Cytotoxicity and metal ions removal using antibacterial biodegradable hydrogels based on N-quaternized chitosan/poly(acrylic acid)

Riham R. Mohamed, Mahmoud H. Abu Elella, Magdy W. Sabaa*

Chemistry Department, Faculty of Science, Cairo University, Giza, 12613, Egypt

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Physically crosslinked hydrogels resulted from interaction between N,N,N-trimethyl chitosan chloride (N-Quaternized Chitosan) (NQ) and poly(acrylic acid) (PAA) were synthesized in different weight ratios (3:1), (1:1) and (1:3) taking the following codes Q3P1, Q1P1 and Q1P3, respectively. Characterization of the mentioned hydrogels was done using several analysis tools including; FTIR, XRD, SEM, TGA, biodegradation in simulated body fluid (SBF) and cytotoxicity against HepG-2 liver cancer cells. FTIR results proved that the prepared hydrogels were formed via electrostatic and H-bonding interactions, while XRD patterns proved that the prepared hydrogels –irrespective to their ratios- were more crystalline than both matrices NQC and PAA. TGA results, on the other hand, revealed that Q1P3 hydrogel was the most thermally stable compared to the other two hydrogels (Q3P1 and Q1P1). Biodegradation tests in SBF proved that these hydrogels were more biodegradable than the native chitosan. Examination of the prepared hydrogels for their potency in heavy metal ions removal revealed that they adsorbed Fe (III) and Cd (II) ions more than chitosan, while they adsorbed Cr (III), Ni (II) and Cu (II) ions less than chitosan. Moreover, testing the prepared hydrogels as antibacterial agents towards several Gram positive and Gram negative bacteria revealed their higher antibacterial activity as compared with NQC when used alone. Evaluating the cytotoxic effect of these hydrogels on an in vitro human liver cancer cell model (HepG-2) showed their good cytotoxic activity towards HepG-2. Moreover, the inhibition rate increased with increasing the hydrogels concentration in the culture medium.

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1. Introduction

Environmental and microbial pollution are the most global serious problems threatening the human health [1–3]. Environmental pollution is caused by heavy metal ions including Cadmium (Cd), Chromium (Cr), Iron (Fe), Cobalt (Co) and Nickel (Ni) ions that are industrial waste products drained into water streams [3–8]. Heavy metal ions are toxic, carcinogenic and non-biodegradable; accordingly they cause hazardous effects on human health due to their bioaccumulation in living cells [8,9]. There are different techniques for removing toxic heavy metal ions from industrial waste water including; coagulation, precipitation, ion-exchange, electro-dialysis, electro-winning, electro coagulation, solid phase extraction, reverse osmosis and membrane process [3,8]. However, these techniques have drawbacks such as; using high energy and inefficient heavy metal ions removal. Recently, the adsorption technique is the most effective method for removing heavy metal ions from waste water because it is eco-friendly, low cost and high performance technique [3,9–11]. The microbial pollution is another problem resulted by pathogenic microorganisms such as Escherichia coli (E. coli), Pseudomonas aeruginosa (P. aeruginosa) and Staphylococcus aureus (S. aureus). They cause dangerous effect on human health, so great efforts have been exerted to produce new and effective antimicrobial agents [2]. Nowadays, natural polysaccharides, such as chitosan and its derivatives, and synthetic polymers, such as poly (acrylic acid) (PAA) which is a water soluble polymer, are used as adsorbents and antimicrobial agents [12–16].

Hepatocellular carcinoma (HCC) is one more problem, which is the most common type of cancer among the Egyptian population. There are many strategies for treating HCC, but all of them are very expensive and require long term treatment and have several side effects. Therefore another approach is needed for managing such an aggressive type of cancer [17].

Chitosan (Ch) is a chemically modified, biodegradable linear polysaccharide derived by partial alkaline hydrolysis of N-acetyl groups of chitin [18] which is extracted from the shell of shrimps and other crustaceans. It is considered as a copolymer of glucosamine and N-acetyl glucosamine linked via β-(1→4). Ch is used
for many biomedical applications such as; drug delivery systems, gene delivery, wound dressing and tissue engineering [19–22]. Ch applications are very limited due to its very poor solubility as it is only soluble in acids [23–26]. For this, chemical modification of Ch was an urgent need to increase both its solubility and antimicrobial activity due to the permanent positive charge on its backbone.

NN, N-Trimethyl ammonium chitosan, is a water soluble Ch derivative over a wide pH range, it was first prepared by converting the primary amino groups (–NH₂) on C₂ of Ch to N-quatery ammonium (–N(CH₃)₃) groups [27]. It is used in different applications such as drug or protein delivery, food applications and antimicrobial fields [28–34].

Hydrogels are three-dimensional networks, composed of physical or chemical crosslinked homopolymers or copolymers, so they imbibe large amounts of water or biological fluids without being soluble. Recently, the prepared hydrogels from polysaccharides are applied for antimicrobial fields [35] and biomedical applications as they play a crucial role in the cytotoxic profile and targeting of cancers that are characterized by rapid division and aggressive growth [36]. Also, they are used as adsorbent agents for heavy metal ions removal from sewage water [37].

In a previous publication of our research group [38], we have prepared chemically crosslinked hydrogels between N-quatnized chitosan (NQC) and poly(vinyl alcohol) (PVA) using glutaraldehyde and these hydrogels also were characterized via several analysis tools. Different applications have been done on those hydrogels including; metal ions uptake, swellability in different buffer solutions, also biodegradation studies in simulated body fluid (SBF) solutions and antimicrobial activity towards various bacteria and fungi.

The objective of the present work was to synthesize physically crosslinked hydrogels composed of N-quatnized chitosan (NQC) and poly(acrylic acid) (PAA) in different weight ratios. Characterization of the prepared hydrogels was done using different analyses; FTIR, XRD, biodegradation in simulated body fluid (SBF), SEM and cytotoxicity against HepG-2 liver cancer cells. Moreover, the thermal stability of hydrogels was studied using TGA. Some applications were done on the prepared hydrogels including; the antibacterial activity against Gram positive and Gram negative bacteria and removal of some heavy metal ions from their salt solutions.

2. Experimental

2.1. Materials

Chitosan (Ch) (degree of deacetylation 90–95%) was purchased from Oxford-London, UK. poly(acrylic acid) (PAA) (25 wt% aqueous solution) (average molecular weight = 21 × 10⁴ g mol⁻¹) was purchased from Alfa Aesar®-Germany. Sodium chloride, sodium hydroxide and hydrochloric acid were purchased from Merck-Germany. Dimethylsulfate was obtained from Loba chemi Pvt. Ltd., Mumbai, India. Chromium (III), iron (III), nickel (II), copper (II) and cadmium (II) chloride salts were purchased from Sigma-Aldrich–Germany. Visking® dialysis tubing regenerated cellophane (molecular weight cut off 12,000–14,000 g mol⁻¹) was purchased from Serva Electrophoesis, Heidelberg-Germany.

2.1.1. Cytotoxicity assay materials

HepG-2 (liver cancer) cell line, Phosphate buffer saline, pH 7.2 (1×), calcium and magnesium free (Gibco). 0.5% Trypsin–EDTA (10×) (Gibco) diluted to 1× by PBS. (DMEM) Dulbecco’s Modified Eagle Medium, high glucose (Lonza). Complete media: DMEM supplemented with 10% Fetal bovine serum (SeraLab)+1% antibiotic (penicillin G potassium+ streptomycin (Sigma)), 0.4% (wt/vol) Trypan blue (Sigma). Neutral red stock solution 4 mg ml⁻¹: 40 mg neutral red dye was dissolved in 10 ml PBS protected from light by foil. Neutral red medium (40 µg ml⁻¹): the neutral red stock solution diluted with culture medium and incubated overnight at the same temperature of cultured cells. Neutral red destain solution: 50% ethanol 96%(Riedel-de-Häen), 48% deionized water, 1% glacial acetic acid (Sigma).

2.2. Methods

2.2.1. Synthesis of N-Quaternized chitosan (NQC)

N-Quaternized chitosan (N, N, N-Trimethyl ammonium chitosan chloride) (NQC) was prepared by suspending 1 g of Ch in 16 ml of dimethylsulfate and 4 ml of distilled water then adding NaOH and NaCl (1.2 g and 0.88 g, respectively) and stir well for 6 h at room temperature (∼25 °C) [39]. Use dialysis tubing regenerated cellophane (molecular weight cut off 12,000–14,000 g mol⁻¹) for cleaning up the resulting product in distilled water for three days. The final product was obtained by precipitation in acetone then filtration. Finally, the product was dried under vacuum. The structure of NQC was elucidated with ¹H NMR and FTIR techniques.

2.2.2. Synthesis of hydrogels between NQC and PAA

Definite weight of NQC was dissolved in distilled water and was mixed well with a certain amount of aqueous solution of PAA (25 wt%) under stirring for 2 h at room temperature (∼25 °C) to obtain a white gel NQC/PAA in different weight ratios; 3:1 (Q3P1), 1:1 (Q1P1), 1:3 (Q1P3), where Q is NQC and P is PAA. Films of the different hydrogels were obtained by casting gel of the mixed polymers in Petri dishes and were left to dry in vacuum oven till constant weight [40].

2.2.3. Biodegradation studies in SBF solution

Biodegradation studies of Ch and NQC/PAA hydrogels were carried out in vitro by placing the samples in a protein free acellular simulated body fluid medium (SBF or Kokubo solution with pH 7.4), this solution was prepared according to literature [41] and its ionic composition is equal to those in blood plasma. Its composition is reported as follows: Na⁺ = 142.0 mmol L⁻¹, K⁺ = 5.0 mmol L⁻¹, Mg²⁺ = 1.5 mmol L⁻¹, Ca²⁺ = 2.5 mmol L⁻¹, Cl⁻ = 147.8 mmol L⁻¹, HCO₃⁻ = 4.2 mmol L⁻¹, HPO₄²⁻ = 1.0 mmol L⁻¹, SO₄²⁻ = 0.5 mmol L⁻¹. Therefore, the definite weights of Ch and NQC/PAA hydrogels were soaked in SBF solution at pH 7.4 and incubated at 37 ± 1 °C for different time periods (0.5, 2, 4, 24, 96, and 192 h). The samples were removed from the recipient then dried in vacuum oven at 40 °C for 24 h till constant weight. The degradation index (Dₜ) % was calculated based on the weight loss using the following equation [42]:

\[ Dₜ% = \left(\frac{W₀ - Wₜ}{W₀}\right) \times 100 \]

Where, W₀ weight of dry sample, Wₜ is the weight after the immersion time (t). Each degradation experiment was repeated three times and the average value was considered. Biodegradation studies were not done on both NQC and PAA individually due to their water solubility.

2.2.4. Cell viability/cytotoxicity assay

In vitro cytotoxicity assay was done in the cell culture lab, Cairo University Research Park, Faculty of Agriculture, Cairo University. Cell viability (Cytotoxicity assay) of NQC/PAA hydrogels in different ratios (Q3P1, Q1P1 and Q1P3) was done in estimated against HepG-2 liver cancer cell line using neutral red dye (NRD), as described by Repetto et al. [43]. Cells were grown under aseptic conditions with complete medium in a 25 ml cell culture flask with humidified atmosphere and 5% CO₂ at 37°C. Cultured monolayer at 90% confluence subjected to wash with PBS then trypsinized by 2 ml
(0.25%) trypsin–EDTA solution, incubated for 2 min. Then flask was lightly tapped to detach the cells, the reaction stopped by adding 5 ml complete culture medium. The cell suspension counted using hemocytometer and cell viability checked by trypan blue (100% viability). The cells suspension was diluted with complete medium to have approximately 100,000 cells/ml, agitated gently and placed in a sterile reservoir. 200 µl of the cell suspension (containing ~20,000 cell per well) was dispensed by multichannel pipette into the inner 60 wells of the 96 well plate, the peripherals wells were filled with PBS, then the plate incubated for 24 h. After cell seeding and attachment, the media discarded gently and different concentrations of tested samples (Q3P1, Q1P1 and Q1P3) (1, 50, 100, 200, 300, 400 and 500 µg ml⁻¹ using DMSO as solvent) were prepared by diluting with Dulbecco’s modified eagle medium. 200 µl of treatment media was dispensed in 4 replicates for each concentration, other wells were filled with media only (as a negative control) and wells filled with media containing Doxorubicin HCL (4 µg ml⁻¹) as a positive control. After that the 96 well plate covered by lid, incubated at 37°C for 24 h. After the incubation period, the cultures were examined under inverted microscope, recording changes in morphology of the cells due to cytotoxic effects of the test chemical and photos were taken then the medium was decanted from the wells gently without disturbing the materials. 100 µl of Neutral red medium (which was prepared and incubated at 37°C for 24 h and centrifuged at 1800 rpm for 10 min to remove any precipitated dye crystals) was added into each well and incubated again for 3 h at 37°C. After incubation, the dye containing medium was decanted and each well was rinsed gently for two times with 150 µl PBS solution to remove the unabsorbed neutral red dye contained in the wells. 150 µl of destain solution added and incubated for 10 min with shaking.

The absorbance of acidified ethanol solution containing extracted neutral red dye was measured. The viability% and dose response curve were calculated for having the concentration of the test chemical reflecting the half maximum inhibitory concentration of the cell proliferation (IC50).

2.3. Applications for NQC/PAA hydrogels

2.3.1. Heavy metal ions removal

A definite weight of Ch and NQC/PAA hydrogels were immersed in 20 ml of 0.03 mol L⁻¹ of chloride salt solutions of metal ions (Cr³⁺, Fe²⁺, Ni²⁺, Cu²⁺ and Cd²⁺) at room temperature (~25°C) till reached equilibrium (~24 h). After filtration, the concentration of the remaining metal ion solutions was estimated using atomic absorption technique and consequently, the concentration (mg L⁻¹) of adsorbed metal ions can be calculated by difference [44]. Metal ions uptake was not done on both NQC and PAA individually due to their water solubility. Removal of heavy metal ions (%) and Adsorption capacity (Q) were calculated using the following equations [45]:

Removal of heavy metal ions% = ([(Mp²⁺)/[M²⁺]])X100

Where, [Mp²⁺] is conc. of adsorbed metal ions in polymer (mg L⁻¹), [M²⁺] is initial conc. of metal ions (mg L⁻¹)

Q(mg g⁻¹) = V[(Mp²⁺)]/W

Where, [Mp²⁺] is conc. of adsorbed metal ions in polymer (mg L⁻¹), V (L) is the volume of metal solutions and W (g) is the weight of the dry hydrogel.

2.3.2. Antimicrobial activity

Antimicrobial activity of NQC, PAA and NQC/PAA hydrogels were evaluated against S. aureus, B. subtilis and S. faecalis as examples of Gram positive bacteria and E. coli, P. aeruginosa and N. gonorrhoeae as examples of Gram negative bacteria. Dimethylsulphoxide (DMSO) was used as solvent control. Antibacterial test was carried out using the agar disc diffusion method. The concentration of the tested sample was 1 mg mL⁻¹. Norfloxacin was used as a reference drug (standard) against both Gram positive and Gram negative bacteria (Wide spectrum antibiotic). The plates were incubated at 37°C for 24 h for bacteria. After incubation, antibacterial activity was evaluated by measuring the inhibition zones diameter against the test organisms and was compared with that of the standard. Antimicrobial activity was expressed as inhibition diameter zones in millimeters (mm). The experiment was carried out in triplicates, the average inhibition zone diameter was calculated [46].

2.3.3. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) of antibacterial activity was determined by microdilution susceptibility test in Muller–Hinton Broth (Oxoid) and Sabouraud Liquid Medium (Oxoid). Stock solutions were prepared by dissolving the tested samples in DMSO. Stock solutions were diluted with standard method broth to prepare two-fold serial dilutions of the broth containing about 10⁶ CFU ml⁻¹ of test bacteria then it was added to each well of 96-well microtiter plates. The microplates were incubated for 24h at 37°C for antibacterial activity. MIC values were determined by recording the lowest concentration of the substance that had no visible turbidity. Control set containing DMSO was performed parallel to the test samples under the same conditions [46].

2.3.4. Instrumentation

Proton Nuclear Magnetic Resonance (¹H NMR). ¹H NMR spectra of Ch and NQC were scanned by Varian Mercury VX-300 NMR Spectrometer. ¹H spectra were run at 300 MHz in DMSO/trifluoroacetic acid (TFA) as solvent for Ch and NQC. Chemical shifts are quoted in and were related to that of the solvents.

Fourier Transform Infrared (FTIR), FTIR spectra of samples were recorded in the frequency range of 600–4000 cm⁻¹ using Jasco FTIR 4100 spectrophotometer [Japan]. Scanning Electron Microscopy (SEM), Surface morphology of samples was obtained using JEOL (JSM-5200). Samples were prepared by placing a small part of the film on a carbon tape on a stub, which was coated with a thin layer of gold. X-Ray Diffraction (XRD): XRD of samples were obtained using an X-ray powder diffractometer (a Philips Xpert MPD PRO) with Ni-filtered Cu-Kα radiation (λ = 0.154 nm) at an accelerating voltage/current of 50 KV/40 mA. The relative intensity was recorded in the scattering range 2θ, varying from 3° to 60° at a scan speed of 1 step s⁻¹. Thermogravimetric Analysis (TGA), TGA spectra of samples were done on TGA-50H Shimadzu Thermogravimetric Analyzer. The measurements were done over temperature ranges from room temperature to 700°C and the reference material was alumina with a dynamic heating rate 10°C min⁻¹ under nitrogen atmosphere. The sample weights in all experiments were taken in the range 4–5 mg. Atomic Absorption, Concentration of the remaining metal ions in the Ch and hydrogels liquor was estimated by using A Analyst 100 win lab-Perkin Elmer. Cytotoxicity assay absorbance was measured using spectrophotometer (BioTek, ELX808).

3. Results and discussion

3.1. Proton nuclear magnetic resonance spectrum (¹H NMR)

¹H NMR spectrum – Fig. 1- has proven the preparation of NQC and its comparison with Ch. It showed two singlet signal at δ= 2.0 and 2.9 ppm corresponding to protons of the methyl (–CH₃) of the remained N-acetyl (–NHCOCH₃) groups and protons bonded to C₂ of N-glucosamine and N-acetyl glucosamine, respectively [47,48].
In addition to that, multiplet signal appeared at $\delta = 3.4–3.6$ ppm which assigned to protons bonded to C3, C4, C5 and C6 of glycopyranose unit [H3, H4, H5 and H6], two singlet signal appeared at $\delta = 4.5$ and $7.5$ ppm assigned to proton bonded to anomeric C1 of glycopyranose unit [H1] and protons of amino (-NH$_2$) groups [49,50]. Moreover, two sharp signals appeared at $\delta = 2.5$ and $8.6$ ppm related to dimethylsulphoxide (DMSO) and trifloroacetic acid (TFA), respectively, as solvents.

The degree of N-quaternization percentage (DQ%) was calculated from $^1$H NMR spectra according to the following equation:

$$\text{DQ} (%) = \frac{(-N^+\text{CH}_3)_i / 9}{[\text{H}_1]}$$

Where: $[(-N^+\text{CH}_3)_i]$ was the integral area under the signal of the $N,N,N$-trimethyl protons at $\delta = 3.2$ ppm and [H1] was the integral area under the signal of H1 proton at $\delta = 4.8–5.6$ ppm [50]. DQ% of NQC was determined as 42%.

3.2. Fourier transform infrared (FTIR) spectroscopy

FTIR spectrum (Fig. 2a) had proven the preparation of NQC and its comparison with Ch. The spectrum showed the main peaks for Ch such as the broad peak at 3435 cm$^{-1}$ due to stretching vibration of O–H and N–H bonds and intra-hydrogen bond between them. Also peaks at 2918 and 2879 cm$^{-1}$ related to C–H symmetric stretching vibration of alkyl groups, absorption peaks at 1653 cm$^{-1}$ was corresponding to the stretching vibration of C–O bond of the remaining N-acetyl group (–NHCOCH$_3$). Also, absorption peak at 1597 cm$^{-1}$ assigned to N–H bending vibration of the amino (–NH$_2$) groups. Absorption peaks around 1155 and 895 cm$^{-1}$ corresponded to C–O–C bending vibration in saccharide repeated unit in Ch [51,52]. Broad peaks at 1080 and 1031 cm$^{-1}$ assigned for C–OH stretching vibration of 2nd and 1º alcohol on the backbone, respectively [51,52]. However, some changes have been observed in NQC spectrum. The FTIR spectrum of NQC showed absorption peak at 3435 cm$^{-1}$ corresponded to O–H and N–H stretching vibration.
was lower intensity than this peak in Ch due to converted amino (−NH2) groups to monomethyl amino (−NH(CH3)2), dimethyl amino (−N(CH3)2) and quaternized amino (−N(CH3)3) groups, so the H-bonding interactions decreased in NQC structure. In addition to that, the absorption peaks at 1653 cm−1 assigned to the carbonyl (C=O) stretching vibration of remaining N-acetyl (−NHCOCH3) groups [51]. The presence of absorption peaks at 1470 cm−1 corresponded to the asymmetric stretching of C−H, had confirmed the presence of the methyl groups on the nitrogen atoms of the amino groups in Ch such as ((−NHCH3), (−N(CH3)2), and (−N′(CH3)3)) groups.[52]

While, FTIR spectrum of PAA revealed that the strong absorption peak that appeared at 1720 cm−1 related to the stretching vibration of carboxylic acid (−COOH) groups, also peaks at 1247 and 1179 cm−1 corresponded to the stretching vibration of (C=O) and (C−O−C), respectively [53]. The broad peak from 3576-2560 cm−1 was due to the stretching vibration of the hydroxyl (−OH) groups of carboxylic acid [54]. Fig. 2b illustrates FTIR spectra of NQC/PAA hydrogels (Q3P1, Q1P1, and Q1P3). It showed broad peak near 3438 cm−1 due to stretching vibration of the hydroxyl (−OH) groups of both NQC and carboxylic acid groups of PAA and the amino (−NH) groups of NQC. This peak was broader than the one in PAA and NQC, individually, because of the intermolecular H-bonding interactions between NQC and PAA. In addition to that, absorption peak that appeared at 2933 cm−1 is related to (C−H) stretching vibration in alky groups, peak at 1720 cm−1 corresponded to the stretching vibration of carbonyl (C=O) groups of (−COOH) groups, the intensity of this peak increased with increasing the% of PAA. A peak appeared at 1630 cm−1 attributed to stretching vibration of the carboxylate (−COO−) groups [55,56]. Furthermore, peak of asymmetric stretching of (C=H) at 1470 cm−1 in NQC shifted to 1450 cm−1 due to the hydrogen bonding interactions between N-alkyl groups of NQC and the carboxylic acid groups of PAA.

The results of FTIR analysis had proved the formation of the hydrogels through secondary binding forces such as electrostatic interaction between N-quatarnary ammonium (−NH4+) groups of NQC and carboxylate (−COO−) groups of PAA, and H-bonding interaction between NQC and PAA (Scheme 1). This scheme is similar to formation of polyelectrolyte complex between Ch and PAA scheme which was reported in literature through ionic and H-bonding interactions [57].

3.3. Scanning electron microscopy (SEM) (Magnification ×1000)

Scanning electron microscopy (SEM) micrographs of Ch, NQC, PAA and NQC/PAA hydrogels of different weight ratios (Q3P1, Q1P1 and Q1P3) are illustrated in Fig. 3. The images showed that the flaky nature of Ch surface was completely changed into a surface covered with lumps in case of NQC due to the introduction of extensive methyl groups. While SEM micrographs of PAA surface showed the presence of aggregations on its surface due to the presence of carboxylic groups on its surface. The images of NQC/PAA hydrogels showed that their surface is porous, irregular in shape and have some aggregation.

3.4. X-Ray diffraction (XRD)

X-ray diffraction (XRD) patterns of Ch and NQC are illustrated in Fig. 4a. Ch exhibited three diffraction peaks, a sharp diffraction peak at 2θ = 20.0°, a broad diffraction peak at 2θ = 9.5° and a small diffraction peak at 2θ = 30° which confirmed the crystalline structure of Ch. These crystallinity was attributed to the ordered regions due to the intra- and intermolecular H-bonding interactions among the amino (−NH2), the remaining N-acetyl (−NHCOCH3) and the hydroxyl (−OH) groups on Ch chains [58,59]. While, XRD pattern of NQC showed diffraction peak at 2θ = 20° but less intense than its corresponding peak in Ch, hence, NQC is less crystalline than Ch due to the presence of methyl groups on its chains leading to the destruction of the crystallinity of Ch. Moreover, XRD patterns of NQC, PAA and NQC/PAA hydrogels (Q3P1, Q1P1 and Q1P3) are represented in Fig. 4b.

The XRD pattern of PAA showed two small broad diffraction peaks at 2θ = 18° and 37°. These peaks showed the amorphous nature of PAA [61,62]. In addition to that, the XRD pattern of NQC/PAA hydrogels showed broad diffraction peak at 2θ = 19° but more intense than those found in both PAA and NQC, so hydrogels are more crystalline than both PAA and NQC, individually. The intensity of the diffraction peak at 2θ = 19° increased with increasing PAA content (%) due to the more H-bonding between NQC and PAA chains. The crystallinity of hydrogels increased according to the following order:

Q1P3 > Q1P1 > Q3P1
3.5. Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) thermograms of Ch, NQC, PAA and NQC/PAA hydrogels of different weight ratios (Q3P1, Q1P1 and Q1P3) are represented in Fig. 5. The TGA thermograms of Ch and NQC showed that the initial decomposition temperatures (IDT) of Ch and NQC were found to be; 240 and 214°C, respectively. NQC had the least IDT, so NQC was the least thermally stable one, due to the extensive methyl groups of N-quaternized salts which led to an increase in the intermolecular spaces between the chains, which decreased the H-bond interactions between the chains [63]. TGA thermogram of PAA showed that PAA degraded on two steps; the first weight loss step was below 180°C (about 7%) due to the loss of the absorbed water, while the second weight loss step was in the...
IDT, so it was higher thermal stability than NQC due to the intra- and intermolecular H-bonding interactions among PAA chains leading to strong PAA–PAA chains interaction, also, the hydrogels (Q3P1, Q1P1 and Q1P3) had higher IDT and were thermally more stable than NQC due to the more intra- and intermolecular H-bonding interactions among PAA and NQC chains.

Moreover, Q1P3 hydrogel had the highest IDT, so it was the highest thermally stable one due to more intermolecular H-bonding interactions among NQC and PAA chains leading to strong NQC-PAA chains interaction than the other hydrogels. Thus, the thermal stability increased according to the following order:

\[
Q1P3 > Q1P1 > PAA > Q3P1 > NQC
\]

3.5.1. In vitro biodegradation studies in simulated body fluid (SBF) solution

Biodegradation studies of Ch and NQC/PAA hydrogels of different weight ratios using the SBF solution (pH 7.4) at 37 ± 1 °C for different immersion time (0.5, 2, 4, 24, 96, 192 h) are illustrated in Fig. 6. Biodegradation studies were not done on both NQC and PAA individually due to their water solubility. The results showed that Ch was the least biodegradable one due to the intra- and intermolecular H–bonding between the hydroxyl (–OH), amino (–NH2) and residual acetamido groups. Also, the results showed that the degradation index (weight loss) % of hydrogels increased with the
increase of NQC content in the hydrogels, so Q3P1 was the most degradable one because Q3P1 surface composed of porous and irregular zones (as it was mentioned previously in SEM section – Fig. 3). When the samples immersed in SBF solution, the solution could easily diffuse into the inner side of hydrogels, resulting in a rapid degradation from the outside to the inner side, as reported by Zhuang et al. [64]. So Q3P1 had the highest weight loss%. The degradation index (weight loss)% increased with increasing the immersion time. The degradation index (weight loss)% increased according to the following order:

Q3P1 > Q1P1 > Q1P3 > Ch

3.5.2. Cell viability/cytotoxicity assays

Cytotoxicity effect of NQC/PAA hydrogels on cell viability of HepG-2 liver cancer cell was illustrated in Fig. 7a and the half maximal inhibitory concentration (IC50) was calculated in every case. The cytotoxic effect of hydrogels was shown at various concentrations of hydrogels (500, 400, 300, 200, 100, 50 and 1 µg ml⁻¹) towards HepG-2 cell cultures. Results showed that 400 µg ml⁻¹ of Q1P1 killed 87.5% of the cells after 48 h of cell exposure and IC50 reached 275 µg ml⁻¹ while 400 µg ml⁻¹ of Q1P3 killed 89.3% of the cells after 48 h of cell exposure and IC50 reached 319 µg ml⁻¹ and 400 µg ml⁻¹ of Q3P1 killed 85.7% of the cells after 48 h of cell exposure and IC50 reached 326 µg ml⁻¹. Moreover, the inhibition rate increased with increasing hydrogels concentration in the culture medium-whatever their ratio-. This indicates that the inhibition of cell viability by NQC/PAAA hydrogels was clearly dose and time dependent. Cytotoxicity data of NQC/PAA hydrogels towards HepG-2 is better than others reported in literature [63].

After the incubation period, cultures were examined under inverted microscope, recording changes in morphology of cancer cells due to cytotoxic effects of NQC/PAA hydrogels. Fig 7b showed the various morphological changes that occur after treatment with 500 µg ml⁻¹ concentrations of the hydrogels. Cell shrinkage occurred and the cells became smaller in size and the cytoplasm became condensed and more tightly packed.
Table 1
Concentration of metal ions uptake (Cr³⁺, Fe³⁺, Ni²⁺, Cu²⁺ and Cd²⁺), percentage of metal ions uptake (metal ions removal%) and adsorption capacity (Q) of Ch and NQC/PAA hydrogels samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Cr³⁺</th>
<th>Fe³⁺</th>
<th>Ni²⁺</th>
<th>Cu²⁺</th>
<th>Cd²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Metal ions uptake (mg L⁻¹)</td>
<td>Metal ions uptake (%)</td>
<td>Q (mg g⁻¹)</td>
<td>Metal ions uptake (mg L⁻¹)</td>
<td>Metal ions uptake (%)</td>
</tr>
<tr>
<td>Ch</td>
<td>772</td>
<td>44.0</td>
<td>332.0</td>
<td>1618</td>
<td>91.8</td>
</tr>
<tr>
<td>Q1P3</td>
<td>714</td>
<td>43.5</td>
<td>275.9</td>
<td>1543</td>
<td>59.7</td>
</tr>
<tr>
<td>Q1P1</td>
<td>691</td>
<td>42.2</td>
<td>269.5</td>
<td>817</td>
<td>63.1</td>
</tr>
<tr>
<td>Q3P1</td>
<td>601</td>
<td>36.7</td>
<td>233.5</td>
<td>290</td>
<td>59.0</td>
</tr>
</tbody>
</table>

Fig. 8. Antibacterial activity of NQC/PAA hydrogels of different weight ratios towards Gram positive bacteria.

3.6. Application of NQC/PAA hydrogels

3.6.1. Removal of heavy metal ions

Adsorption capacity (Q) of toxic heavy metal ions such as Cr³⁺, Fe³⁺, Ni²⁺, Cu²⁺ and Cd²⁺ by NQC/PAA hydrogels are tabulated in Table 1. Adsorption capacity investigations were not done on both NQC and PAA individually due to their water solubility.

The metal ions adsorption by Ch is due to the presence of many available chelating sites on its chains such as the nitrogen atoms of the amino (−NH₂) groups and the oxygen atoms of the hydroxyl (−OH) groups. While NQC/PAA hydrogels metal ions adsorption might be due to the available chelating sites on its chains like nitrogen atoms of both the N-monomethyl and N-dimethyl groups and the oxygen atoms in both the hydroxyl and methoxy groups in NQC. Also the carbonyl groups (−C=O) of carboxylic acid in PAA. The results illustrated that the prepared hydrogels adsorbed more metal ions than Ch in case of Fe³⁺ and Cd²⁺ ions, while Ch adsorbed more metal ions than the hydrogels in case of Cr³⁺, Ni²⁺ and Cu²⁺ ions.

The adsorption of metal ions depends on both the attributes of metal ions (ionic sizes, electro positivity, and reactivity) and the different structural aspects of the polymer itself [65].

3.6.2. Antibacterial activity

Antibacterial activity of NQC, PAA, and NQC/PAA hydrogels of different weight ratios (Q3P1, Q1P1 and Q1P3) were investigated against three Gram negative bacteria (E. coli, P. aeruginosa and N. gonorrhoeae) and three Gram positive bacteria (S. aureus, B. subtilis and S. faecalis) in presence of Norfloxacin (wide spectrum antibiotic) as standard using the disk diffusion method. The results of antibacterial activity of these samples – tabulated in Table 2- had shown that the hydrogels had higher antibacterial effect toward both Gram positive and Gram negative bacteria than both of NQC and PAA, individually. Also the agar plates were shown in Figs. 8 and 9 indicating the inhibition zones diameter (mm) against the test organisms.
Table 2
Antibacterial activity of NQC, PAA and NQC/PAA hydrogels of different weight ratios (Q3P1, Q1P1 and Q1P3).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Inhibition zone diameter (mm)</th>
<th></th>
<th></th>
<th>Gram- positive bacteria</th>
<th></th>
<th></th>
<th>Gram-negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E. coli</td>
<td>P. aeruginosa</td>
<td>N. gonorrhoeae</td>
<td>S. aureus</td>
<td>B. subtilis</td>
<td>S. faecalis</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>34.83 ± 0.62</td>
<td>34.33 ± 1.25</td>
<td>35.17 ± 0.62</td>
<td>35.0 ± 0.82</td>
<td>33.33 ± 1.25</td>
<td>34.83 ± 0.62</td>
<td></td>
</tr>
<tr>
<td>NQC</td>
<td>25.33 ± 0.24</td>
<td>25.17 ± 0.24</td>
<td>25.33 ± 0.24</td>
<td>22.50 ± 0.41</td>
<td>22.0 ± 0.82</td>
<td>22.17 ± 0.94</td>
<td></td>
</tr>
<tr>
<td>PAA</td>
<td>23.17 ± 0.27</td>
<td>23.83 ± 0.62</td>
<td>25.0 ± 0.41</td>
<td>22.50 ± 0.41</td>
<td>20.67 ± 0.47</td>
<td>22.0 ± 0.71</td>
<td></td>
</tr>
<tr>
<td>Q1P3</td>
<td>29.0 ± 0.41</td>
<td>29.0 ± 0.82</td>
<td>28.17 ± 0.24</td>
<td>28.17 ± 0.24</td>
<td>26.67 ± 0.47</td>
<td>28.83 ± 0.85</td>
<td></td>
</tr>
<tr>
<td>Q1P1</td>
<td>27.50 ± 0.41</td>
<td>26.17 ± 0.24</td>
<td>32.50 ± 0.41</td>
<td>29.50 ± 0.41</td>
<td>26.50 ± 0.41</td>
<td>24.67 ± 0.47</td>
<td></td>
</tr>
<tr>
<td>Q3P1</td>
<td>24.83 ± 0.24</td>
<td>22.50 ± 0.41</td>
<td>27.67 ± 0.24</td>
<td>25.0 ± 0.82</td>
<td>24.83 ± 0.24</td>
<td>24.0 ± 1.08</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 9. Antibacterial activity of NQC/PAA hydrogels of different weight ratios towards Gram negative bacteria.

and compared with that of the standard. The exact mechanisms of antibacterial activities of Ch and its derivative have not been known yet [66]. Several mechanisms elucidating the antimicrobial activity of Ch have been postulated, the mechanism is based on the electrostatic forces between the protonated amino groups 

(-N+$\text{H}_3$) of Ch and the electronegative charges on cytoplasmic membrane of the bacterial cell surface [66]. The main constituents of the cytoplasmic membrane are proteins and phospholipids, the phospholipids of bacteria are phosphoglycerides (fatty acid with long chains hydrocarbons) [67].

Also, polymer might cause strong attachment through the bacterial surface by H-bonding interaction [66]. Table 2 showed that PAA and NQC had higher antibacterial activity because PAA has free carboxylic acid groups which could interact with the bacterial cell surface through H-bonding interaction [15], while NQC synthesized with degree of quaternization (DQ 42%) and with better solubility in aqueous media has high cationized ammonium (-N+$\text{H}_3$) groups which could strongly interact by electrostatic forces with the cytoplasmic membrane of bacterial cell surface [60]. Also, it was shown that the NQC/PAA hydrogels had higher antibacterial activity than the parent NQC and PAA, due to the interaction between NQC and PAA which led to the increase in the number of cationized ammonium (-N+$\text{H}_3$) groups on NQC chains, thus, leading to a better antibacterial activity. On the other hand, as the% of PAA increased, the antibacterial activity of hydrogels increased due to the increasing of the free carboxylic acid groups which could interact with bacterial cell surface through H-bonding, so Q1P1 and Q1P3 had the highest antibacterial activity. Minimum inhibitory concentration (MIC), which is the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation. The Q1P1 and Q1P3 hydrogels were measured against N. gonorrhoeae and S. aureus and the values were compared to Norfloxacin (Standard). The results were represented in Table 3. It had been shown that Q1P3 had higher antibacterial activity (low MIC value) against S. aureus than Q1P1, while Q1P1 had
higher antibacterial activity (low MIC value) against N. gonorrhoeae than Q1P3.

4. Conclusion

Synthesis of hydrogels based on NQC with polyacrylic acid (PAA) from different weight ratios (3:1, 1:1, and 1:3) (Q1P1, Q1P1, and Q1P3) – where Q is NQC and P is PAA- has been studied, and were characterized via several analysis tools such as: FTIR, XRD, SEM and TGA. Different applications had been performed on NQC/PAA hydrogels. The following results were obtained:

- TGA results revealed that PAA had high IDT, so it had higher thermal stability than NQC due to the intra- and intermolecular H-bonding interactions among PAA chains interaction, also the prepared hydrogels had higher thermal stability than NQC due to the more intra- and intermolecular H-bonding interactions among PAA and NQC chains.
- Biodegradation in SBF results showed that the weight loss of hydrogels increased with increasing NQC content, so Q3P1 was the most biodegradable one due to the fact that its surface contained large halo zones, which facilitated the diffusion of SBF solution into the inner side of the hydrogel, resulting in a rapid biodegradation from outside to inside.
- Evaluating the cytotoxic effect of the prepared hydrogels on an in vitro human liver cancer cell model (HepG-2) showed that they have a good cytotoxic activity towards HepG-2. The half maximal inhibitory concentration (IC50) was calculated for Q1P1 275 µg ml⁻¹ while for Q1P3 reached 319 µg ml⁻¹ and for Q3P1 reached 326 µg ml⁻¹. Moreover, the inhibition rate increased with increasing hydrogels concentration in the culture medium.
- Adsorption of heavy metal ions (Cr⁺³, Fe⁺³, Ni²⁺, Cu²⁺ and Cd²⁺) results by the prepared hydrogels were compared to the parent Ch. They showed that hydrogels adsorbed more Fe⁺³ and Cd²⁺ ions than the parent Ch, while Ch adsorbed more Cr⁺³ and Ni²⁺ and Cu²⁺ ions as hydrogels.
- Antibacterial activity results revealed that the investigated hydrogels had higher antibacterial activity towards both Gram positive and Gram negative bacteria than both NQC and PAA individually. The antibacterial activity of the hydrogels increased due to the increase in the free carboxylic acid groups, which could interact with the bacterial cell surface through H-bonding interaction.

References
