

# PVA/CMC/Attapulgate clay composite hydrogel membranes for biomedical applications: Factors affecting membranes crosslinking and bio-evaluation tests

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## Research Article

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# Abstract

Herein, polyvinyl alcohol-carboxymethyl cellulose (PVA-CMC) composite hydrogel membranes were prepared using solution-casting method, where citric acid (CA) was added as crosslinker in different ratios of (7, 10 and 12 wt.%). Attapulgite clay extracted from Northwestern Desert of Borg El-Arab, Egypt; was incorporated as nanofiller (1, 2, 4, and 5 wt.%) into membranes for improving their mechanical/thermal stability. Results revealed that, physicochemical properties of membranes *e.g.* swelling%, tensile strength and morphology of membranes affected significantly by different clay concentrations and citric acid crosslinker. Also, attapulgite clay with concentration 1 (wt.%) enhanced mechanical strength of composite membranes, compared to other clay concentrations. Furthermore, protein adsorption %, hydrolytic degradation, hemolysis (%) and antimicrobial activity significantly affected by clay contents and CA concentrations. Four bacterial pathogens *e.g. Candida albicans, Escherichia coli, Klebsiella pneumoniae, and Bacillus cereus* were used for testing antimicrobial activity of prepared membranes. Results referred to increasing of clay contents led to a high hemolysis %; however, increasing CA concentration significantly reduced hemolysis %. Meanwhile, membranes with low clay contents offered the most effective resistance against tested microbes. These findings are referring to the ability of using PVA-CMC-attapulgite composite membranes crosslinked by CA as good candidate of biomaterials for dermal wound dressings.

## 1. Introduction

Wound healing is one of the most important and complex processes occurs to human involving interaction between body cells and its extracellular cover to protect skin hurt zone. Recently, wound dressings examples like hydrogels have specific properties required for wound closure such as keeping humidity and cooling sensation for the wound, preventing bacterial infections, permitting gas exchange and biocompatible to be easily removed from wounds [1] hydrogels are highly hydrated three-dimensional crosslinked polymeric network with porous molecular structure that can absorb wound exudates and retains moisture making them suitable candidate for wound dressing applications [2]. Last decade, many wound dressings have been prepared using natural and synthetic polymers.

Polyvinyl alcohol (PVA) is hydrophilic synthetic polymer that easily soluble in water, slightly soluble in ethanol, but insoluble in other organic solvents, it was prepared by the polymerization of vinyl acetate to form Poly (vinyl acetate) followed by partial hydrolysis [3]. PVA hydrogel is considered an effective agent in biomedical and pharmaceutical applications due to its excellent chemical and physical properties such as biocompatibility, non-toxicity, non-carcinogenic, high flexibility, high tensile strength, film forming, emulsifying, and adhesive properties. However, PVA hydrogel possesses high water holding capacity or biological fluids due to its rubbery or elastic structure. As result of these advantages PVA hydrogel can stimulate natural tissue and can be accepted in the body which make it suitable for wound dressing application [4].

Carboxymethyl cellulose (CMC) is a natural polymer derived from cellulose, it is biodegradable, has high swelling ability, and hydrophilic carboxylate groups in its backbone. So, it is suitable for forming hydrogel but with low mechanical strength and stability [5]. Thus, blending with other high mechanical polymers, PVA to obtain composite hydrogel is inexpensive and advantageous way to prepare new structural material combined properties of both polymers [6].

Citric acid is used as popular chemical crosslinker because it is cheap, nontoxic, and antimicrobial ability. Citric acid crosslinked PVA-CMC hydrogel films were prepared by esterification reaction in which citric acid forms a cyclic anhydride at high temperature followed by esterification of the hydroxyl group of the adjacent polymer chains leads to the formation of crosslinks [7]. Previously, blending of PVA-CMC polymers lead to semitransparent, flexible, and highly efficient hydrogel with excellent physical and mechanical properties suitable for wound dressing as can absorb wound exudates due to their highly swelling capacity [8, 9]. Herein, an extended research work of using attapulgite clay as antimicrobial clay was recommended for incorporation with PVA/CMC composite hydrogel membranes as promising biomaterials for wound dressing applications. It is well known that pristine hydrogel with high strength and elasticity will be better as dressing material however, reinforcing agents *e.g.*, naturally favored for incorporation into membrane composition [10]. Attapulgite clay extracted from Borg Al-Arab desert, Egypt; was first purified and incorporated into hydrogel membranes for avoiding the quick deterioration of hydrogel membranes due to its high swelling capacity to wound exudates. Furthermore, clay was incorporated into hydrogel membranes to adjust the swelling degree, prolonging the degradation rate and for enhancing the mechanical and thermal stability of hydrogel membranes. This work aims to preparation of effective CA crosslinked PVA-CMC-attapulgite composite hydrogel membranes for wound dressings. Both the effect of CA concentration and clay contents were studied intensively and their influence on physicochemical properties of PVA-CMC-attapulgite composite hydrogel members. The prepared composite membranes were instrumentally characterized by FTIR and SEM, while the bio-assessment test *e.g.* hemolysis % and antimicrobial activity were carried out *in vitro*.

## 2. Materials And Methods

### 2.1. Materials

Polyvinyl alcohol, PVA (typically average  $M_{wt} \sim 72,000$  g/mol; 98.9% hydrolyzed) and high viscosity carboxymethyl cellulose, CMC sodium salt (degree of substitution 0.6-0.95) were purchased from Biochemika, Germany. Citric acid anhydrous (CA) (purity > 96%) was purchased from Sigma-Aldrich, Germany. Attapulgite clay was obtained from Northwestern desert of Borg El-Arab city, Alexandria, Egypt. Attapulgite clay was purified from sands and rocks before use. Distilled water was used during this research. All other chemicals were used without further purification.

Four bacterial pathogens; *Candida albicans* ATCC 700, *Escherichia coli* NCTC10418, *Klebsiella pneumoniae* ATCC13883, and *Bacillus cereus* ATCC6633 were kindly provided by National Institute of

Oceanography and Fisheries (NIOF), Alexandria, Egypt. Luria-Bertani (LB) Broth powder was obtained from Bio Basic, Canada.

## **2.2. Preparation of PVA-CMC-attapulgitite hydrogel membranes**

PVA-CMC hydrogel membranes were prepared by solution-casting method according to previous reported study by Hussein et al. [11], with some modifications. Briefly, aqueous solution of 10 (% w/v) PVA and 1 (% w/v) CMC were carefully dissolved in 15 mL distilled water with stirring at 60°C for 4h. After getting a clear polymeric PVA-CMC mixture solution, it was cooled to the room temperature, then 10 (% w/v) of citric acid was added as crosslinker at room temperature with gentle stirring for 15 min to prevent quick premature crosslinking. Purified attapulgitite clay was added as nano-filler followed by ultrasonic in water bath for grantee the clay well dispersion in polymeric solution, and then 1 mL of glycerol was added as plasticizer and 0.25 g of polyethylene glycol (PEG) was added as pore forming agent. The mixture solution was left under stirring at room temperature for two hours to remove the entrapped air bubbles. Finally, the solution was casted in (20 mm×100 mm) petri dish and dried in oven overnight at 60°C to evaporate excess water, and then dried films were transferred into 80°C for two hours to complete the esterification reaction. Different concentrations of PVA (5,7 and 10%, w/v), CMC (0.25, 0.5, 1.0 and 2.0% w/v), citric acid (7, 10 and 12%, w/v) and attapulgitite clay contents (1, 2, 4 and 5%, w/v) were incorporated and tested for optimizing of composite hydrogel membranes.

## **2.3 Instrumental characterization**

### **2.3.1. FT-IR**

Vacuumed and dried samples of PVA, CMC, and PVA–CMC hydrogel were analyzed by FTIR on an EQUINOX 55 instrument (BRUKER, Germany). Translucent KBr-disks were prepared by grinding the dried sample materials together with infrared grade KBr and then pressing. The FTIR spectra were obtained by recording 64 scans between 4000 and 400  $\text{cm}^{-1}$  with a resolution of 2  $\text{cm}^{-1}$ . All samples were freeze-dried using liquid nitrogen, crushed to a fine powder (KBr: sample = 140 mg: 2 mg), and pressed by applying a force 105 N into a transparent disk (maximum disk weight = 145 mg) with a diameter of 13 mm. All samples were measured in transmission mode.

### **2.3.2. SEM**

The surface and internal structure of the hydrogel membrane samples were investigated by Analytical-SEM (JEOL, JSM-6360LA, Japan) with 15 kV voltage for secondary electron imaging. The hydrogel membranes were 11425, USA dehydrated by freeze-dryer and coated with Au using an ion sputter coater in (model: 11430, USA, combined with vacuum base unit or SPi module).

### **2.3.3. TGA**

The thermal decomposition of vacuumed-dried PVA–CMC membranes was measured using TGA thermogram. The thermo-gravimetric analysis (TGA) was performed on a 204 Phoenix TGA instrument (NETZSCH, Germany) from 50-600°C at a heating rate of 10°C/min. TGA thermographs calculated the onset temperature  $T_{\text{onset}}$  which known as the temperature at the intersection of the baseline mass and the tangent attracted to the mass curve at the point of inflection or point of maximum mass loss percentage.

## 2.3.4. Mechanical properties

To measure maximum tensile strength and degree of elongation to break PVA-CMC composite hydrogel membranes were drawn on a tensile testing machine (model: AG-I/50–10KN, Japan). PVA–CMC membranes were cut into a rectangular or plate shape (5 cm long, 1 cm width). The analysis was carried out at a stretching rate of (20 mm/min) with preload (0.5N) for each sample [12].

## 2.4. Physicochemical characterization

### 2.4.1. Swelling ratio (%)

Dried PVA-CMC membrane samples were cut into (2×2 cm) pieces and its dry weight was determined ( $W_d$ ) and then was soaked in distilled water, the weight of swelled samples ( $W_s$ ) was determined at different specific time intervals till saturation or equilibrium. Water uptake was measured according to the following formula:

$$\text{Swelling ratio or Water uptake (SR)} = [(W_s - W_d) / W_d] \times 100. \text{ Eq. (1)}$$

Where,  $W_d$  is weight of dry sample and  $W_s$  is weight of swollen sample at specific time intervals [13].

### 2.4.2. Protein adsorption

Bovine serum albumin (BSA) was used as a medication or protein model in this study. Weighed BSA (25 mg/mL) depending on the weight of samples tested) it was fine dissolved at pH 7.4 at 37 °C in phosphate buffered saline (PBS) solution. PVA-CMC hydrogels with specific weights were immersed in BSA-PBS solution overnight at 37°C. Overall sum of BSA adsorbed on the hydrogel surface was calculated by removing the hydrogel bits from BSA solution and then calculate the BSA concentration before/after the immersion of hydrogel in BSA solution. The adsorbed BSA on hydrogels surface was measured spectrophotometer at 280 nm [14].

### 2.4.3. Hydrolytic degradation

Hydrolytic degradation or weight loss (%) of PVA-CMC-CA hydrogel membranes, as a function of citric acid different concentrations, prepared membranes were immersed in phosphate buffered saline (PBS) solution (pH 7.4, at 37°C for 1–3 weeks). The degradation or weight loss (%) of hydrogels was measured after equilibrium swelling weight of swollen hydrogels. At specific time intervals, samples were removed

from PBS solution and were softly wiped with filter papers. The weight loss (%) of hydrogels was detected as a function of time of incubation using the following equation.

$$\text{Weight loss (\%)} = [(W_0 - W_t)/W_0] \times 100. \text{ Eq. (2)}$$

Where,  $W_0$  is the hydrogel's initial weight,  $W_t$  is the removed PBS hydrogel and weighed at specific incubation time intervals [15].

## 2.5. Bio-assessment tests

### 2.5.1. Disc diffusion assay

Antimicrobial activity of prepared composite hydrogel membranes was tested using disc-diffusion assay [16]. The surfaces of LB agar plates were inoculated with the microbial pathogens using sterile cotton swabs after their dilution to 0.5 McFarland standard. A single disc (0.7 mm diameter) of each membrane was aseptically added to the plates' surface using tip-ignited forceps. All the plates were then incubated at 30°C for 24h. After incubation, the plates were checked for the formation of clear zones which were measured and recorded [17].

### 2.5.2. Hemolysis assay

Hemolysis test is an investigating assay that is widely used to determine the biocompatibility of a certain compound or a polymeric material. It depends on testing the ability of that compound to destroy the human RBCs and releasing their hemoglobin content. It started by the collection of a healthy blood sample in EDTA containing tube to avoid its clotting. A net volume of 700  $\mu\text{l}$  of  $\text{Ca}^{+2}$ - $\text{Mg}^{+2}$  free Dulbecco's phosphate-buffered saline (DPBS) buffer was mixed with 10  $\mu\text{l}$  of the blood sample with gentle mixing. After that, 100 mg of each tested sample were separately added. Both of Triton X-100 and DMSO (0.5%) were used as the positive and negative controls, respectively. The tested and controls tubes were incubated for 3.5h at 37°C with gentle interval mixing followed by centrifugation at 10,000 rpm for 15 min. Equal volumes of the supernatants and the reagent (cyanmethemoglobin) were mixed, followed by spectrophotometric measuring of each mixture at 540 nm against the blank ( $\text{Ca}^{+2}$ - $\text{Mg}^{+2}$  free DPBS buffer plus the sample without blood [18]).

## 3. Results And Discussion

### 3.1. Instrumental characterization

Polyvinyl alcohol-carboxymethyl cellulose blended hydrogel membranes were prepared using citric acid as chemical crosslinker through esterification reaction as displayed in scheme 1., where the feature peaks verifying the chemical crosslinking is proven by FTIR analysis. While physical interaction between PVA and CMC as is explained in scheme1 is also verified by FTIR analysis.

Scheme 1

### 3.1.1. FTIR analysis

FTIR spectra of pure PVA, pure CMC, PVA-CMC non-crosslinked membrane and citric acid crosslinked hydrogel membrane are represented in Fig. 1a. The peaks associated with PVA shows C-H alkyl-asymmetric stretching vibration at  $\nu$  2850  $\text{cm}^{-1}$ . A notable stretching band for  $-\text{CH}_2$  appears at  $\nu$  1560 – 1450  $\text{cm}^{-1}$  was found. Also, C-O stretching band appeared approximately at  $\nu$  1100  $\text{cm}^{-1}$  [19].

For CMC,, aliphatic C-H band appears approximately at  $\nu$  2900  $\text{cm}^{-1}$ , a new peak represents asymmetric stretching vibration of carbonyl group of  $\text{COO}^-$  at  $\nu$  1600-1640 $\text{cm}^{-1}$  and symmetric stretches at approximately 1400  $\text{cm}^{-1}$ , also a band at 1070  $\text{cm}^{-1}$  is assigned to C-O-C stretching, were clearly detected [20].

FT-IR spectrum of PVA/CMC membranes crosslinked by 10 (wt. %) of CA shows abroad band appeared at  $\nu$  3200–3600  $\text{cm}^{-1}$ , which represents O-H stretching vibration [21] also  $-\text{OH}$  peak intensity decreased with the crosslinking reaction. This is due to the esterification reaction with citric acid and formation of ester bonds. In addition, new peaks are observed at  $\nu$  1750 and 1300  $\text{cm}^{-1}$ , which represent the formation of C = O and C-O stretching of ester bonds, respectively; because of the crosslinking reaction [22]. However, IR spectra were difficult to be evaluated due of the peaks overlapping occurred with carboxylic group bands of CMC, and addition of citric acid protonation reaction of carboxymethyl groups of CMC [23].

Figure 1

Figure 1b represents the effect of citric acid concentration, when CA concentration increases from 7, 10, to 12 (wt.%) in membrane composition, the intensity of carbonyl band increases. This is owing to a result of increase the number of ester crosslinks and also free  $-\text{COOH}$  groups are associated to the increase of CA concentration, where it can't be significantly determined [24]. Interestingly, esterification mechanism leads to the formation of crosslinks between PVA and CMC, whereas a cyclic anhydride intermediate is formed by post heat-treatment to complete the crosslinking cycle. This intermediate is responsible for the formation of crosslinks with CMC by esterification of  $-\text{OH}$  group of CMC resulting in another carboxylic acid occurring intramolecular cyclic anhydride with neighboring carboxylate group [25].

Figure 2

### 3.1.2. SEM investigation

The surface morphology of crosslinked PVA-CMC composite hydrogel membranes with different CA concentrations (7, 10 and 12 wt.%) were investigated using SEM, as represented in Fig. 2. SEM micrographs showed that, citric acid content is clearly responsible for changing the surface morphology of tested membrane, since the amount of PVA and CMC were uniform in all films. At membrane of (7 wt.%) CA concentration, few pores with smooth, regular and homogenous surface is observed due to low crosslinking degree in the obtained composite membranes. By increasing the CA concentration up to (10

wt.%), the film exhibits more porous network that enables the diffusion of water through the pores. These results might be attributed to the presence of three (-COOH) groups for each CA molecule. Thus, it was found with increasing CA percentage, increases the number of -COOH groups available for crosslinking with -OH group of PVA leading to increasing of the crosslinking density and hence, promoting the porosity. This process decreases the water absorption of PVA composite hydrogels. Further, increase of CA ratio up to (12 wt.%), hyper-crosslinking was triggered due to further increasing available free -COOH groups of CA for crosslinking process, causing much pores forming and limited water uptake of hydrogel membranes occurred. These results are supporting swelling findings which were discussed below and consistent with previous findings of Nataraj et al. [26].

Figure 3

Figure 3 displays the surface morphology alternations due to incorporation of attapulgite clay in different contents (1, 2, 4 and 5 wt. %) into PVA-CMC composite hydrogel membranes. As clearly seen, the low attapulgite contents (*i.e.* 1 and 2 wt.%) showed compacted, smooth and uniform shape structure. However, high incorporated clay contents (4 and 5 wt.%) showed clay aggregation onto the membrane surface. The high clay incorporation behavior is similar and consistent with previous results obtained by Hussein et al. [15], regarding the aggregated high concentrations of CNCs onto PVA/HA hydrogel membranes.

Table 1

Table 1  
Effect of citric acid concentration on the mechanical properties of crosslinked PVA-CMC hydrogel membrane.

CA concentration (wt. %)	Elongation to break (%)	Max. stress (N/mm <sup>2</sup> )	Max. force (N)
7	372.2	4.9	15.8
10	340.6	4.85	15.5
12	270	3.7	11.7

### 3.1.3. Mechanical properties

To assess the mechanical properties of crosslinked PVA/CMC membranes with different CA concentrations (7, 10 and 12 wt.%), the maximum tensile strength and elongation to break were tested and obtained data was listed in Table 1. Unexpectedly, maximum tensile strength and elongation to break decreases with increasing the citric acid concentration and they show the same mechanical pattern behavior. These results refer to addition of citric acid to PVA-CMC hydrogel membranes might destabilize and accelerate the elongation-to-break of membranes resulting insignificant decreasing in the maximum tensile strength. These results are almost matched with results obtained by Jiang et al. [27], who demonstrated that increasing CA concentration up to 9 wt.% boosted the CA molecules available for

crosslinking leading to an increase in mechanical strength. However, further increase of CA concentration triggered the hyper-crosslinking reaction resulting in sudden mechanical deterioration of CA crosslinked zein NFs [27].

Table 2

Table 2  
Effect of attapulgite clay contents on the mechanical properties of PVA/CMC/Clay composite hydrogel membrane.

Clay content (wt.,%)	Elongation to break (%)	Max. stress (N/mm <sup>2</sup> )	Max. force (N)
0	123.4	2.7	8.9
1	249.3	5.8	15.2
2	140.9	6.4	10.2
4	188	10.5	10.5
5	128.6	10.2	10.2

Different attapulgite clay contents (1, 2, 4, and 5 wt.%) were incorporated into PVA-CMC composite hydrogel membranes and their mechanical stability were tested and displayed in Table 2. It was found that, addition of clay up to 1(wt. %) significantly increased the entire parameters of mechanical properties of PVA/CMC/clay membrane (Table 2). Further increase in attapulgite clay resulting in agglomeration of clay occurring a weak interaction between PVA-CMC matrix, which might reduce the crosslinking degree followed by lowering the mechanical properties of hybrid hydrogel membranes [28]. Hence, 1wt.% clay was considered as optimal concertation in terms of cost and high mechanical stability of composite membranes. Similar results were previously reported by Du et al.[29]. They have tested mechanical properties of high Laponite clay contents in Laponite-poly (acrylic acid) nanocomposite membranes.

Figure 4

### 3.1.4. Thermal properties (TGA measurements)

Thermal stability of PVA/CMC hydrogel membranes crosslinked with different CA concentration (7, 10, and 12 wt.%) were conducted by TGA measurement (Fig. 4). It was shown that, (9.5, 8.1 and 2.4% of weight loss (%) of PVA/CMC membranes crosslinked with CA (7, 10 and 12 wt.%), respectively occurred up to 66–107 °C, owing to evaporation of free water, bounded water and stored humidity. The highest crosslinked membranes with 12 (wt.%) of CA was stable until 180 °C, then a steep and sharp weight loss (%) occurred until 459 °C, as known  $T_{onset}$  or the second decomposition stage. This biggest weight loss (%) around 79% was allied with organic matters decomposition and destruction of PVA/CMC chain linkage. Meanwhile, in this second thermal decomposition; some volatile compounds *e.g.* CO<sub>2</sub> were thermally-decomposed. Finally, the pyrolysis or the third complete volatilization stage is detected from

459–550 °C with total weight loss (%) of 82% was detected (Fig. 4). Overall, increasing of used CA for crosslinking PVA/CMC membranes sharply improved crosslinking degree, accompanied with enhancing the thermal stability of membranes.

Figure 5

As shown in Fig. 5, the weight loss of PVA/CMC/clay composite membranes sharply reduced from 20–2% with increasing the incorporated attapulgite clay contents from 1 to 5 (wt.%) owing to evaporation of free water, bounded water and stored humidity. While  $T_{\text{onset}}$  of the beginning of second decomposition stage increased from 166 to 193°C, when the incorporated clay content was increased from 1–5 (wt.%), respectively. Notably, the total weight loss % after pyrolysis thermal decomposition stage (i.e. third decomposition stage) decreased from 92–81% with increasing the incorporated clay contents in membranes from 1–5 (wt.%). These findings refer to the addition of attapulgite clay into PVA-CMC membranes remarkably the entire thermal stability behavior of composite membrane, in addition prolonged the time of decomposition and the total weight loss decreased due to existing of inorganic matters. These results are consistent with obtained results of Du et al. [29], where who demonstrated that thermal stability of poly (acrylic acid) nanocomposite membranes were sharply enhanced with increasing Laponite clay contents in membranes.

## 3.2. Physicochemical characterization

### 3.2.1. Swelling ratio % of hydrogel membranes

It was found, citric acid concentration affects significantly on the swelling ratio or water uptake of prepared PVA/CMC composite hydrogel membranes, as shown in Fig. 6a. It was proven previously that, swellability of hydrogel membranes increases with increasing the concentration of citric acid, due to increase the hydrophilicity which associated with increasing the carboxyl content in membranes [30]. Notably, the swelling ration of membranes decreased with increase the used concentration of CA, due to increase the crosslinking degree of membranes. However, membranes crosslinked with  $\geq 12$  wt.% of CA show an opposite swelling effect, where the swelling increased significantly owing to imaginably termination reaction occurred. Thus, 10 wt.% of CA was chosen as an optimum concentration for further experiments. Our results also matched with swelling results obtained by Demitri et al. [31], who reported that the optimal degree of swelling for practical applications was achieved using low CA concentrations.

Figure 6

As presented in Fig. 6b, it was observed that with increasing attapulgite clay contents, the swelling ability of hydrogel membranes decreases. This might be ascribed to the blocking available pores of membranes and formation of a very compacted interior-structure of hydrogel network causing swelling reduction. In the same context, Mahdavinia et al. [32] reported that the incorporation of Laponite clay reduced dramatically the swelling ability. Because of, addition of clay acts as additional physical crosslinker

preventing from water absorption. Despite decreasing water uptake, but the results are still with acceptable for water uptake capacity to absorb wound exudates and keeping high water resistance, in case employing the prepared membranes as topical wound dressings.

Figure 7

## 3.2.2. Protein adsorption (%)

The assessing of adsorbed BSA onto the surface of composite hydrogel membranes is a real significant factor for the ability of physiological and cells attachment manner, which also verify the biocompatibility of tested biomaterials. The influence of both CA crosslinker concentrations and contents of incorporated clay on the amounts of adsorbed BSA onto the surface of membranes after incubation for 48 hours, were assessed and shown in Fig. 7. As seen, the adsorbed BSA onto the membrane surfaces generally increased significantly with the low CA concentration and low contents of incorporated attapulgite clay (7 wt.% CA and 1 wt.% clay, respectively). It is due to the fact that, the adsorbed BSA onto membrane surface increases significantly with the highest swollen composite hydrogel membranes, or with other mean the lowest crosslinked membranes. Notable, the presented findings here are fully consistent with the presented results of swelling measurements in Fig. 6. Also, the speculation of reduction of adsorbed protein was found with the highest crosslinked membranes and lowest values of swollen membranes, which is fully consistent with our previous and reported findings [14, 17, 18, 30].

Figure 8

## 3.2.3. Hydrolytic degradation

The hydrolytic degradation or weight loss (%) of crosslinked PVA/CMC hydrogel membranes were assessed as function of different CA concentrations (7, 10, and 12 wt.%) and different attapulgite clay contents (1, 2, 4 and 5 wt.%), is shown in Fig. 8. It was obviously shown that, both the high CA concentration and attapulgite clay content, might hinder and resist the degradation behavior of hydrogel membranes, compared to the low CA concentration and clay content, respectively. Notably, CA concentration  $\geq 10$  (wt. %) and incorporated clay  $\geq 4$  (wt.%) have progressively kept the mechanical stability of crosslinked membranes, where membranes lost almost 40% and 25–30% of their weights after 15 days, respectively. This implies that, both using CA as crosslinker and incorporation of attapulgite clay have improved dramatically mechanical stability and hindered the hydrolytic degradation behavior of crosslinked PVA/CMC/attapulgite composite membranes. The current degradation results are entirely consistent with our previous reported results [14, 17, 18].

## 3.3. Bio-assessment tests

### 3.3.1. Antimicrobial activity

The antimicrobial activity of crosslinked PVA/CMC and PVA/CMC/clay hydrogel membranes was investigated using disc diffusion assay against different human pathogens, as function of different CA

and clay contents; was presented in Fig. 7 and Table 3.

Table 3

The inhibition zones of crosslinked PVA/CMC and PVA/CMC/attapulgitic clay composite hydrogel membranes against human pathogens using disc diffusion assay; antimicrobial activity of membranes was tested as function of different CA concentrations and different clay contents.

CA	Conc./ Content (wt. %)	Inhibition zones (mm)			
		<i>Candida albicans</i>	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Bacillus cereus</i>
	7	18	20	20	17
	10	20	12	19	15
	12	25	16	22	11
<b>Clay</b>	0	25	15	24	17
	1	21	13	15	11
	2	23	13	21	13
	4	17	13	22	12
	5	13	20	15	10

Figure 9

Table 3

Crosslinked PVA/CMC hydrogel membranes show remarked resistances against *Candida albicans* with all used CA concentrations (Fig. 9 and Table 3). It was observed that, increasing the concentration of citric acid was matched with the increase of the measured inhibition zones (Table 3). When the CA concentration was raised from 7 to 12 (wt. %), shows an increasing in the inhibition zones from 18 to 25 mm. On the other hand, prepared membranes show the lowest resistance against *Bacillus cereus* microbe (Table 3). However, increasing CA concentrations from 7 to 12 (wt.%) results in decreasing in the measured inhibition zones from 17 to 11 mm, respectively. While prepared hydrogel membranes exhibit a moderately resistance toward *Escherichia coli* and *Klebsiella pneumoniae*. A maximum inhibition zone was recorded of 22 mm with 12 (wt.%) CA against *Klebsiella pneumoniae* and 12 mm as the lowest measured inhibition zone with 10 (wt. %) CA was applied against *Escherichia coli*. These results are consistent with obtained results by Siregar et al.[33], who reported that CA crosslinked PVA-CMC films have powerful antimicrobial properties. These results could be attributed to citric acid possesses non dissociated form (-COOH) that can permeate the bacterial cell membrane, and allowing to donate hydrogen ions to the system. To maintain the intracellular pH, H<sup>+</sup> ions are released resulting in weak pH, these acidic conditions lead to deformation and damage to cells also damage enzymatic activity, protein structure and DNA of the microorganism [34].

Also, crosslinked PVA/CMC/clay composite hydrogel membranes show remarked resistances against all tested microbes (Table 3). *Candida albicans* was the most affected microbe by hydrogel membranes, while *Bacillus cereus* was the lowest affected one. It was observed that, the membranes that lack clay was generally most effective against the tested microbes than clay-containing membranes. The clay-lacking membrane succeeded to affect the pathogens *Candida albicans*, *Klebsiella pneumonia* and *Bacillus cereus* and show inhibition zones measuring of 25, 24, and 17 mm, respectively. However, the pathogen of *Escherichia coli* is highly affected by 5% incorporated clay that resulted in 20 mm inhibition zone, compared to 15 mm inhibition zone of clay-lacking membrane was applied. Thus, it could be concluded that each prepared membrane resulted in the formation of clear zones that are considered pathogen dependent. These results are consistent with results obtained by El-Bassyoni et al.[35], who demonstrated that PVA-HES-attapulgitic composite hydrogel membranes tested for wound dressing showed significant inhibition zones with antimicrobial potency. It was varied according to the tested microbe and these results might be attributed to the presence of some metals in attapulgitic clay composition which have antimicrobial character.

### 3.3.2. Hemolysis test

The effect of citric acid concentration on the biocompatibility of prepared membranes was investigated according to the hemolysis percentage of a tested healthy blood sample. As shown in Table 4 (up), the percentage of citric acid is significantly affecting the blood hemolysis percentage. It was found that the lowest used concentration of citric acid (7 wt.%) results in the highest hemolysis of tested membrane at (66%). Increasing citric acid concentration to 10 (wt.%) shows a slightly reduction of hemolysis at (64%). However, the lowest hemolysis percentage is recorded as 35.5%, when the citric acid concentration is elevated to 12 (wt. %). It could be concluded that the descending order of hemolysis would be summarized as: CA concentrations of 7 > 10 > 12 (wt. %). Our results are matched with the biocompatible nature of citric acid as previously reported by Salihi et al. [36]. Also, Mali et al.[37] demonstrated that, hemolysis study of CMC composite hydrogel films crosslinked with citric acid indicated their hemocompatibility making them effective for drug delivery application. These results are attributed to the presence of three carboxylic groups in CA structure, so crosslinking of polymers with CA provides some pendant free carboxylic groups which is responsible for enhancing biocompatibility [38].

Table 4

Hemolysis percentage of crosslinked PVA/CMC and PVA/CMC/attapulgitic clay composite hydrogel membranes, biocompatibility of membranes was tested as function of different CA concentrations and different clay contents.

Tested parameters	Concentration/content (wt. %)	OD <sub>540nm</sub>	Hemolysis (%)
CA	7	0.924	66
	10	0.901	64.3
	12	0.496	35.4
Clay content	0	0.931	66.5
	1	1.038	74
	2	0.732	52.3
	4	1.060	75.7
	5	1.136	81
	Positive control	1.4	100
<b>List of Figure captions</b>			

Table 4

Different clay contents were tested for their biocompatibility depending on their behavior towards a healthy blood sample. As depicted in Table 4 (down), the percentage of blood hemolysis was varied according to the tested clay concentration. It was shown that, the lowest clay content in membranes of (1 wt. %) results in a hemolytic percentage at 74%. While, increasing clay concentration to 4 and 5 (wt. %) results in a gradual increase of hemolysis percentages of 75.7 and 81%, respectively. However, 2 (wt. %) of clay provides the lowest blood hemolysis of 52.3%. According to these results, the ascending order of the clay concentration that resulted in high percentages of blood hemolysis could be summarized as: clay contents in membranes (2 < 1 < 4 < 5 (wt. %)). Our results are consistent with results of Golafshan et al. [28], who demonstrated that the hemolysis ratio of PVA-alginate-Laponite clay hydrogel was promoted by increasing Laponite clay content for wound healing application. Also, it was reported that the clay incorporation into PVA hydrogels improves the hemolysis percentage and coagulation activity of blood making the hydrogel potential candidate for wound healing application [38, 39]. Moreover, gelatin-PVA-hydroxyapatite composite hydrogel membranes have high hemocompatibility making them suitable for biomedical applications [40].

## 4. Conclusions

In conclusion, PVA-CMC-CA composite hydrogel membranes were successfully prepared and tested for wound dressing's application, due to their unique features such as porosity, biocompatibility, non-cytotoxicity, and high thermal and mechanical stability. The prepared hydrogel membranes were crosslinked *via* esterification mechanism using citric acid as a safe chemical crosslinker. Membranes were mechanically supported by incorporating of attapulgite mineral clay as additional physical crosslinker. PVA/CMC/attapulgite clay composite hydrogel membranes were characterized by FTIR, SEM, swelling tests, mechanical/thermal stability, as well as antimicrobial activity. The obtained results showed that, the properties of composite hydrogel membranes have significantly affected by the used CA concentration and incorporated attapulgite clay contents. It was concluded that, high CA concentration and high attapulgite clay contents in membranes kept the mechanical/thermal stability of crosslinked membranes, and resisted the degradation growth of crosslinked membranes; compared to low concentration and content of CA and clay, respectively. While, optimized CA concentration and attapulgite clay content in PVA/CMC membranes showed remarked antimicrobial activity, adequate swelling behavior, and high mechanical and thermal stability. Biocompatibility of PVA/CMC-attapulgite clay films was determined using hemolysis test which indicated that, CA crosslinked PVA-CMC-attapulgite clay hydrogels are safe for employing as a potential wound dressings and promising biomaterials for versatile biomedical purposes.

## Declarations

### Ethical statement

All experiments were performed in accordance with the Guidelines of World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects, and approved by the ethics committee at Alexandria University and The British University in Egypt (BUE).

### Conflicts of interest

The authors report no financial or nonfinancial conflict of interest.

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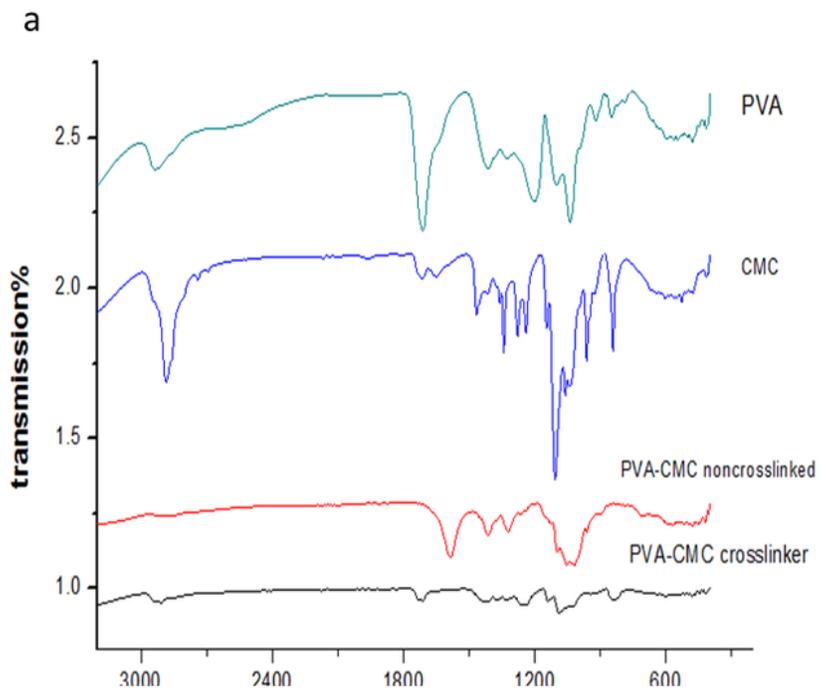
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## Scheme

Scheme 1 is available in Supplementary Files section.

## Figures



**Figure 1**

FTIR spectra of pure PVA, pure CMC, uncrosslinked PVA/CMC membranes and crosslinked PVA/CMC hydrogel membranes (**a**) and crosslinked PVA/CMC hydrogel membranes using different citric acid concentration at 7, 10, and 12 wt.% (**b**).

## **Figure 2**

SEM micrographs of crosslinked PVA/CMC hydrogel membranes, using different citric acid concentration at 7, 10, and 12 wt.%, (original magnifications at 1000X, scale 10  $\mu\text{m}$ , and applied voltage 10 kV).

## **Figure 3**

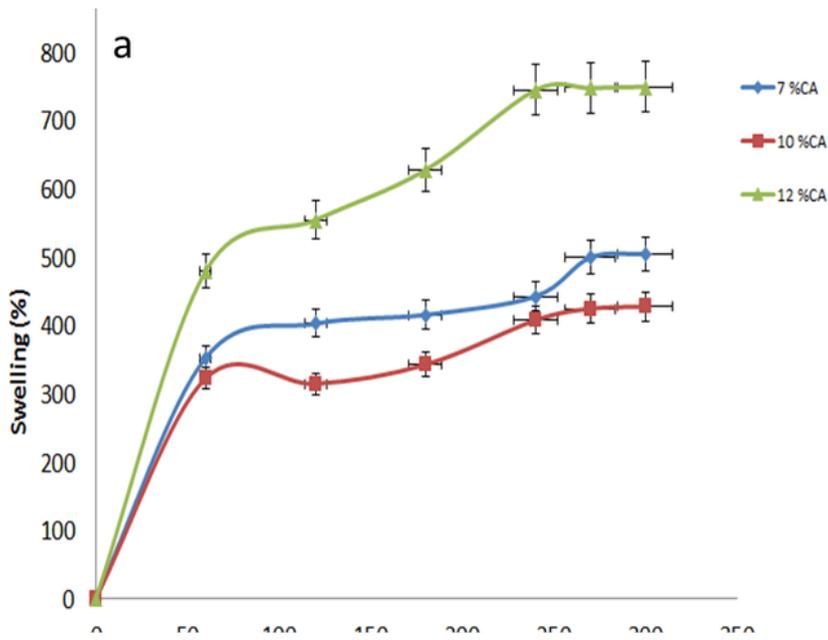
SEM micrographs of crosslinked PVA/CMC/attapulgitite clay composite hydrogel membranes, using different clay concentration at (1, 2, 4, and 5 wt.%), (original magnifications at 1000X, scale 10  $\mu\text{m}$ , and applied voltage 10 kV).

## **Figure 4**

TGA results of crosslinked PVA/CMC hydrogel membranes, using different citric acid concentration at 7, 10, and 12 wt.%.

## **Figure 5**

TGA results of crosslinked PVA/CMC/attapulgitite clay composite hydrogel membranes, using different attapulgitite clay contents in membranes at 1, 2, 4 and 5 wt.%.

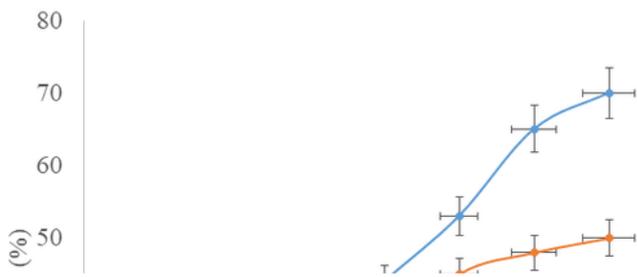
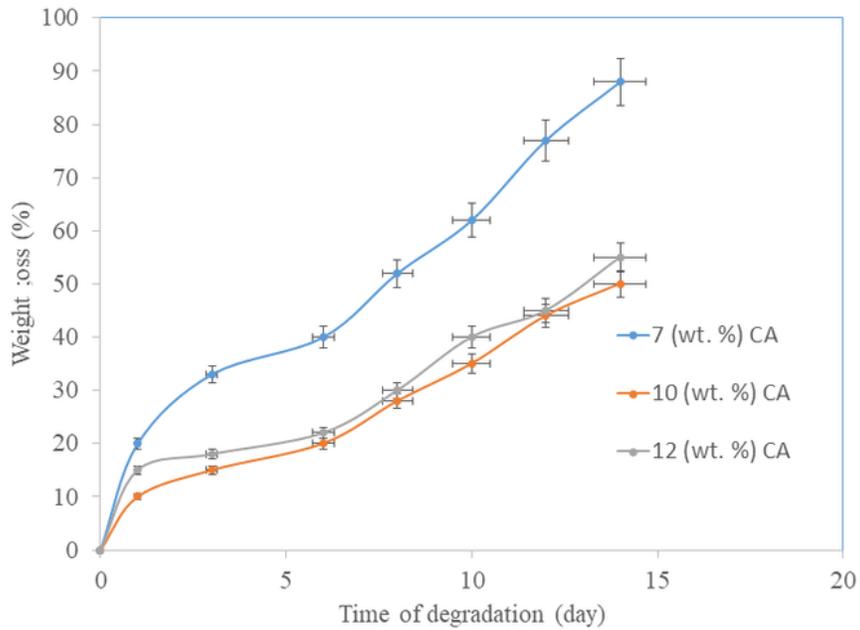


**Figure 6**

Swelling ratio % of crosslinked PVA/CMC hydrogel membranes, using different citric acid concentration at 7, 10, and 12 wt.% (a) and using different incorporated attapulgite clay contents (1, 2, 4, and 5 wt.%) (b).

**Figure 7**

Protein adsorption (%) onto the surface of crosslinked PVA/CMC hydrogel membranes, using different citric acid concentration at 7, 10, and 12 wt.% (up) and using different incorporated attapulgite clay contents (1, 2, 4, and 5 wt.%) (down).



## Figure 8

Hydrolytic degradation or weight loss (%) of crosslinked PVA/CMC hydrogel membranes, using different citric acid concentration at 7, 10, and 12 wt.% (up) and using different incorporated attapulgite clay contents (1, 2, 4, and 5 wt.%) (down).

## Figure 9

Antimicrobial activity images representing the inhibition zones of crosslinked PVA/CMC hydrogel membranes, using different citric acid concentration at 7, 10, and 12 wt.% (up) and using different incorporated attapulgite clay contents (1, 2, 4, and 5 wt.%) (down).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Scheme1.tif](#)