Synthesis of novel grafted hyaluronic acid with antitumor activity

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ABSTRACT

In our study, we aimed to synthesize novel grafted hyaluronic acid with cationic biodegradable polymer, poly (N-vinyl imidazole) (PVI), through free radical polymerization using potassium persulfate as initiator. The effect of various grafting factors including initiator and monomer concentrations, reaction time and temperature was studied on the percentage of grafting parameters such as; graft yield (% GY), grafting efficiency (% GE) and amount of homopolymer formation (% H). Maximum grafted HA was % GY = 235% and %GE = 83% obtained on optimum conditions at [In] = 17.5 mmol L⁻¹, [M] = 1.25 mol L⁻¹, Temp. = 50 °C, time = 1.5 h and [HA] = 0.025 mol L⁻¹. The structure of grafted HA (HA-g-PVI) was elucidated via various analysis tools such as; elemental analyses, FTIR, ¹H NMR, XRD, TGA and Field emission scanning electron microscopy (FE-SEM). Hepatic and breast cancers are two common cancer types threatening people worldwide, so, the antitumor activity of two grafted HA samples (% GY = 155% and 235%) was studied against hepatic cancer (HepG-2) and breast cancer cell lines (MCF-7) compared to unmodified HA and PVI. The results showed that antitumor activity of grafted samples was more than unmodified HA and increased with increasing the grafting percentage of PVI onto HA chains, also, the antitumor activity of tested samples against HepG-2 cell lines was higher than MCF-7 cell lines.

1. Introduction

Cancer is a worldwide problem threatening human life. It involves an altered DNA sequence leading to abnormal cell division expressed in tumors, organs dysfunction and finally death. Cancer is the second disease that leads to death in United States. In 2016, a total number of cancer patient is 1,685,210 and cancer death cases are 595,690 (Debele, Mekuria, & Tsai, 2016; Siegel, Miller, & Jemal, 2016). Cancer is a disease that leads to death in United States. In 2016, a total number of cancer patient is 1,685,210 and cancer death cases are 595,690 (Debele, Mekuria, & Tsai, 2016; Siegel, Miller, & Jemal, 2016). Cancer disease includes different types such as; hepatic and breast cancers. Hepatic and breast cancer are two common cancer types threatening people worldwide. Hepatic cancer is chronic disease and causes serious health problem, so it is the third common type of cancer related to death worldwide, while, breast cancer is the second common type of cancer that causes death among women (Ma, 2013; Salama et al., 2010; Tang, Wang, Kiani, & Wang, 2016; Torre et al., 2015).

Polysaccharides are polymeric carbohydrates formed through glycosidic linkages between monosaccharides as repeating units (Debele et al., 2016). Recently, they are used in different biomedical and pharmaceutical fields due to their abundance in nature and low cost processing and their physicochemical properties including nontoxicity, biocompatibility and biodegradability (Debele et al., 2016; Jian et al., 2012; Rodrigues, Cardoso, da Costa, & Grenha, 2015). Hyaluronic acid (HA) is a water-soluble anionic polysaccharide. Its structure composed of N-acetyl-β-D-glucosamine and β-D-glucuronic acid as repeating disaccharide units linked by (1→3) and (1→4) glycosidic bonds (Fig. 1). HA has anionic character because it is in the form of sodium salt. Consequently, HA is highly hydrophilic. HA has a high molecular weight (10⁶–10⁷ Da) and is represented in extracellular matrix of cartilage. It has many properties as; non-toxicity, biocompatibility, biodegradability and non-antigenic, so it is widely used in biomedical applications (Laurent & Fraser, 1992; Nakagawa, Nakasako, Ohta, & Ito, 2015; Schanté, Zuber, Herlin, & Vandamme, 2011).

Compounds containing imidazole moiety are used in biomedical applications due to its biodegradability, biocompatibility, cationic nature and thermal stability. N-vinyl imidazole is used as a good grafting agent due to its water solubility. N-vinyl imidazole can be grafted by free radical polymerization (Anderson & Long, 2010; Caner, Yilmaz, & Yilmaz, 2007). The imidazole ring biocompatibility provides a scaffold for biomimetic applications (Le Poul et al., 2007), including the use of imidazoles as DNA sequence targets for alkylating DNA and suppressing gene expression (Bando & Sugiyama, 2006). Not only are imidazoles biocompatible, but they are also antimicrobial (Luca, 2006).

The aim of this study was to synthesize novel grafted hyaluronic acid (HA) with synthetic biodegradable polymer, poly (N-vinyl imidazole) (PVI) and characterize the grafted structure (HA-g-PVI) via different analysis tools (FTIR, ¹H NMR, XRD, FE-SEM, TGA and elemental analyses), then examine the antitumor activity of the grafted sample...
against two different cancer cell lines namely HepG-2 and MCF-7 as compared to unmodified HA and PVI.

2. Materials and experimental methods

2.1. Materials

Sodium salt of hyaluronic acid (Mwt = (1.0–1.8) X 10^6 Da) was purchased from Euromedex- France. N-vinyl imidazole, potassium persulfate (K2S2O8), absolute ethanol, aceton, crystal violet and trypan blue dyes were purchased from Sigma-Aldrich, Germany. Human hepatocellular cancer cell line (HePG-2) and human breast cancer cell line (MCF-7) were obtained from VACSERA-Tissue Culture Unit, Egypt. Fetal bovine serum, L-glutamine, gentamycin, Dulbecco’s modified Eagle’s medium (DMEM) and 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) buffer solution were purchased from Lonza, Basel, Switzerland.

3. Experimental methods

3.1. Grafting of PVI onto HA

PVI was grafted onto HA via free radical polymerization reaction in presence of potassium persulfate (PPS) as initiator under nitrogen atmosphere (Scheme 1). Briefly, 0.25 g (0.025 M) of HA (Wo) was dissolved in 25 mL of distilled water under continuous stirring for 24 h then different amounts of initiator solution (2.5–22.5 mmol L\(^{-1}\)) were dropwisely charged to the above solution. Various amounts of N-vinyl imidazole (0.25–1.75 mmol L\(^{-1}\)) (W1) were added dropwisely to the previous mixture under nitrogen atmosphere. The reaction was done at different reaction temperatures (30–60 °C) and time period (1–2.5 h). The grafted mixture was precipitated in cold acetone under vigorous stirring, then the precipitate was separated via filtration using a G2 sintered glass funnel and washed three times with acetone then was dried in vacuum oven at 50 °C till constant weight (W2). The grafted products were purified from homopolymer with absolute hot ethanol using soxhlet for 8 h, then the purified products were dried in vacuum oven at 50 °C till constant weight (W3).

The percentage of grafting parameters such as: grafting yield (%GY), grafting efficiency (%GE) and homopolymer (%H) were calculated according to the following Eqs. (1)–(3) (Abu Elella, Mohamed, ElHafeez, & Sabaa, 2017; Badwaik, Sakure, Alexander, Dhongade, & Tripathi, 2016; Caner et al., 2007).

\[
\% \text{GE} = \left(\frac{W_1 - W_0}{W_0}\right) \times 100
\]  

\[
\% \text{H} = \left(\frac{W_2 - W_1}{W_3}\right) \times 100
\]  

\[
\% \text{GY} = \left(\frac{M_o \times GY}{M_g \times 100}\right)
\]  

Where: \(W_0\) is weight of HA, \(W_1\) is weight of grafted product after purification, \(W_2\) is weight of grafted product before purification and \(W_3\) is weight of N-vinyl imidazole. All experiments were done in triplicates and the mean average was taken.

The number of grafted PVI chain (Ng) per repeated HA chain is known as grafting frequency (GF) and was calculated according to the following Eq. (4) (Gurdag & Sarmad, 2013).

\[
\text{GF} = \left(\frac{M_o \times GY}{M_g \times 100}\right)
\]  

Where: \(M_o\) is the molecular weight of HA, \(M_g\) is the molecular weight of grafted copolymer and GF is the percentage of grafting yield. Molecular weight of HA and grafted copolymer (%GY = 235%) was determined by viscometric method (Podzimek, Hermannova, Bilero, Bezakova, & Velebny, 2010) and it was found 1.49 × 10^6 g mol\(^{-1}\) and 2.55 × 10^6 g mol\(^{-1}\), respectively. The grafting frequency (GF) for grafted copolymer (%GY = 235%) was calculated as 1.37.

3.2. Antitumor activity

Antitumor activity (cytotoxicity assay) was done in Cell Culture Laboratory, Regional Center for Mycology and Biotechnology, Azhar University, Egypt. Cytotoxicity assay of tested samples (HA, PVI and two grafted products with %GY 155 and 235%) was evaluated against human hepatic and breast cancer cell lines (HePG-2 and MCF-7, respectively) according to literature (Gomha, Riyadh, Mahmmoud, & Elasser, 2015; Gomha, Salaheldin, Hassaneen, Abdel-Aziz, & Khedr, 2015; Mesmann, 1983). HePG-2 and MCF-7 cell lines were propagated in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, HEPES buffer and 50 μg mL\(^{-1}\) gentamycin. The propagated cells were maintained at 37 °C in a humidified atmosphere with 5% CO\(_2\) then were subcultured 2–3 times a week. For cytotoxicity assay, the cells were seeded in 100 μL of growth medium in 96 well plates at a cell concentration of 10^4 cells/well. After 24 h of seeding, fresh medium contains different concentrations of tested samples using water as a solvent was charged to previous seeded cells. Two-fold serial dilutions (3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, 1000 μg mL\(^{-1}\)) of the tested samples were added to confluent cell monolayers dispensed into 96 well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette which were incubated for 48 h in a humidified incubator at 37 °C with 5% CO\(_2\). Three wells were used for each tested sample concentration. Control cells were incubated without test polymeric sample. After incubation of the cells, various concentrations of tested polymeric samples were added, and the incubation was continued for 24 h at 37 °C and cell viability yield was determined by a colorimetric method. After incubation, media were aspirated and 1% crystal violet solution was added to each well for 30 min then removing the stain and rinsing the plates using water until all excess stain is removed. 30% of glacial acetic acid was added to all wells. The plates were shaken on Microplate reader then the absorbance was measured at 490 nm. All experiments were carried out in triplicate and percentage of cell viability of each tested sample was calculated according to its optical density according to the following Eq. (5):

\[
\text{GF} = \left(\frac{M_o \times GY}{M_g \times 100}\right)
\]  

Scheme 1. Preparation of grafted HA with PVI (HA-g-PVI).
% Cell Viability = \[1 - \frac{OD_t}{OD_c}\] \times 100 \quad (5)

Where: OD<sub>t</sub> = the mean optical density of wells treated with tested samples and OD<sub>c</sub> = the mean optical density of untreated cells. The half maximum inhibitory concentrations (IC<sub>50</sub>) of tested samples is defined as the sample concentration required to cause toxic effects in 50% of intact cells, was estimated in every case from dose response curve, it is the relation of surviving cells (% cell viability) and tested samples concentrations, using Graph pad Prism software.

4. Characterization

4.1. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of HA, HA-g-PVI and PVI were done on Jasco FTIR 4100 spectrometer (Japan) using KBr pellets within the wavenumber range of 4000–600 cm<sup>-1</sup> at 25 °C.

4.2. Proton nuclear magnetic resonance (1H NMR) spectroscopy

1H NMR spectra HA, HA-g-PVI and PVI were determined by Varian Mercury (VX-300) NMR Spectrometer at 300 MHz in D<sub>2</sub>O as solvent.

4.3. X-ray diffraction (XRD)

XRD patterns of HA, HA-g-PVI and PVI were recorded on X-ray powder diffractometer (a Philips Xpert MPD Pro) using Ni-filtered Cu K<sub>α</sub> radiation of the wavelength (λ) of 0.154 nm over scattering range 20 from 3° to 60° at a scan speed of 1 step s<sup>-1</sup>.

4.4. Thermogravimetric analyzer (TGA)

Thermal stability of tested samples was carried out using thermogravimetric analyzer (TGA) (TGA-50H Shimadzu). 4–5 mg of tested samples was heated at 25–700 °C and the reference material was alumina with a dynamic heating rate 10 °C min<sup>-1</sup> under nitrogen atmosphere.

4.5. Elemental analysis (EA)

EA of HA and HA-g-PVI was done using Elementar Vario ELIII Analyzer.

4.6. Field emission scanning electron microscope (FE-SEM)

Surface morphology images of HA, HA-g-PVI and PVI were scanned on FE-SEM (Quanta 250 FEG).

5. Results & discussion

5.1. Effect of various reaction conditions on the grafting process of PVI onto HA

Graft reactions were done under N<sub>2</sub> atmosphere. The effect of various graft copolymerization reaction conditions of PVI onto HA including the initiator and monomer concentrations, reaction temperature and time were studied on the percentage of graft yield (% GY), grafting efficiency (% GE) and amount of homopolymer (% H). Optimum reaction conditions were recorded by changing one of the reaction conditions, while keeping constant all the other factors. The results are illustrated in Fig. 2.

5.1.1. Effect of initiator concentration

The concentration of initiator (PPS) affecting all grafting parameters (%GY, %GE and %H) was varied from 2.5–22.5 mmol L<sup>-1</sup>, in presence of 0.25 mol L<sup>-1</sup> N-vinyl imidazole, reaction temperature was kept at 50 °C, reaction time was 1.5 h and HA concentration was 0.025 mol L<sup>-1</sup>. The results are shown in Fig. 2a and illustrated that %GY and %GE increased with increasing PPS concentrations to reach a maximum value at 17.5 mmol L<sup>-1</sup> due to the increase in the number of active centers along HA chains that followed by grafting of monomer onto HA chains. While, increasing the initiator concentration (above 17.5 mmol L<sup>-1</sup>) led to increase in %H and decrease in other grafting parameters due to the competition between initiation and termination steps through chain transfer reaction to initiator or due to reacting PPS with monomer that led to formation of homopolymer (PVI) which took place on the expense of the grafting process, so %GY and %GE decreased. These results were in agreement to other data reported in literature (Badwaik et al., 2016; Caner et al., 2007).

5.1.2. Effect of N-vinyl imidazole concentration

Fig. 2b illustrates the effect of monomer (N-vinyl imidazole) concentrations [M] onto grafting parameters with varying from 0.25 to 1.75 mmol L<sup>-1</sup> and keeping the initiator concentration constant at 17.5 mmol L<sup>-1</sup> and the reaction temperature and time at 50 °C and 1.5 h, respectively, the concentration of HA was kept 0.025 mol L<sup>-1</sup>. The results exhibited that %GY and %GE values increased by increasing [M] from 0.25 to 1.25 mmol L<sup>-1</sup> to give %GY = 235% and% GE = 83% at 1.25 mmol L<sup>-1</sup> because the increase of monomer concentration led to increase in its amount during propagation step in the free radical polymerization mechanism. Increasing the monomer concentration (above 1.25 mmol L<sup>-1</sup>) led to a decrease in %GY and %GE values and an increase in % H value due to degradative chain transfer reaction to monomer as proposed in literature (Bamford & Schofield, 1981) that generated unreactive species with low propagation tendency that retard further grafting or consuming the monomer in homopolymer formation which led to an increase in the viscosity of the reaction medium. These findings were also in good agreement to others reported in literature (Abu Elella et al., 2017; Caner et al., 2007).

5.1.3. Effect of reaction temperature

The effect of reaction temperature range (30–60 °C) on grafting parameters was studied and the results are shown in Fig. 2c. While, the other parameters were kept constant ([I<sub>n</sub>] = 17.5 mmol L<sup>-1</sup>, [M] = 1.25 mol L<sup>-1</sup>, reaction time = 1.5 h and [HA] = 0.025 mol L<sup>-1</sup>). The results revealed that grafting parameters (%GY and %GE) increased with increasing the temperature range from 30 °C to 50 °C to reach an optimum grafting conditions at 50 °C that increased the decomposition of PPS to potassium sulfate radicals (KSO<sub>4</sub>) in the initiation step and generated more active sites on HA backbone that increased the percentage of grafting parameters when monomer was added to HA solution. At elevated temperature (above 50 °C), the activity of PPS decreased because it decomposed to O<sub>2</sub> and H<sub>2</sub>O as reported in literature (Kumar, Srivastava, & Behari, 2009), so it caused less efficient initiation to HA backbone. Consequently, the number of active sites on HA backbone decreased that led to a decrease in grafting parameters. These data were also in agreement to others reported in literature (Lv et al., 2009; Trivedi, Kalia, Patel, & Trivedi, 2005).

5.1.4. Effect of reaction time

The influence of the reaction time on grafting parameters is shown in Fig 2d. The grafting time was varied from 1 to 2.5 h, while keeping the other parameters constant ([I<sub>n</sub>] = 17.5 mmol L<sup>-1</sup>, [M] = 1.25 mol L<sup>-1</sup>, reaction temperature was kept at 50 °C and [HA] = 0.025 mol L<sup>-1</sup>). The obtained results exhibited that grafting increased with increasing time up to 1.5 h due to an increase in the active sites on HA backbone. Above 1.5 h, the % grafting began to level off and reached a plateau because of the depletion of the monomer and initiator concentration that led to depletion of grafting sites on HA backbone. These observations were in agreement to others reported in literature (Caner et al., 2007; Kumar et al., 2009).
5.2. Characterization of HA-g-PVI composites

5.2.1. FTIR spectroscopy

FTIR spectra of HA, PVI and HA grafted sample, HA-g-PVI, (%GY = 235%) are shown in Fig 3. HA spectrum showed a broad peak at 3430 cm$^{-1}$ related to stretching vibration of hydroxyl (−OH) and amino (−NH−) groups. Also, it showed an absorption peak at 2904 cm$^{-1}$ corresponding to stretching SP3 hydrocarbon (C−H) groups. Moreover, two sharp peaks appeared at 1630 cm$^{-1}$ and 1420 cm$^{-1}$ assigned to symmetric stretching vibration of carbonyl (C=O) groups in both N-acetyl (−COCH$_3$), carboxylate (−COO$^-$) groups and asymmetric stretching vibration of carboxylate (−COO$^-$) groups, respectively. Furthermore, new peaks appeared at 1156 cm$^{-1}$ and 1044 cm$^{-1}$, respectively, assigned to glycosidic (ether) (C−O−C) bonds in HA structure (Kutlusoy, Oktay, Apohan, Süleymanoğlu, & Kuruca, 2017; Lee, Ahn, & Park, 2009; Lu et al., 2016; Mičochová et al., 2006; Vafaei et al., 2016). While, PVI spectrum showed two absorption peaks at 3111 cm$^{-1}$ and 2918 cm$^{-1}$ corresponded to the stretching vibration of SP2 olefin (≡C−H) bonds in imidazole ring and SP3 hydrocarbon (≡C−H) bonds in main backbone chains, respectively. The absorption peak at 1630 cm$^{-1}$ assigned to C≡C groups in imidazole ring. In addition, absorption peak is due to C≡N stretching groups in imidazole ring appeared at 1504 cm$^{-1}$. Also, two peaks appeared at 1415 cm$^{-1}$ and 1083 cm$^{-1}$ corresponded to in-plane bending vibration of C−H groups. Moreover, three absorption peaks appeared at 1282 cm$^{-1}$, 1229 cm$^{-1}$ and 662 cm$^{-1}$ referred to the stretching vibration of C−N groups. Furthermore, two peaks for out-of-plane bending vibration of C−H groups in imidazole ring appeared at 822 and 754 cm$^{-1}$.
743 cm\(^{-1}\) (Pekel & Güven, 2002; Unal, Erol, & Gumus, 2014).

On the other hand, the FTIR spectrum of HA-g-PVI exhibited broad absorption peak at 3430 cm\(^{-1}\) due to the stretching vibration of hydroxyl and amino (\(-\text{NH}\)) groups in HA structure, this peak was more broad than that found in HA due to the H-bonding interactions between PVI and HA. Also, it showed absorption peak at 3111 cm\(^{-1}\) due to the stretching vibration of SP\(^2\) olefin (\(\text{C} \equiv \text{H}\)) bonds in imidazole moiety that found in PVI structure. The absorption peak of C=\(\equiv\)C and C=\(\equiv\)N stretching groups in imidazole ring overlapped with the absorption peak of the stretching vibration of carbonyl groups in HA at 1630 cm\(^{-1}\), so, this peak was more broad and more intense than the same peak in HA spectrum. Moreover, broad absorption peaks appeared at 1422 cm\(^{-1}\) related to asymmetric stretching vibration of carboxylate (\(-\text{COO}\)) groups in HA and in-plane bending vibration of C=\(\equiv\)H groups in PVI. In addition, a sharp absorption peak appeared at 1105 cm\(^{-1}\) which is related to in-plane bending vibration of alkyl (C=\(\equiv\)H) bonds inside the imidazole ring and glycosidic bonds in HA structure. Two absorption peaks appeared at 840 and 754 cm\(^{-1}\) corresponded to out-of-plane bending of alkyl (C=\(\equiv\)H) bonds in the imidazole ring. Furthermore, a sharp peak appeared at 618 cm\(^{-1}\) assigned for \(\text{C} \equiv \text{N}\) bonds of the imidazole ring (Abu Elella et al., 2017; Ramasamy, 2015; Unal et al., 2014).

5.2.2. \(^1\)H NMR spectroscopy

The structure of HA, PVI and grafted copolymer (HA-g-PVI, \%GY = 235\%) was confirmed by \(^1\)H NMR technique ~ Fig. 4. \(^1\)H NMR of HA exhibited a sharp singlet signal at \(\delta = 2.1\) ppm related to methyl protons of N-acetyl (\(-\text{COCH}_3\)) group and broad multiplet signals at \(\delta = 3.4\)–3.9 ppm assigned to protons in HA structure (\(\text{H}_2\)-\(\text{H}_6\)). In addition, doublet signals referred to anumeric protons (\(\text{H}_1\)) overlapped with sharp singlet signal of \(\text{D}_2\text{O}\) solvent at \(\delta = 4.8\) ppm (Nakagawa et al., 2015; Schanté et al., 2011; Vafaei et al., 2016). While, the \(^1\)H NMR spectrum of PVI showed doublet signals at \(\delta = 2.1\)–2.2 ppm assigned to methylene protons (\(-\text{CH}_2\)) in the backbone chains. Also, it exhibited splitting triplet signals at \(\delta = 2.8\)–3.8 ppm related to methine protons (\(-\text{CH}\)) in backbone chains. In addition, multiplet signals at \(\delta = 6.8\)–7.2 ppm corresponded to methine protons in imidazole moiety. While, a sharp signal appeared at \(\delta = 4.8\) ppm referred to \(\text{D}_2\text{O}\) solvent (Pekel, Rzaev, & Güven, 2004). On the other hand, the \(^1\)H NMR spectrum of HA-g-PVI showed characteristic signals of PVI and HA. Characteristic signals of PVI are multiplet signals at \(\delta = 7.2\)–8.7 ppm and triplet signal at \(\delta = 3.8\)–4.0 ppm corresponded to the three protons of methine \((-\text{CH})\) groups in imidazole ring and in the backbone chain, respectively. While, a signal of methylene protons in the backbone of PVI at \(\delta = 2.1\) ppm overlapped with a signal of methyl protons of N-acetyl (\(-\text{COCH}_3\)) group in HA backbone, so, the intensity of the obtained
signal increased. Furthermore, $^1$H NMR of HA-g-PVI illustrated characteristic signals of HA such as a singlet signal at $\delta = 2.1$ ppm corresponded to methyl protons of N-acetyl (−COCH$_3$) group, multiplet signal at $\delta = 3.4$–3.7 ppm related to protons of HA backbone and doublet signal at $\delta = 4.6$ ppm assigned to anemic protons (H$_1$). While, a sharp signal of D$_2$O solvent appeared at $\delta = 4.8$ ppm. Fig. 4 had proven the successful synthesis of the grafted product HA-g-PVI.

5.2.3. XRD patterns
The crystallinity of HA, PVI and HA grafted sample (%GY = 235%) are illustrated by XRD technique and results are shown in Fig. 5. XRD pattern of HA showed three diffraction peaks at $2\theta = 28^\circ$, $47^\circ$ and $56^\circ$ that indicated the semi crystalline nature of HA due to the hydrogen bonding interactions between hydroxyl, acetyl and carboxylate groups in HA chains, these results were similar to others reported in literature (Anirudhan, Vasantha, & Sasidharan, 2017). While, XRD pattern of PVI exhibited many diffraction peaks at $2\theta = 22^\circ$, $30.7^\circ$, $32^\circ$, $37^\circ$, $39^\circ$, $44^\circ$, $55.7^\circ$ and $56.2^\circ$ that indicated the semi crystalline nature of PVI structure. These results were previously mentioned by the same authors (Sabaa, Magid, & Mohamed, 2017). On the other hand, XRD pattern of HA-g-PVI exhibited higher crystallinity than HA and PVI individually. The pattern showed two sharp diffraction peaks at $2\theta = 28^\circ$, this peak included diffraction peak of PVI at $2\theta = 30.7^\circ$ that shifted to $28^\circ$ and overlapped with the previous peak of HA) at $32^\circ$ with higher intensity. Also, three small diffraction peaks at $2\theta = 37^\circ$, $39^\circ$ and $44^\circ$ related to the semi crystalline PVI that formed H-bonding interactions with the hydroxyl groups in HA chains – Scheme 2. These results investigated that HA-g-PVI had higher ordered crystalline structure than HA and PVI individually.

5.2.4. TGA analysis
Thermal stability of HA, PVI and grafted copolymer (HA-g-PVI, %GY = 235) was investigated by TGA and the results are shown in Fig 6. The results indicated that HA, PVI and HA-g-PVI lost about 9%, 11% and 7.5%, respectively, from their weight till 150 °C due to the loss of absorbed water. TGA thermogram of HA exhibited thermal decomposition of HA that took place in one major step and its initial decomposition temperature (IDT) was at 230 °C. Moreover, increasing temperature led to an increase in the weight loss%. So, at 300 °C the weight loss of HA was 52% and 68% at 700 °C, these observations were in agreement to others in literature (Lewandowska, Sionkowska, Grabska, & Kaczmarek, 2016). While, PVI thermogram illustrated that thermal degradation of PVI occurred in only single step and its IDT was 375 °C due to the decomposition of PVI chains (Fodor, Bozi, Blazsó, & Iván, 2012; Unal et al., 2014). However, TGA thermogram of HA-g-PVI showed that its degradation occurred in two steps while the IDT was 200 °C for the first degradation step (specific for degradation of HA chains) and 300 °C for the second degradation step (specific for PVI chains degradation). Moreover, its thermal degradation rate is less than the degradation rate of HA.

At 300 °C, the weight loss of HA-g-PVI was only 32%, while at 700 °C, its weight loss was 49%. Therefore, the previous results confirmed that grafted copolymer (HA-g-PVI) was more thermally stable than HA and PVI individually due to H-bonding interactions between HA and PVI.

5.2.5. Surface morphology analysis
Surface morphology of HA, PVI and grafted sample (%GY = 235) was observed through FE-SEM images with a magnification of 1000 and are shown in Fig 7. HA surface showed high porosity roughness shape with irregular lobules (Park, Ma, Higaki, & Takahara, 2016), While, the surface of the PVI exhibited highly irregular cavities and porous structure that changed to compact surface with small pores in the grafted sample. So, SEM images proved the modification of HA with PVI.

5.2.6. Elemental analysis
Elemental analysis of HA was performed and the results showed that % C, H, O and N were: 33.56, 5.70, 58.07 and 2.67, respectively. Also, the structure of HA-g-PVI (%GY = 235) was elucidated with elemental analysis and the results showed that % C, H, O and N were: 44.49%, 5.63%, 34.43% and 15.45%, respectively. These results showed that...
%C and %N increased due to grafting of PVI onto HA backbone that contains C and N atoms in the imidazole ring, while O% decreased due to the dilution of HA chains by grafting by PVI.

5.2.7. Antitumor activity

The antitumor activity (cytotoxicity assay) of two grafted samples (%GY = 155 and 235%) was tested against two different cancer cell lines (HepG-2 and MCF-7) compared to the unmodified HA and PVI, in presence of doxorubicin as reference drug and the results are represented by dose response curve – Fig. 8.

IC$_{50}$ for tested samples was calculated from dose response curve. The results showed that cytotoxicity assay (cell viability %) of tested samples decreased with increasing the concentration of investigated samples. Also, it showed that grafted HA samples more sensitive to hepatic cancer cell line (HepG-2) than breast cancer cell line (MCF-7). IC$_{50}$ of doxorubicin was estimated as 0.71 μg mL$^{-1}$ and 0.27 μg mL$^{-1}$ against HepG-2 and MCF-7, respectively. Low values of IC$_{50}$ of doxorubicin are in agreement with previous studies in literature (Eldehna et al., 2017; Lee, Na, & Bae, 2005; Gomha, Riyadh et al., 2015; Gomha, Salaheldin et al., 2015; Refaat, 2010).

At the concentration of 1000 μg mL$^{-1}$ of grafted samples, a strong decrease occurs in cell viability %, so, Fig 8a (cytotoxic effect on HepG-2 cell line) illustrated that HA killed 39.6%, while, grafted samples, %GY = 155 and 235%, killed 61.1% and 70.5%, respectively, while PVI killed 60.0%. Moreover, IC$_{50}$ of HA was more than 1000 μg mL$^{-1}$, PVI was 488 μg mL$^{-1}$ and grafted samples 155 and 235%, was 446 and 215 μg mL$^{-1}$, respectively. However, Fig 8b (cytotoxic effect on MCF-7 cell line) showed that HA killed 12.7% and PVI killed 78.7% while, grafted samples 155% and 235% was 960 and 580 μg mL$^{-1}$, respectively.

Cytotoxicity assay data showed that grafted samples had more antitumor activity against HepG-2 cell line than MCF-7 cell line compared with unmodified HA and PVI. The data exhibited that hepatic cancer cell line is more sensitive to grafted samples (HA-g-PVI) than PVI alone may be due to dual effect of PVI and HA on cancer cells through electrostatic and H-bonding interactions. Also, it showed that sensitivity of breast cancer cell line depended on the percentage of PVI, so MCF-7 cell line is more sensitive to PVI than grafted samples may be only due to the cationic nature of imidazole ring in PVI that interacts with negative charges on the cancer cells through electrostatic interactions.
6. Conclusion

Grafted HA using PVI was synthesized via free radical polymerization using potassium persulfate as initiator. The optimum grafting conditions were 235% (GAM) and 83% (GAM) that was obtained at reaction conditions; [In] = 17.5 mmol L−1, [M] = 1.25 mol L−1. Temp. was adjusted at 50°C, reaction time was kept at 1.5 h and [HA] = 0.025 mol L−1. The structure of (HA-g-PVI) was elucidated by FTIR, 1H NMR and elemental analysis techniques. While, the crystallinity of HA-g-PVI was illustrated with XRD technique. The results showed that grafted HA was higher in crystallinity than unmodified HA and PVI due to H-bonding interactions between the nitrogen atoms in imidazole rings in PVI and the hydroxyl groups in HA chains. Moreover, thermal stability of HA, PVI and grafted sample was examined with TGA technique and the results exhibited that grafted HA was thermally more stable than unmodified HA and PVI at high temperatures. Furthermore, SEM technique showed the variation in surface morphology from roughness as lobules in native HA and high porosity surface in PVI to compact surface with small pores in HA-g-PVI. The anticancer activity of tested samples (unmodified HA, PVI and two grafted HA with different C%GAM (155 and 235%)) was studied against two cancer cell lines (HepG-2 and MCF-7). The results showed that cell viability % decreased with increasing the percentage of grafting on HA chains, so, modified samples had more anti-cancer activity than unmodified HA.

References

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