Chapter

Wound Dressing Application of Ch/CD Nanocomposite Film

Ranju Kandra and Sunil Bajpai

Abstract

In this work, carbon dots (CDs), obtained through microwave assisted synthesis from butane tetra carboxylic acid (BTCA), was introduced into chitosan film via simple solvent casting approach. The CDs had an average diameter of 40 to 60 nm as determined by Transmission Electron Microscopy (TEM) analysis. They possessed a zeta potential of −20.2 mV. The X-ray photon spectroscopy (XPS) confirmed presence of carboxylate groups on the surface of carbon dots. The XRD of both the plain sample Ch/CD (0) and carbon dots loaded sample Ch/CD(2) showed two crystalline sharp peaks at 14.6 and 18.1 degree, along with presence of amorphous region also. The moisture absorption data was well fitted on GAB isotherm and the profiles obtained were sigmoidal. The water vapor permeation rates for the sample Ch/CD(0) and Ch/CD(2) were found to be 1758 and 956 g/m^2 /day respectively. The film samples Ch/CD(0) and Ch/CD(20 expanded 2.8 and 103 times when immersed in 4% gelatin solution for 4 h. The % hemolysis for the samples Ch/CD(0) and Ch/CD(2) was 2.12 and 1.11 respectively, thus indicating biocompatible nature of the films. In the ex-vivo mucoadhesion study, the maximum detachment force ($F_{max)}$ was 88.22 and 46.28 mN for the samples Ch/CD(0) and Ch/CD(2) respectively. Finally, both of the samples, namely Ch/CD (0) and Ch/CD(2) scored "0", suggesting their non-cell cytotoxic nature.

Keywords: chitosan film, carbon dots, biocompatible, percent hemolysis

1. Introduction

In recent past, carbon dots have gained considerable importance as a versatile material formultiple applications [1, 2]. Usually they possess a sp^2 conjugated core with various oxygencontaining functionalities such as carboxyl, hydroxyl, aldehyde groups etc. [3, 4]., ease of functionalization, and production of fluorescence on UV exposure,carbon dots (CDs) find a number of biomedical applications like bioimaging [5–7], targeteddrug delivery [8, 9], wound dressings [10], cancer theranostics [11], screening the purinemetabolic disorders in human fluid etc. [12]. Carbon dots (luminescent) nanoparticles can be usedto track biological processes inside cells. A thorough literature survey reveals that there has not been even a single study which discussesthe changes in physico-chemical properties of chitosan film due to impregnation of carbon dotsinto film matrix. With this objective, we have previously reported synthesis and characterizationof carbon dots from butane tetra carboxylic acid (BTCA) and preliminary investigation of waterabsorption behavior of chitosan/carbon dots nanocomposite film [13]. In continuation, we hereby report a detailed investigation of physico-chemical properties and biocompatibility of

Ch/CD nanocomposite films, taking plain chitosanfilm as control. As chitosan is a biopolymer with a number of biomedical applications, it may beinteresting to see alteration in its properties upon addition of carbon dots [14].

2. Materials and methods

2.1 Preparation of CDs from BTCA

The CDs were synthesized Via microwave method .

2.1.1 Preparation of CD/Chitosan composite film

For fabrication of the chitosan/CDs nanocomposite, 3 wt% homogenous solution was prepared by dissolving chitosan in 0.3 M acid acetic. The sterile square chitosan patterns were produced using a standard square mold with the size of 30 × 40 mm. Various proportions of CDs, including The films were designated as CD/Ch(0), CD/Ch (1), CD/Ch (2) and CD/Ch (3) and Ch/CD(4) were gently added to the chitosan solution to prepare the desirable antibacterial mixture. Respectively, where the number in Parent hesis denotes the volume of CDs solution present in 20 ml of the film forming chitosan solution. Subse- quently, the mixture was injected into the square mold for the formation of composite films. Then, the molds were lyophi- lized in a freeze dryer to complete the molding process.

2.1.2 Characterization of CDs and CD/Ch composite films

The size of the carbon dots was analyzed by Transmission Electron Microscopy (TEM).The TEM samples were made by placing a drop of nanoparticle ethanol suspension on acarbon-coated copper grid. The X-ray photoelectron spectra (XPS) were performed on aVGESCALAB 220-IXL spectrometer using an Al Kα X-ray source (1486.6 eV). The crystallinenature of the plain and CD loaded chitosan film was investigated by X-ray diffraction analysisusing a Rikagu Diffractometer (Cu radiation = 0.1546 nm) operating at 40 kV and 40 mA. The X-ray Photon Spectroscopy (XPS) was also carried out.

2.1.3 Protein adsorption study

In order to study the adsorption of therapeutic protein Bovine serum albumin(BSA) on the filmsurface, the test films, namely Ch/CD(0) and Ch/CD(4), were cut into 1 x 1 cm^2 pieces andimmersed in BSA solution, prepared in phosphate buffer saline(PBS) at a concentration of 5 mg/ml, for a period of 24 h at 37 °C.

2.1.4 Antioxidant properties of hydrogel wound dressings

In order to evaluate the anti-oxidant property of the carbon dots loaded wound dressing film, sample Ch/CD(2) was taken as a representative. We followed two methods to evaluate antioxidant property of the CDs-loaded chitosan film, namely, 2,2-diphenyl-1- picrylhydrazyl(DPPH) radical scavenging assay, and superoxide radical (O_2. $^{\bullet-}$) scavenging activity assay. Inthe first method, definite quantity of powdered sample Ch/CD(2) was added in to methanolicsolution of DPPH radical (100 μM) and allowed to be kept in dark for a period of 18 h [15]. The DDPH

solution, without containing film sample, was taken as control to calculate the percentage scavenging activity [16].

$$\text{DPPH radical scavenging activity}(\%) = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of test solution respectively.

The Fenton reagent was employed to test the hydroxyl radicals scavenging capacity of plain chitosan film Ch/CD(0) and CDs-loaded sample Ch/CD(2), as described elsewhere [17]. In a typical experiment, pre-weighed quantity of grinded film was added in to a solution which contained 50 mL of 1.0 mM $FeCl_2$, 100 ml of 1 mM 1,10-phenanthroline, 3.6 ml of 0.2 M phosphate buffer (pH 7.8), 175 mL of 0.18 M H_2O_2. The reaction was initiated by addition of pre-calculated quantity of hydrogen peroxide. The reaction mixture was incubated at room temperature for a period of 15 min under mild stirring so as to keep the grinded film powder constantly exposed to free radicals generated. Finally, the absorbance was recorded at 560 nm. The film free solution was taken as control.

The activity of plain Ch/CD(0) and CDs-loaded sample Ch/CD(2) to scavenge superoxide free radicals was investigated in riboflavin/methionine-light system [18]. Results were expressed as percent inhibition of superoxide radicals. All the experiments were carried out in triplicate and average data were given.

3. Results and discussion

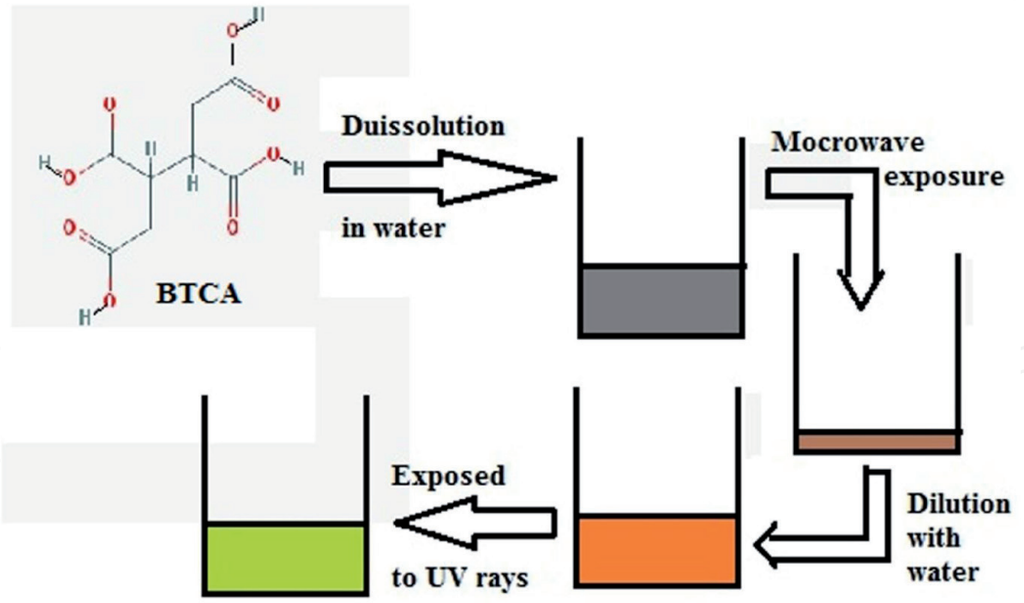
3.1 Preparation of CDs

The microwave assisted synthesis of carbon dots is an effective method to prepare CDs. When a solution of BTCA in aqueous medium, is allowed to get exposure of microwaves for a definite time, there occurs uniform heating and finally the volume of the solution is almost reduced to one tenth of the original volume. Now, the residue is taken out and is diluted by the addition of distilled water. The CDs solution, so obtained is centrifuged under high resolution speed of 10000 rpm and the supernatant is collected. The passage of laser beam through the solution is preliminary indication of the formation of carbon dots. The overall synthetic procedure and passage of laser beam through the solution are shown in **Figure 1(a)** and **(b)** respectively.

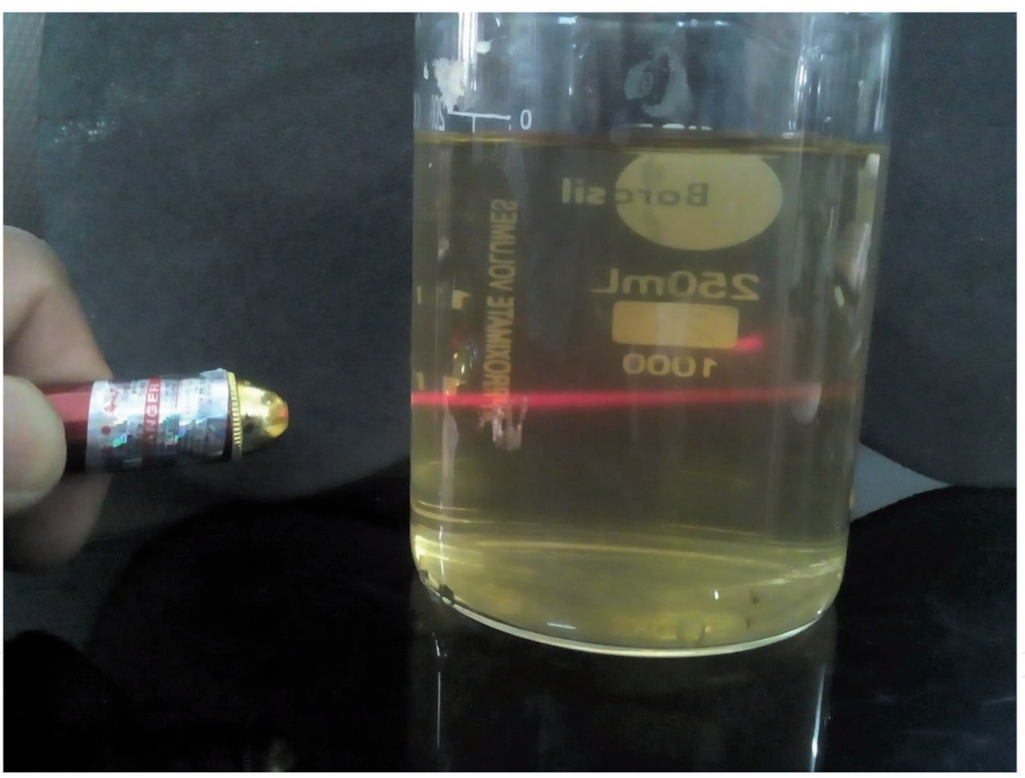
3.2 Size determination of carbon dots

As per conventional definition, carbon nanoparticles with a diameter of less than 10 nm are called carbon dots [19, 20]. Such small carbon dots are required for for cell imaging and other related biomedical applications [21]. However, in the present work, CDs with relatively larger size were required so that they could be used as efficient crosslinker to control the permeation properties of the chitosan film. The results of TEM analysis are shown in **Figure 2**.

It can be seen that the particles are almost spherical and bear size in the range of 40 to 60 nm. The relatively bigger size range could be attributable to the fact that we did not use any stabilizer in the preparation of CDs and hence chances of formation of bigger carbon nanoparticles could not be ruled out. There are



(a)



(b)

Figure 1.
(a) Scheme showing formation of carbon dots, (b) passage of laser beam through the pale yellow solution of CDs.

several reports which describe formation of bigger carbon nanoparticles. For example, Smagulova et al. [22] have synthesized carbon dots from birch soot via hydrothermal approach and reported their size in the range of 10 to 60 nm with a maximum percent of particles with diameter of around 25 nm. Similarly, Hou et al. [23] synthesized carbon dots from human hair for detection of Hg (II) ions and reported their diameter in the range of 29 to 80 nm. Similarly, Runa et al. [24] reported synthesis of carbon dots from spider silk via hydrothermal approach and reported an average diameter of 178 nm. Therefore, it appears that it is possible

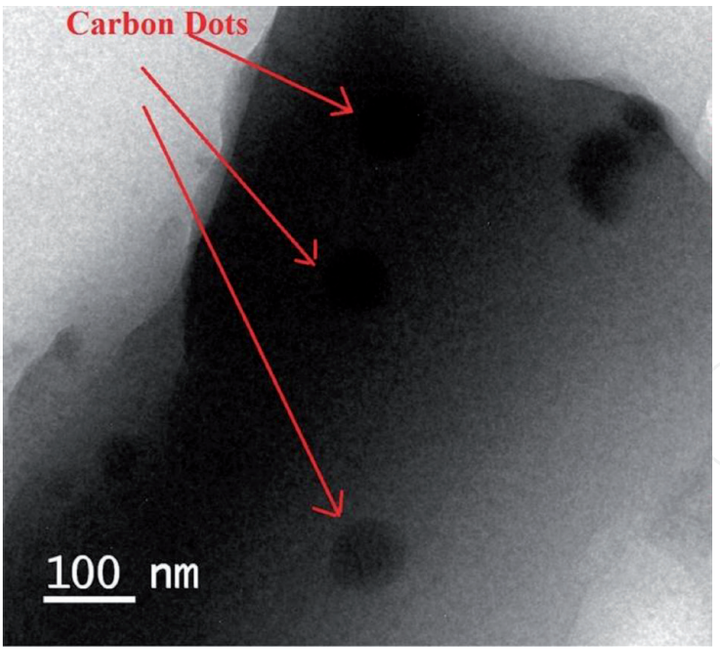


Figure 2.
TEM image of CDs with 100 nm bar length.

to prepare carbon dots of different sizes, depending upon their applicability. For example, CDs mentioned in the aforesaid examples cannot be used for bio imaging and intracellular sensing.

We also measured zeta potential of as –prepared carbon dots which was found to be −20.2 mV, thus indicating the negatively charged surface of carbon dots. This is simply attributable to the presence of carboxylate groups on the surface of carbon dots, thus rendering them negatively charges surface.

3.3 XPS analysis of carbon dots

A survey scan, ranging from 0 to 1200 eV is shown in **Figure 3(a)**. It is evident that two elements, namely C (286 eV) and O (543 eV) are present in the spectrum [25]. The detailed XPS spectra of C 1S and O 1S are shown in **Figure 3(b)** and **(c)** respectively.

The C 1S signal peak can be divided in to three peaks, located at 283.8, 284.83and 287.4 eV. These peaks correspond to C-C, C=C and C=O respectively [26]. Finally, in the XPS spectrum of O1S, the peak at 532.25 eV refers to oxygen singly bound to aliphatic carbon while the smaller or low intensity peak at 535.53 eV refers to COO^- thus confirming the presence of carboxylate groups on the surface of carbon dots.

3.4 Preparation of Ch/CD films

In this work, we prepared Ch/CD films by solvent evaporation method. When the film forming solution is placed in an oven (see experimental section), the solvent is evaporated and semi-transparent film with dark brown appearance is obtained. The crosslinking of chitosan chains by carbon dots may be described as follows: In the film forming solution, chitosan exists in dissolved state with protonated $–NH_3^+$ groups along the macromolecular chains. The final pH of the solution was found to be 6.2. At this pH, -COOH groups present on the surface of the carbon dots, ionize to give negatively charged $–COO^-$ groups, thus rendering negative charges on the surface of CDs. These negative charges bind electrostatically to the protonated amino groups of chitosan chains and a crosslinked network is formed.

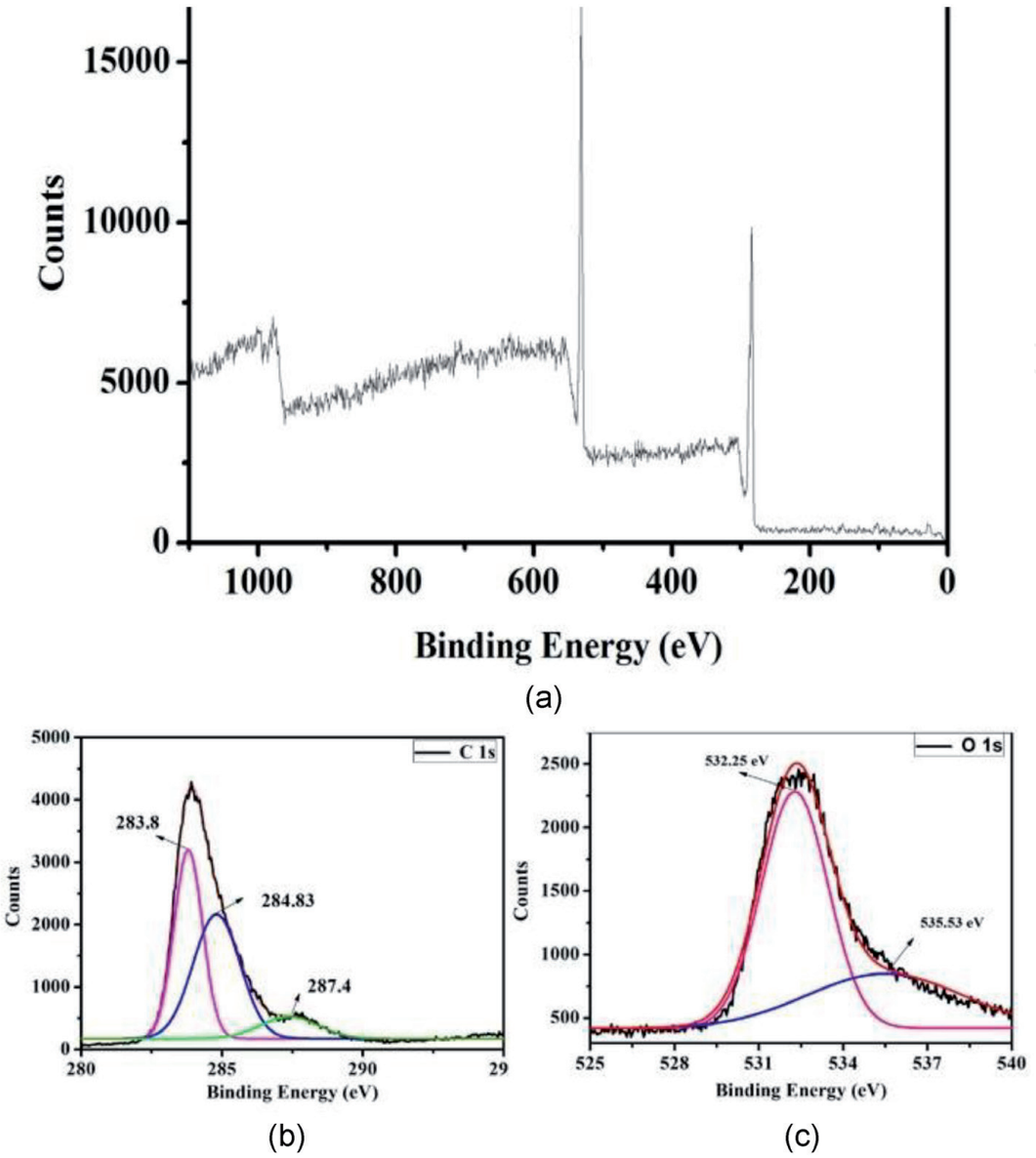


Figure 3.
(a) A survey scan, ranging from 0 to 1200 eV; XPS spectra of (b) C 1S and (c) O 1S.

The plain Ch film appeared to be semi-transparent with pale yellow appearance while the Ch/CD film was dark brownish. An optical photograph of the plain Ch/CD(0) and the Ch/CD(2) films and mode of crosslinking is shown in **Figure 4(a)**.

It is well known that carbon dots emit fluorescence on exposure to UV radiations of suitable Wavelength [27]. This makes them a potential candidate for imaging and other related applications [28]. The optical image of the sample Ch/CD(2), exposed to UV radiations, is shown in **Figure 4(b)**. It can be noticed that the film appears green, due to the fluorescence exhibited by carbon dots present within the film matrix.

3.5 XRD analysis of films

The crystalline nature of the plain and CDs loaded films was investigated by XRD analysis. The XRD patterns of plain film Ch/CD(0) and carbon dots loaded film Ch/CD(2) are shown in **Figure 5(a)** and **(b)** respectively. It can be seen that both of the samples, namely plain sample Ch/CD(0) and CDs-loaded sample Ch/CD(2) exhibit two peaks at 2θ values of 14.6 and 18.1, indicating presence of crystalline region within

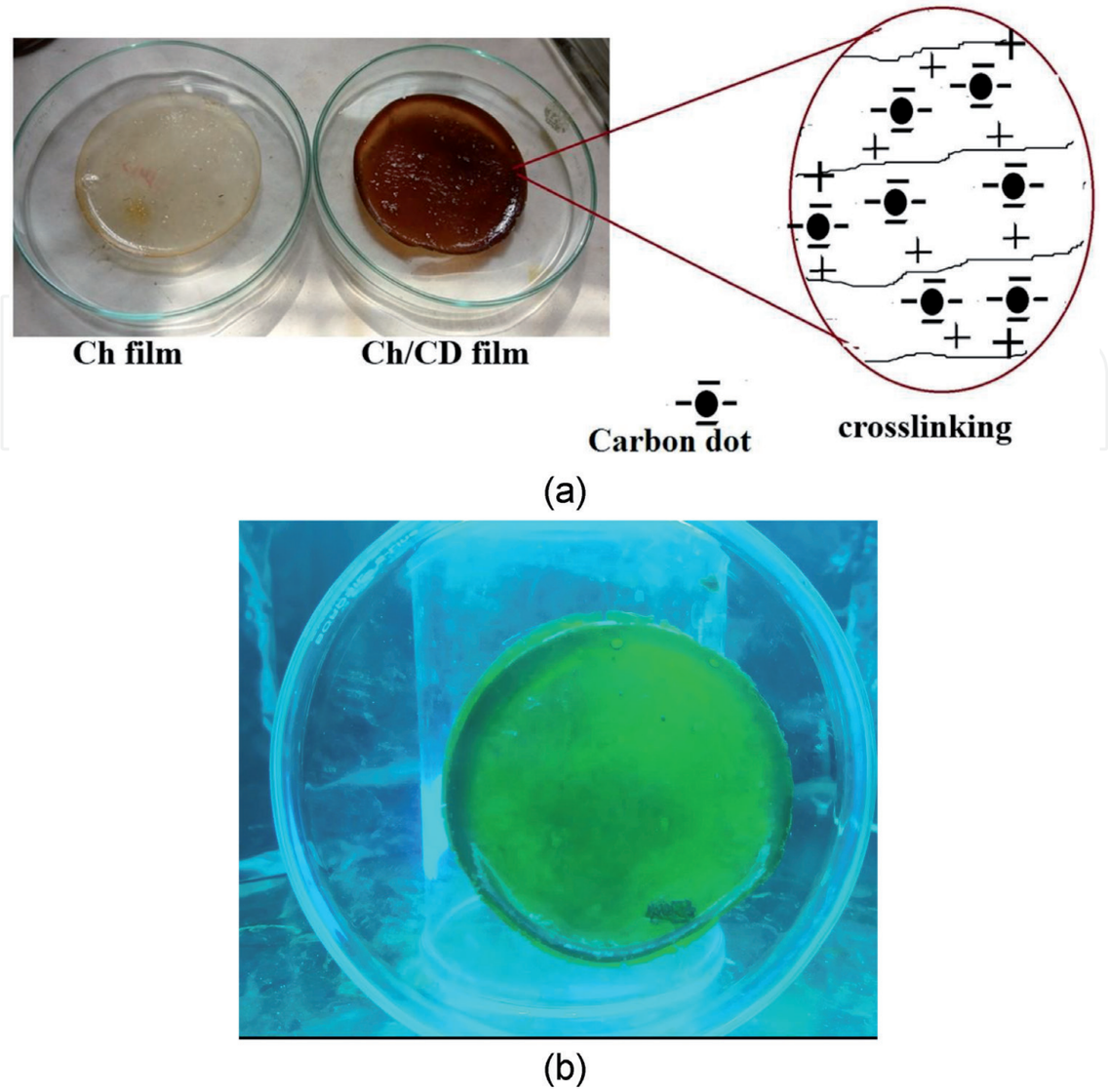


Figure 4.
(a) Optical photographs of the samples Ch/CD(0) and Ch/CreD(2) and mode of crosslinking by carbon dots; (b) optical image of the sample Ch/CD(2) when exposed to UV radiations.

the film matrix. These values are very close to those reported elsewhere [29, 30]. In addition, a scattered broad pattern is also visible, suggesting amorphous region too.

The XRD pattern of the nano-composite film Ch/CD(2), as shown in **Figure 5(b)**, also shows similar pattern with the two peaks, occupying almost the same positions. However, the difference lies in the fact that in the case of CD/Ch(2) film the intensities of the two peakes have decreased remarkably, probably due to presence of amorphous carbon dots within the film matrix. It is also noticable that the amorphous scattered bump is much more pronounced in the XRD pattern of composite film.In this way, it may be concluded that presence of carbon dots within the chitosan film has resulted in increase in the amorphous nature of the composite film.

3.6 Film expansion study

The use of a polymeric film for wound dressing requires fair structural integrity in the presence of exudate coming out from wound. The reason is that when a film is placed over the wound, it comes in contact with the exudate and begins to undergo expansion in its size. If the film expands appreciably, then it may lose its integrity, become soft and sticky, and ultimately may cause inconvenience to the patient. It

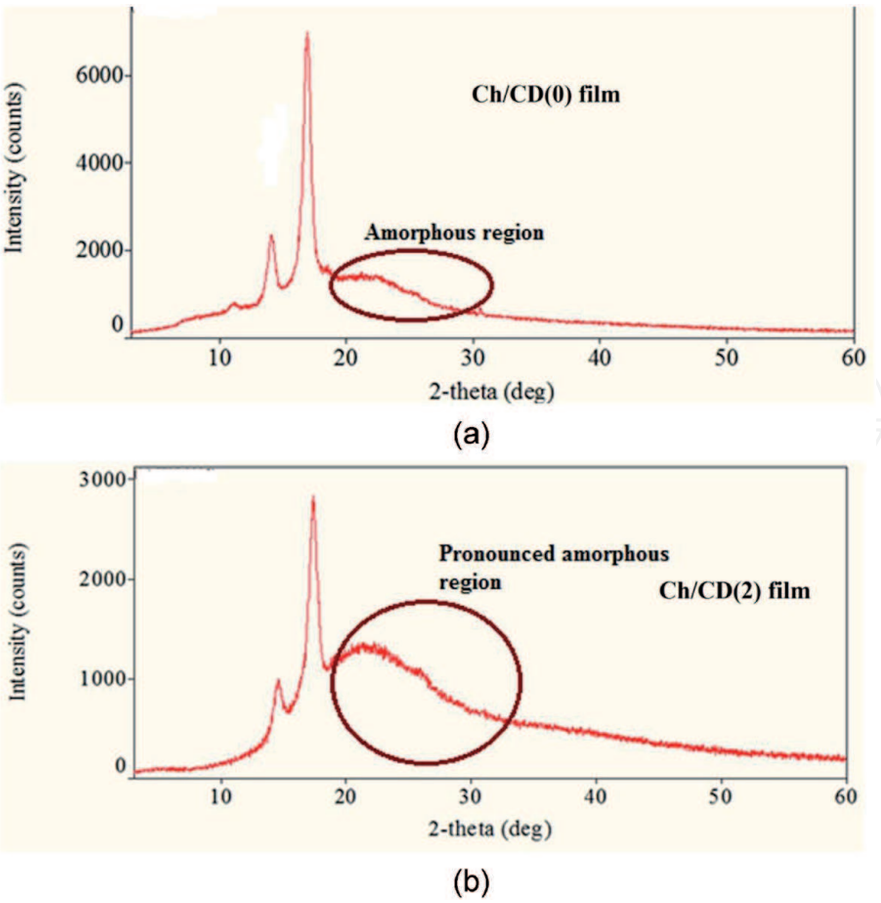


Figure 5.
X-ray diffraction of film samples (a) Ch/CD(0) and (b) Ch/CD(2) .

may also be torn and leave the wound surface. Hence, it becomes essential to test its expansion limit and structural integrity. The results of expansion study are shown in **Figure 6**.

It is noticeable that the plain Ch/CD(0) film undergoes 2.8. Fold expansion in its diameter in duration of 60 min, while in the same time frame, the Ch/CD(2) film expands to only 1.3 times and it maintains its structural integrity throughout. It is also worth mentioning here that the plain film sample Ch/CD(0) gets hydrated, slippery and becomes difficult to handle properly Thus it may be concluded from this study that addition of pre-calculated quantity of carbon dots in to chitosan film can render it enough mechanical strength and control its water absorption capacity as per requirement.

3.7 Biocompatibility tests

Blood compatible nature of the films Ch/CD (0) and Ch/CD(2) was evaluated in the terms of % hemolysis. The % hemolysis of these films was found to be (2.12 ± 0.02) and (1.13 ± 0.18) respectively.

3.8 Protein adsorbed study

The adsorption of protein on a wound dressing film indicates its cell adhesion behavior. Albumin, a multifunctional transporter protein, is the most abundant protein found in the plasma (approx. 50 $mg.ml^{-1}$) [31]. Its adsorption is related to the inhibition of the coagulation cascade and consequently, platelet adsorption. As per reports [32], albumin has high adsorption affinity for hydrophobic surfaces,

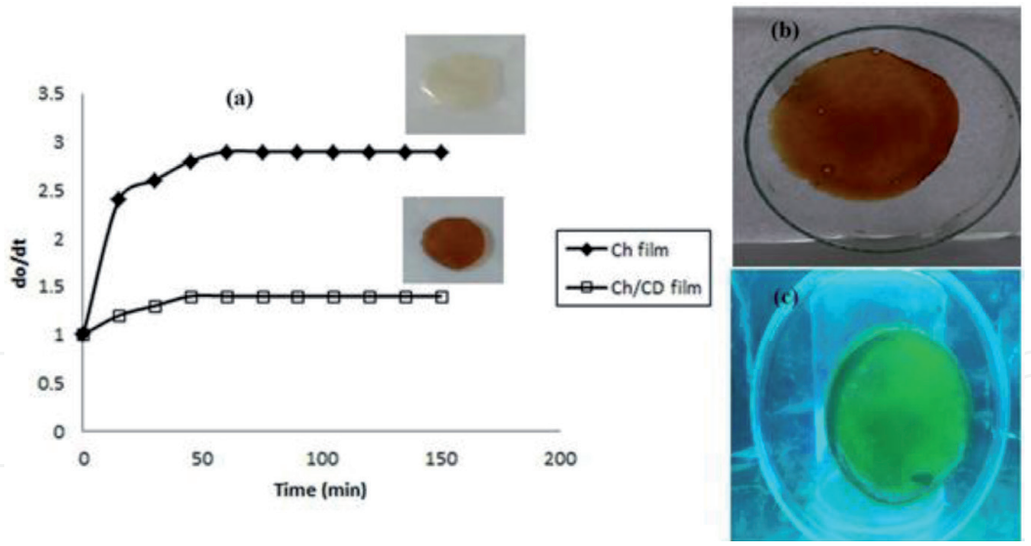


Figure 6.
d/d_o (versus time profiles for the samples) Ch/CD (0) and Ch/CD(2) at 37° C.

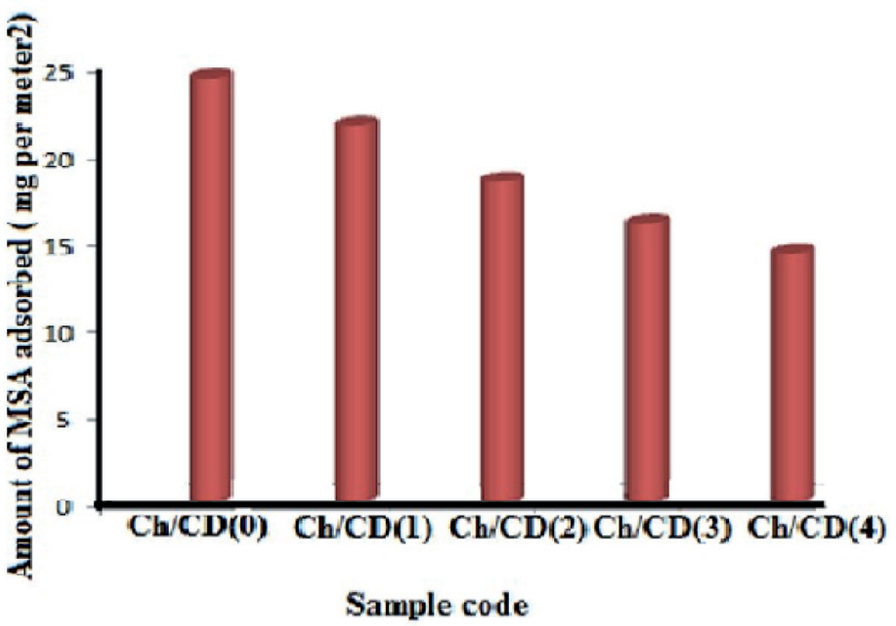


Figure 7.
BSA adsorption on the various samples Ch/CD (0), Ch/CD (1), Ch/CD (2), Ch/CD (3) and Ch/CD (4) at 37° C.

mainly because of hydrophobic interactions between the protein and the surface. Indeed, a wound dressing film with a poor tendency to adsorb albumin is desirable. The results of BSA adsorption study are shown in **Figure 7**.

It can be seen that plain sample Ch/CD(0), and CDs-loaded samples Ch/CD(1), Ch/CD(2), Ch/CD(3) and Ch/CD(4) show BSA adsorption of 24.2, 21.5, 18.3, 15.9 and 14.1 mg/m^2 respectively. The extremely low values of BSA adsorption may be indicative of the hydrophilic nature of the film surfaces for all the samples studied. It has been reported [33] (**Figure 8**).

3.9 Antioxidant properties of hydrogel wound dressings

In this work, % scavenging capacity of the plain film sample Ch/CD(0) and carbon dots loaded samples Ch/CD(1), Ch/CD(2), Ch/CD(3) and Ch/CD(4) for the various free radicals i. e. DPPHR, SOR and HR are shown in **Figure 9**. It can be seen

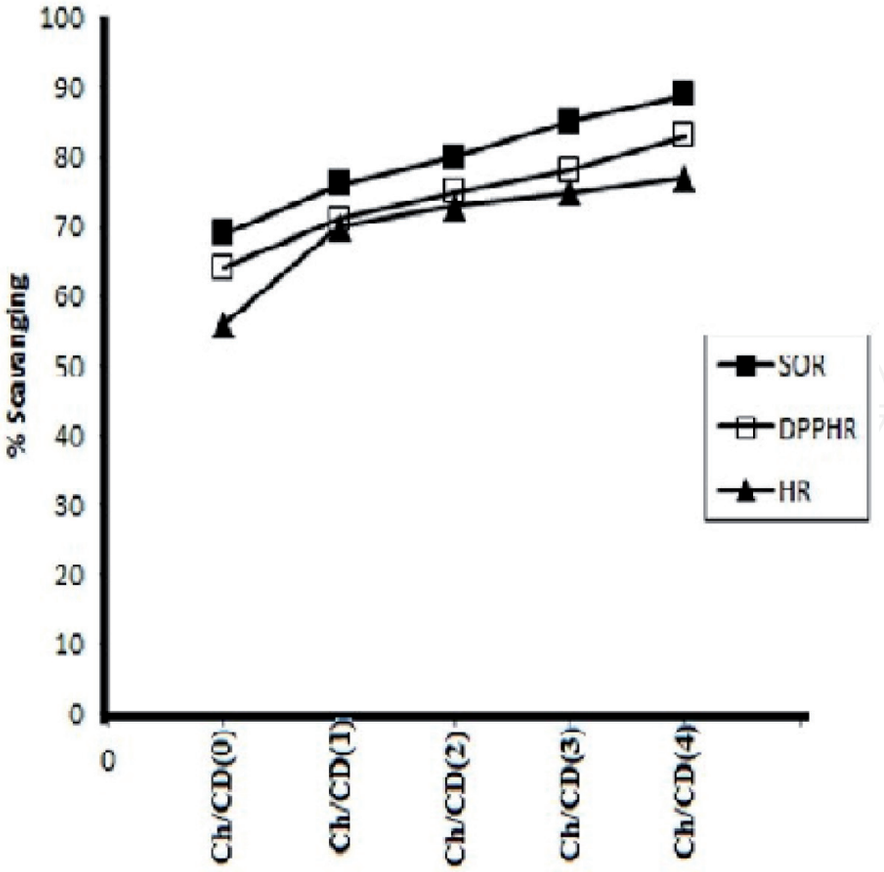


Figure 8.
Percent scavenging for DDPH, superoxide and hydroxyl free radicals by various film samples.

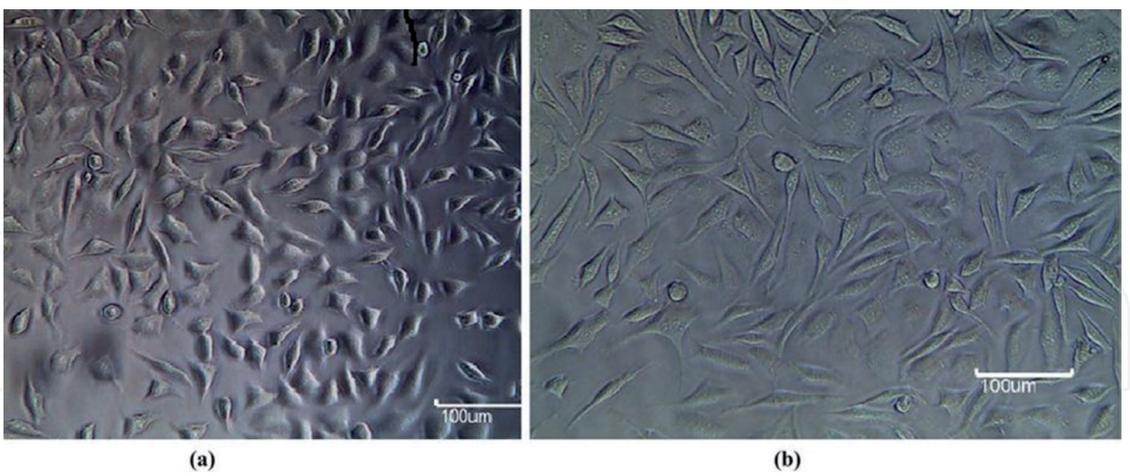


Figure 9.
Images showing completely undamaged L929 cells after 24 h contact with extract of film samples (a) Ch/CD (0) and (b) Ch/CD(2).

that plain chitosan film sample shows fair % Scavenging capacity and it increases slightly due to addition of carbon dots in the film matrix.

Owing to the fair stability, DPPH radical is usually employed to determine the antioxidant or free radical scavenging activity of the wound dressing films. The method involves reduction of methanolic DPPH radical by hydrogen donating antioxidant polymeric film. Topical wound healing formulations with DPPH scavenging ability have been reported to improve skin repair and regeneration [34]. The observed increase may probably be due to the contribution of –COOH groups

present on the surface of carbon dots towards reduction of DPPH radicals. It is here also worth mentioning that chitosan has already been reported to have free radicals scavenging activity [35]. Therefore it appears that addition of functionalized carbon dots into chitosan film matrix improves its antioxidant property.

Hydroxyl, an oxygen centered radical, attacks proteins, DNA, polyunsaturated fatty acid in membranes, and most biological molecules with which it comes in contact [36]. It abstracts hydrogen atoms from membrane lipids [37] and brings about peroxidic reaction of lipids. As per reports [38], the scavenging action of chitosan against hydroxyl radicals may probably be due to following reactions (i) The hydroxyl groups, present within the chitosan macromolecular chains, may react with. OH via hydrogen abstraction, and (ii). OH can react with the residual free amino groups NH_2 to form stable macromolecule radicals. Finally, when superoxide anions come in to contact with a biomolecule, they damage it directly or indirectly by forming H_2O_2, ^{-}OH, peroxy nitrite or singlet oxygen during aging and pathological events such as ischemic reperfusion injury. It is also reported that Super oxide radicals initiate lipid peroxidation [39]. Therefore, scavenging of superoxide radicals by chitosan based wound dressing might be helpful for preventing delayed wound healing induced by superoxide radicals in pathological conditions. The scavenging of SOR will also diminish formation of hydroxyl radicals, which is considered main factor for delayed healing of chronic wounds.

3.10 Ex-vivo mucoadhesion studies of films

The adhesion of a wound dressing film on the wounded skin is a significant parameter and requires a perfect balance between the adhesion capacity of the dressing film and comfort level of patient. In case, the film has a very strong adhesion tendency, it may cause discomfort and pain during removal of the dressing. However, its poor adhering property may also be uncomfortable from the point of view of wound healing. In this work, maximum detachment force (F_{max}) required to detach the film from mucosal surface, was determined for all the film samples namely, Ch/CD(0), Ch/CD(1), Ch/CD(2), Ch/CD(3) and Ch/CD(4). The values of F_{max} were found to be 88.22 ± 11.52, 45.15 ± 8.61, 43.29 ± 7, 44.25 ± 6.97, and 46.22 ± 5.94mN.

In a report, F_{max} value for gum acacia-cl-(poly(HEMA-co-carbopol hydrogel film was found to be 70.20 ± 17.57 m N. The higher value was attributed to the fair hydrophilic nature of the film [39]. In the present work, F_{max} values for various CDs-loaded chitosan films were relatively low, probably due to the presence of functionalized carbon dots within the film matrix. The poor water absorption tendency of these films did not induce polymeric chain relaxation and hence there were no new active sites available for exposure to mucus surface. In addition, the physical crosslinks between carboxylate groups present on the carbon dots surface (as discussed earlier) and protonated amino groups on chitosan chains also made the chitosan chains rigid and prevented them from relaxation.

A plausible explanation for mucoadhesion behavior of plain chitosan film Ch/CD(0) and CDs-loaded sample Ch/CD(2) may be given on the basis of the most commonly proposed Diffusion-Interpenetration Theory [40, 41]. In the case of pure chitosan film sample Ch/CD(0), an appreciable water content within the film matrix causes polymeric chains to relax or un-fold,thus resulting in exposure of new active sites. These polar segments come in contact with mucuschains and get entangled with them. This results in stronger bio-adhesion as shown in **Figure 10(a)**.

However, in the case of CDs-loaded sample Ch/CD(2), the film absorbs very small quantity of water due to physical crosslinks and therefore polymeric chains remain folded or non-relaxed. This minimizes the interaction with mucus chains as shown in **Figure 10(b)**. As a result, value of F_{max} is quite low.

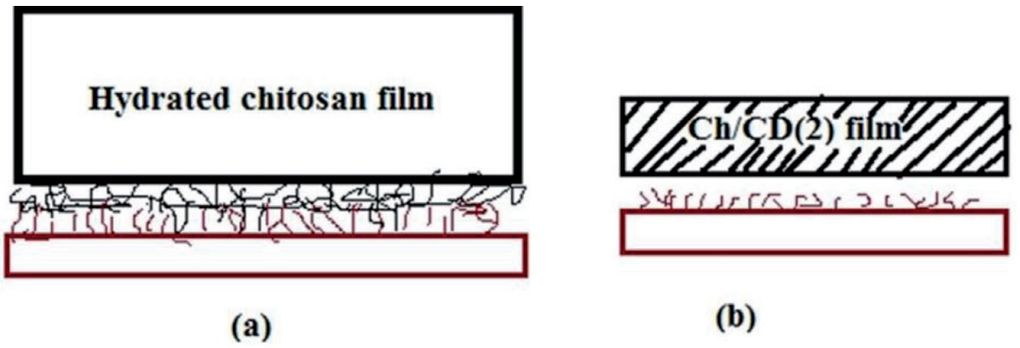


Figure 10.
Bio adhesion in the film samples Ch/CD(0) and Ch/CD(2).

3.11 Cell cytotoxicity studies

The results of cell cytotoxicity for the plain sample Ch/CD (0) and the CDs loaded sample Ch/CD (2) are shown in **Figure 9(a)** and **(b)** respectively.

As per ISO 10993-5, the achievement of numerical grade more than 2 is considered as toxic. The score was obtained as 0 for both of the samples, thus indicating non-cell cytotoxic nature of both of the film samples. It can be seen that there are discrete intra cyto plasmatic granules, no cell lysis and there is no reduction of cell growth.

4. Conclusions

It may be concluded from the above study that BTCA, a tetra carboxylic acid compound, can conveniently be used as precursor to synthesize negatively charged carbon dots. These carbon dots, when loaded into plain chitosan film, cause a drastic fall in the water absorption capacity of the resulting nanocomposite films. A detailed investigation of these CDs-loaded chitosan film is under progress and a detailed report shall be documented soon.

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