum of BSA loaded P5 illustrated all the characteristic absorption peaks of both BSA and unloaded graft P5 with little shift in wavenumber such as the absorption peaks of hydroxyl and carboxylate groups (amide I) in BSA which were shifted to 3427 and 1634 cm$^{-1}$, respectively, however, they were broader than those found in FTIR spectrum of unloaded graft P5. In addition, the absorption peaks of amide II and III in BSA structure were shifted to 1634, 1453 and 1212 cm$^{-1}$. Moreover, the absorption peak of carbonyl (–C=O) groups of acetate groups in unloaded sample structure was shifted to 1723 cm$^{-1}$. The shifted absorption peaks may be due to H-bonding interactions between BSA and the functional groups on grafted chains. These observations indicated that XG-g-PVI/MBA could encapsulate BSA through physical (H-bonding) interactions.

Fig. 2. FTIR of a) XG, PVI and P5 b) BSA and BSA loaded graft P5.
12.4. FE-SEM techniques

The surface morphology of tested samples was examined by FE-SEM unit and the results were shown in Fig. 3. It illustrated the surface morphology of XG, PVI, unloaded P5, BSA and BSA loaded graft P5. The surface of XG appeared as irregular lobules. PVI surface appeared with small pores and irregular cavities, while unloaded graft P5 surface exhibited heterogeneous, irregular and highly porous surface [63]. In addition, the surface morphology of BSA has irregular lobules [62]. While, FE-SEM images showed that BSA loaded graft P5 surface was rough and porous filled with lustrous particles. Consequently, the surface morphology of loaded graft confirmed that BSA was diffused into P5 matrix within many pores that were found on its surface leading to occupy most of the polymer surface with BSA. These previous observations supported the fact that BSA was successfully loaded onto crosslinked XG grafts.

On the other hand, the elemental composition -using EDX analysis (Fig. 3f)- for BSA loaded graft P5 showed that its spectrum showed six peaks at 0.275, 0.380, 0.533, 1.04, 2.313 and 3.695 keV corresponding to six different elements including C, N, O, Na, S and Ca, respectively. The appearance of S peak in EDX spectrum of BSA loaded graft (P5/BSA), indicated the successful loading of BSA within P5 graft.

Fig. 3. FE-SEM images of a) XG, b) PVI, c) unloaded P5, d) BSA, e) BSA loaded P5 and f) elemental composition of BSA loaded P5.
Furthermore, additional peak corresponding to gold (Au) was observed at 2.14 keV in EDX spectra of BSA/P5 due to coating of all samples with Au before examining them onto FE-SEM technique.

12.5. X-ray diffraction

The crystallinity nature of tested samples was examined with XRD technique and the results were shown in Fig. 4. Fig. 4a illustrated XRD patterns for XG, PVI and unloaded P5. It exhibited that XG has completely amorphous nature due to a weak broad diffraction peak at $\theta = 20^\circ$, and PVI pattern showed that PVI has partially crystalline nature because it showed two broad diffraction peaks at $2\theta = 12^\circ$ and $21.7^\circ$, while crosslinked grafted sample in absence of BSA exhibited one diffraction peak broader than that found in XG pattern at $2\theta = 20.7^\circ$ supporting the successful grafting process onto XG (XG-g-PVI) and formation of intermolecular H-bonding interactions between –OH groups of XG chains and nitrogen atom on PVI chains [63]. On the other hand, Fig. 4b represented XRD patterns of BSA and BSA loaded P5 matrix. XRD pattern of BSA exhibited two broad diffraction peaks at $2\theta = 9^\circ$ and $20.5^\circ$, indicating the crystalline structure of BSA due to the H-bonding interactions between BSA chains which is similar to other data reported in literature [52]. While, the XRD pattern of BSA loaded P5graft showed one broad diffraction peak at $2\theta = 21.2^\circ$, however, it is broader and more intense than that found in XRD pattern of unloaded P5 graft. Also, the diffraction peak of BSA at $2\theta = 9^\circ$ disappeared indicating the homogenous dispersion of BSA among P5 graft chains and formed more H-bonding between BSA and P5 chains than those found in unloaded P5 graft.

12.6. Kinetic swell ability study of crosslinked XG grafts

The drugs release from the polymer/drug matrix is controlled by the swelling behavior of the polymer [59]. The swelling ability of crosslinked grafts was kinetically studied in two different buffered solutions (pH 1.2 and 7.4) at various time intervals, from 15 to 420 min and at body temperature (37 °C). Moreover, the effect of the two variables the yield (%) and MBA concentration were studied on the extent of swelling rate of grafts and the results were shown in Fig. 5. It was obvious that the swellability of XG grafts increased with the increase in swelling time to reach equilibrium at around 360 min. Also, the swellability of all grafted XG matrices (from P2 to P7) was higher than the swellability of crosslinked XG/MBA matrix (P1) due to the introduction of the hydrophilic PVI in all grafted samples.

In addition, the swelling rate of the grafts in phosphate buffered saline (pH 7.4) was higher than that in acidic buffered solution (pH 1.2). At acidic medium (pH 1.2), most of the imidazole moieties were protonated by hydrogen ions that may interact with carboxylate anions through electrostatic interactions (free space between crosslinked graft chains decreased) and the electrostatic (anion-anion) repulsive interactions were eliminated, thus the swelling ability of crosslinked grafts decreased. However, at pH 7.4 medium, the ionized imidazole moieties exert electrostatic repulsive force with each other, that increases the free space between crosslinked graft chains and resulting in increased % of water uptake that led to increase the concentration of counter ion inside the polymeric matrix and the presence of osmotic pressure difference between the internal and external buffered solution of the crosslinked graft. The swellability of crosslinked XG grafts balanced the increase in osmotic pressure [66]. The water uptake % of the grafts increased with the increase in graft yield % at constant MBA concentration (5 wt. %) to reach a maximum value of 571% and 253% in pH 7.4 and pH 1.2, respectively as shown in Fig. 5.

The increase in GY % led to increase in the imidazole moieties (hydrophilic groups) on the copolymer chains that increased the swellability % of grafted matrices (P2, P3 and P4) more than the un-grafted one (P1). Also, it showed that the water uptake % of grafts increased with the decrease in MBA concentrations from 2.5% to 10 wt %, keeping the graft yield % constant (95 wt. %) to reach the maximum value 610 and 320% in pH 7.4 and 1.2, respectively. The increase in MBA concentrations from 2.5 (P5) to 10% (P7) may result in a decrease in the intermolecular space between grafts chains (increased the crosslinked grafts rigidity) and consequently led to a decrease in the swellability. While, graft with less amount of MBA concentration (2.5%, P5) showed higher water uptake % than with other MBA ratios, as the rigidity of grafts decreased that resulted in the increase in swellability of P5graft. The more swellability of the polymeric matrices led to more released BSA % from their chains. The previous results confirmed that in-vitro release BSA rate in pH 7.4 was higher than that in acidic buffered solution according to kinetic swelling study behavior of XG and similar results were reported in literature [51,59].

12.7. In-vitro release of BSA

Fig. 6 shows the cumulative release of BSA from loaded crosslinked XG grafts at 37 °C in two different pHs solutions (pH 1.2 and 7.4) through different incubation time intervals from 12 h to 120 h under the effect of different variables: graft yield % (20–95%), MBA concentration (2.5–10%), BSA concentration (1–3 mg mL$^{-1}$) and hydrogel concentration (5–15 mg mL$^{-1}$). The data revealed that BSA release rate
from all the samples was gradually increased with the increase in the incubation time to reach the maximum value within 120 h.

In addition, BSA release rate in pH 7.4 was faster than that in pH 1.2, which was similar to kinetic studies for swell ability of crosslinked XG grafts. This observation was similar to BSA release from some hydrogels as reported before [67,68]. The release rate depends on the dissolution and diffusion of the drug within the polymer [67]. In addition, the release of drug from hydrophilic matrices depends on both the rigidity and the type of matrix [51]. The effect of graft yield % on BSA accumulative release from XG-g-PVI/MBA/BSA matrix, prepared with different %GY (0, 20, 63 and 95% which have the following codes; P1, P2, P3 and P4, respectively) keeping all the other parameters constant ([MBA] = 5% (w/w), BSA concentration (1 mg mL⁻¹) and grafts concentration (10 mg mL⁻¹), is pH 7.4 and 1.2 solutions through different incubation time intervals from 12 h to 120 h is represented in Fig. 6a.

The observed data revealed that BSA release % was gradually increased with increase in the incubation time to reach a peak of 69.7%, 89.2%, 92.6% and 97.6% for P1, P2, P3 and P4, respectively, in PBS (pH 7.4) and 10.5%, 12.8%, 15.1% and 16.4% for P1, P2, P3 and P4, respectively, in pH 1.2. The data showed that the increase in %GY led to maximum BSA release at 120 h.

Moreover, the effect of MBA concentration on BSA accumulative release was studied in two different pHs media (7.4 and 1.2) using 95% graft yield, BSA concentration (1 mg mL⁻¹) and MBA concentrations (1 mg mL⁻¹, 2 mg mL⁻¹ and 3 mg mL⁻¹) in presence of 2.5–10% MBA concentrations, respectively, in PBS (pH 7.4) and pH 1.2, and the data is exhibited in Fig. 6c. It showed that initial BSA concentration had significant effect on the BSA release from XG-g-PVI/MBA/BSA matrix. In addition, it was noted that the increased amount of loaded BSA from 1 mg mL⁻¹ to 3 mg mL⁻¹ decreased the BSA release in both release media. Thus, at 1 mg mL⁻¹ of BSA (P5), the accumulative release was 99.6% and 17.0% in pH 7.4 and pH 1.2, respectively at 120 h, while at 3 mg mL⁻¹, the release of BSA was 91.3% and 10.5% in pH 7.4 and pH 1.2, respectively at 120 h. The percentage of the drug release is controlled by the drug diffusion through the polymer matrix and the relaxation process of the polymer on solvent penetration [51]. At the least amount of BSA loaded (1 mg mL⁻¹) in XG matrix, large pore fraction was formed leading to higher swelling that led to fast BSA release in both release media. On the other hand, at the highest amount of the loaded BSA (3 mg mL⁻¹), high crystalline domain was formed that caused the reduction and shrinkage of small pores and cavities within XG/BSA matrix and resulted in a decrease in BSA release. The mentioned observation was similar to another drugs release rate in literature [51,58,69,70].

Furthermore, release of BSA from XG-g-PVI/MBA/BSA matrix, prepared using different graft concentrations (5, 10 and 15 mg mL⁻¹)
which have the following codes, P10, P5 and P11, respectively) keeping all the other parameters constant: %GY = 95%, BSA concentration = 1 mg mL\(^{-1}\) and 2.5% MBA, in release media against various incubation time intervals are represented in Fig. 6d.

The data showed that BSA was released gradually with increasing the incubation time. Also, the accumulative release rate of BSA from high graft concentration (15 mg mL\(^{-1}\)), P11 matrix, was slower than low concentration of graft (5 mg mL\(^{-1}\)), P10 matrix, due to the increase in the interaction between the grafted copolymer and BSA, consequently, the rigidity of the matrix increased leading to a decrease in the free volume of the matrix leading to a decrease in BSA release. Thus, BSA released from P10 (less graft concentration) reached 100% at 120 h, while the release of BSA from P11 (high graft concentration) was 89.2% at 120 h. BSA released from XG-g-PVI/MBA/BSA matrix was similar to ibuprofen release from sodium carboxymethyl xanthan/sodium alginate interpenetrating network beads and diclofenac sodium release from sodium carboxymethyl locust bean gum/sodium carboxymethyl cellulose interpenetrating network microbeads [51,59].

Fig. 6. In-vitro BSA accumulative release from XG/BSA (P1) and XG-g-PVI/MBA/BSA (P2–P11) matrix in two different release media (pH 7.4 and 1.2) at different a) graft yield %, b) MBA concentration, c) BSA concentration and d) polymer (graft) concentration.
12.8. Kinetic studies of in-vitro release mechanism of BSA

The evaluation of in vitro and in vivo release kinetics of active substances from drug systems plays an important role in predicting and management of both efficacy and safety. Kinetics is more than a scientific goal; it is an essential quality parameter of all type of drugs. Keeping in mind the high diversity of supramolecular drug systems and apparently huge number of phenomenological local characteristics, a classification of models seems to be an impossible task. On the other hand, in practically all these release processes, the diffusion phenomenon is involved, described by the diffusion equation \[71\]. The modeling of release kinetics consists of identifying the most appropriate mathematical conditions connected with the implied physicochemical phenomena.

The present data of in-vitro BSA release from BSA loaded XG-g-PVI matrix was fitted to Koresmeyer model and it is illustrated in Table 1. The obtained data showed that the diffusion exponent values (n) in range 0.53-0.86, so the mechanism of BSA release from BSA loaded XG-g-PVI matrix followed non-Fickian release mechanism. That means, the BSA release from matrix was retarded by a combination of diffusion and erosion-controlled release. The tabulated data are in accordance with the BSA and diclofenac sodium kinetic release \[55,59,72\].

12.9. Cytotoxicity assay of the crosslinked graft copolymer

Cytotoxicity is a significant factor to use the proper vectors for protein delivery \[73\]. The crosslinked XG-g-PVI hydrogel itself must have a low toxicity. Herein, the cytotoxicity of the unloaded crosslinked XG-g-PVI hydrogel (P5) was investigated against human lung normal cell lines using neutral red uptake assay. Different concentrations of tested matrix were incubated with human lung normal cell lines. The viability percentage was calculated and plotted against different concentrations of the tested matrix (SFig. 1a). It showed that concentration of the crosslinked graft (\( \leq 200 \mu g/mL \)) showed nearly no cytotoxicity against human lung normal cell lines. While, at concentration 300 \( \mu g/mL \), crosslinked graft showed less cytotoxicity effect, the % cell viability decreased to 83.5%, on human lung normal cell lines. Therefore, the prepared graft possesses good biocompatibility and could be considered as safe system for drug delivery. Also, to further elucidate the low cytotoxicity of the prepared graft, the morphological alterations that occurred for the tested cell lines after treatment with \( \geq 50 \mu g/mL \) of tested graft sample, compared with untreated cells (negative control) were visualized via inverted microscope and were presented in SFig. 1b.

12.10. SDS-PAGE studies

The structural integrity of BSA released from BSA loaded P5graft into PBS medium (pH7.4) was examined by SDS-PAGE technique. The prepared graft was visualized by the Coomassie brilliant blue staining –SFig. 2. SDS-PAGE method is considered to be the most principal method that widely used for separating proteins according to differences in their molecular weights \[41\]. It was observed that each of the molecular weight marker and BSA standard were shown in Lanes 1 and 2, respectively, showing a band at 66 KD. Similarly, the released BSA from the loaded sample into PBS medium after incubation time; 24 h and 120 h were shown in lanes 3 and 4, respectively. Moreover, the clear bands that appeared in lanes 3 and 4 are of the same size of the bands of BSA standard in lane 2 at 66 KD, these results confirmed that the loaded BSA inside the loaded graft XG-g-PVI/MBA/BSA did not suffer from any fragmentation or aggregation through its release out of the loaded matrix. In addition, they confirmed that the structural integrity of BSA had not been affected by the entrapment procedure or release conditions. The obtained results were similar to previously reported in literature \[40,74\].

12.11. Antimicrobial activity

Antimicrobial activity results of XG, PVI and crosslinked grafted XG samples (P1-P7) and reference drugs against S. aureus and A. niger pathogenic microorganisms using agar well diffusion method are represented in SFig. 3 and STable 2. The results showed that XG has no antimicrobial activity against both S. aureus and A. niger; this confirmed the fact that XG is microbially attacked \[75\]. In addition, the inhibitory effect of crosslinked XG in the absence of PVI (P1) was on fungus growth only, however, P1 did not have any inhibitory effect on the growth of bacterium which may be due to interact of MBA with fungi cell wall through H-bonding interactions leading to fungi membranes disruption and fungi cells death.

Moreover, PVI showed good antimicrobial activity. Thus the inhibition zone diameter of PVI sample against S. aureus and A. niger was 24.0 mm and 24.9 mm, respectively. The antimicrobial activity of PVI is due to several mechanisms that have been postulated based on electrostatic interactions between protonated N-vinyl imidazole moieties in PVI structure that carry positive charge and cytoplasmic membrane of microorganisms cell surface with negative charge. In addition, the hydrophobic interactions between alkene groups in N-vinyl imidazole moiety and cytoplasmic membrane of microorganisms that lead to disruption of microorganisms cell membrane and their death \[27,76\]. Consequently, the antimicrobial activity results of crosslinked grafted XG (XG-g-PVI) samples with different graft yield % from 20 to 95% (P2, P3 and P4) in presence of 5% MBA possessed good antimicrobial activities against two tested pathogenic microorganisms and their antimicrobial activity increased with an increase in graft yield % due to the increase in PVI content on XG chains. On the other hand, the anti-microbial activity results of crosslinked grafted XG samples (XG-g-PVI), 95%Gy, using various MBA concentrations (P5, 2.5%), (P4, 5%), (P6, 7.5%) and (P7, 10%) showed increase in antimicrobial activity with increasing MBA concentrations from 2.5% to 5%, due to interaction of MBA with microorganisms cell wall through H-bonding interactions. Meanwhile, the increased MBA concentration (above 5%) led to decrease in the antibacterial activity and increased the inhibition of fungal growth. This occurred due to the increase in MBA concentration led to an increase in the rigidity of the crosslinked grafted samples that hindered the penetration of N-imidazole rings to the bacterial cell membrane. Also, the increase in MBA concentration led to an increase in the H-bonding interactions between–NH groups in MBA and imine groups of imidazole rings of PVI leading to decrease the amount of positive charges of protonated N-vinyl imidazole rings and a decrease in the amount of electrostatic interactions between them and cytoplasmic membrane of bacterial cell surface. Furthermore, the obtained antimicrobial results confirmed that all crosslinked grafted XG hydrogels (P2-P7) could be used as good antimicrobial drug delivery system.

13. Conclusions

BSA was encapsulated successfully inside safe protein carrier crosslinked grafted XG copolymers (XG-g-PVI/MBA). The swelling ability of crosslinked grafts was performed in presence of two different variables such as; graft yield % and MBA concentrations under acidic (pH 1.2) and slightly alkaline (pH 7.4) media. The swelling rate of grafts increased with the increase in GY % and decrease in MBA concentration. In addition, the swelling rate in pH 7.4 medium was faster than that in acidic (pH 1.2) medium. The effect of various parameters: Graft yield % and MBA, BSA and graft concentrations was studied for determining both the drug loading (DL) and the encapsulation efficiency (EE) %of BSA inside the crosslinked graft chains and for studying the in-vitro BSA release from graft matrices in two different released media (pH 7.4 and 1.2). The results indicated that the EE% increased with the increase in GY%, BSA and graft concentration and the decrease in MBA concentration. On the other hand, the accumulative BSA release % in pH 7.4 medium was faster than in pH 1.2
medium, which increased with increasing the GY % and decreasing BSA, MBA and graft concentrations. The mechanism of in-vitro BSA release from crosslinked grafted networks was kinetically studied according to Koresmeier model. The obtained data showed that the mechanism of BSA release in both pHs media followed non-Fickian release mechanism. Moreover, the structure integrity of BSA after release in dissolution medium through different time intervals was studied with SDS-PAGE analysis and the results confirmed that BSA released without any deformation in its structure through two different release times.

In addition, the cytotoxicity of crosslinked grafted networks was studied against human lung normal cell line and the obtained results showed that the prepared crosslinked grafted networks were safe protein carriers. Moreover, the antimicrobial activity results exhibited that all crosslinked grafted XG samples with good inhibitory effect against two pathogenic microorganisms (S. aureus and A. niger) growth and confirmed that they can be used as antimicrobial protein carrier to transfer the proteins through GI tract. CReDiT authorship contribution statement


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References


