Journal of Advanced Zoology

ISSN: 0253-7214

*Volume **45** Issue **3 Year 2024** Page 726-**736***

K^+ Ions Crosslinked Kappa-Carrageenan/Methylene Blue Composite Film. Part-1: Synthesis, Characterization And Application As Controlled Release Device For Photodynamic Therapy Application

Aniteshma Chanpuria[a], M. Bajpai[a], S. K. Bajpai[a], Seema Tiwari[b*], Sonali Bhosle[b]

[a]Polymer Research Laboratory, Department of Chemistry, Govt. Science College, Jabalpur (M.P.)-482001 INDIA

[b]Army Institute of Technology, Dighi Hills, Pune-411015, INDIA

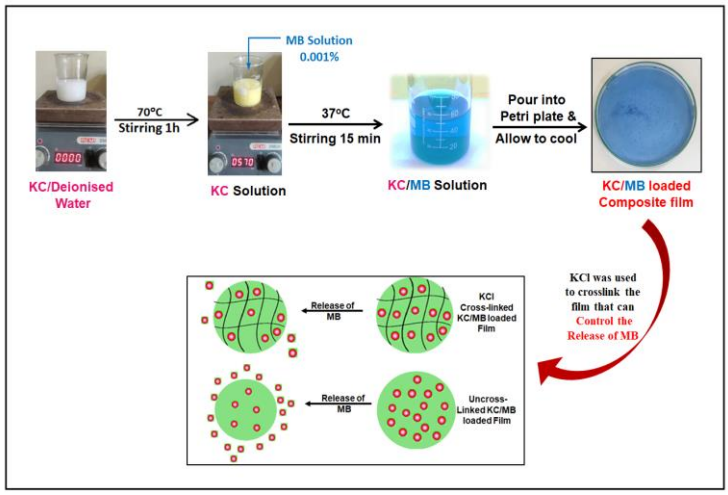
***Corresponding Author:** Dr. Seema Tiwari*

E mail: stiwari@aitpune.edu.in

Abstract:

K^+ ions crosslinked (KC/MB) Methylene blue photosensitizer loaded composite film is prepared for photodynamic therapy (PDT) application as Photosensitizer released by composite film can conveniently be controlled by using different quantities of K^+ ions and this application is based on dynamic interaction between light with suitable wavelength, photosensitizer and molecular oxygen, promoting the death of the target tissue or bacterial cells. In this work, the Methylene Blue (MB) loaded Carrageenan polymeric film was prepared by the method of direct addition of MB into pre-polymerization solution of Carrageenan (KC). This composite film and plain KC film were characterized by FTIR, XRD and SEM analysis. The characteristic peaks of MB photosensitizer were obtained in FTIR spectrum of composite film. The composite film KC/MB shows prominence of amorphous nature may be due to MB is present in very small quantity. The SEM analysis reveals that the composite film (KC/MB) having much more smooth surface texture throughout the film. Finally, the result of effect of variation in degree of crosslinking on amount of MB release is, as the amount of K^+ ions used to crosslink the film increases, MB release decreases.

Keywords: Carrageenan, Methylene Blue, Photosensitizer, Photodynamic therapy.



Graphical abstract

1. INTRODUCTION

The use of photosensitizers such as Methylene Blue (MB), Eosin etc. in management of wounds via Photodynamic Therapy (PDT) is an interesting and significant strategy [1], [2]. This is based on the fact that these dyes, when exposed to visible light of suitable wavelength, get achieved to singlet excited state followed by transition in to more stable triplet state [3], [4]. Now, it interacts with biomolecules to produce Reactive Oxygen Species (ROS). Which, after start a cascade of biochemical events, finally leading to death of microorganisms. Water-soluble hematoporphyrin derivative (HPD) was known as photofrin and it was the first photosensitizer (PS) used for PDT [5], [6]. J. F. Kelly and colleagues reported that Hematoporphyrin (PS) light activation could cure bladder cancer in mice [7], [8]. Since the past 3000 years, Ancient Egyptian, Indian, and Chinese cultures used light with suitable wavelength has been used for PDT to cure numerous cancerous conditions [9]-[11]

In the present study we have taken Methylene Blue photosensitizer as active ingredient to cause bacterial cells death [12]. The MB has been loaded into Carrageenan (KC) based polymer film and crosslinked the film with K^+ ions. In this way the function of MB loaded Carrageenan film performs as the amount of Methylene Blue released from composite film can conveniently be controlled by using different quantities of K^+ ions to crosslinked the film. In other word, the variation in K^+ ions concentration in the film will allow as to deliver a require amount of Methylene Blue dye.

2. EXPERIMENTAL

2.1Materials

Methylene Blue (MB) with molecular mass was 319.85g/mol, was purchased from Hi Media Chemicals, Mumbai, India and used as received. Kappa-Carrageenan (KC) was purchased from Sigma-Aldrich Chemicals, Mumbai, India and used as received and the distilled water was used throughout the experiments.

2.2. Molar Mass of Kappa – Carrageenan

In order to determine the viscosity average molecular mass, viscometrical was followed. In a typical experiment stock solution of KC with concentration of 1.6% (w/v) was prepared in double distilled water and using this solution as a number of KC solutions of varying concentrations were prepared. Each solution was allowed to run through Ostwald Viscometer, and flow time for each solution was recorded using a stop watch [13], [14].

2.3. Moisture Content Determination of Polymer

A pre calculated quantity of KC powder was taken in electric oven at 60°C temperature and its mass measurements were made at a regular time intervals of 30 minutes till the attainment of constant weight. The % moisture content was obtained using the following formula.

$$\text{Percent Moisture Content} = \frac{\text{Initial weight} - \text{Dry weight}}{\text{Initial weight}} \times 100 \quad \text{... (1)}$$

2.4. Preparation of Methylene Blue-loaded KC Film

50 ml of 1.6 % (w/v) solution of KC was taken in a beaker, under the mild stirring at 70^{O}C till a uniform solution was obtained, to this solution 10 ml of 0.001% MB solution was added and stirred for a period of 15 minutes at a room temperature. The resulting solution was poured into Petri plate with the diameter of 10 cm and allowed to cool. After gelation the resulting film was taken out and kept in a dust free chamber to room temperature for further use.

Here, it is worth mentioning that due precautions were taken to ensure that Methylene Blue was not expose to visible light. All the analysis and investigation of transparency were carried out using a representative sample KC/MB $_{(0.08)}$. Where, the number in parenthesis denotes micromole of MB present in 1 gram of wet gel.

2.5 Characterization

2.5.1 Fourier Infrared Spectroscopy Analysis

The infrared spectrum of plain Kappa-Carrageenan film, powder of Methylene Blue and MB photosensitizer loaded Carrageenan film samples were obtained by Fourier Transform Infrared Spectrophotometer, powdered samples were mixed with KBr, the scan was recorded, and the selected spectral range was in between 400 to 4000 cm^{-1}.

2.5.2 XRD Analysis

The X-Ray Diffraction method was used to determine the crystalline nature of plain Kappa-Carrageenan film, powder of Methylene Blue and MB photosensitizer loaded Kappa Carrageenan polymeric film samples, and the Crystallinity Index of these samples were analysed by Rikagu diffractometer running at 40 kV and 40 mA.

2.5.3 SEM Analysis of Film Samples

The surface morphology of the plain Carrageenan film and Methylene Blue loaded composite film was investigated by Scanning Electron Microscope analysis by using JEOL 6400 F microscope. 6 nm layer of gold and palladium layers are coated after the accelerating voltage of 2 KV and a working distance of 4.4 mm with a 50 µL sediment suspension were sprayed silicon wafers to clean them.

2.6. λ_{max} Measurement of Methylene Blue Solution

The visible spectrum of MB solution was investigated using an UV-2371PC UV/VIS Spectrophotometer [15]. The studied range was set to 400–800 nm with 10 nm step width and the concentration of solution was 0.001%. The optical image shown in (Figure 1).

Figure 1. The image of UV/VIS Spectrophotometer (UV-2371PC) for measurement of λ_{max} at room temperature.

2.7. Beer-Lambert Plot for Methylene Blue Solution

In a typical protocol aqueous solution of Methylene Blue of different concentrations were prepared and their absorbance were measured at wavelength of 400-800 nm using an UV-2101PC UV/VIS Spectrophotometer. Finally, a graph was plotted taking percent concentration along X axis and respective absorbance along Y axis.

2.8. Effect of Cross-linking on Methylene Blue Release

As mentioned in the section introduction (page no. 05), the KC/MB $_{(0.08)}$ composite film exhibits bifunctional nature. To investigate the effect of variation in degree of crosslinking on amount of Methylene Blue release, a

representative sample KC/MB $_{(0.08)}$ was selected for this study and a number of samples were prepared having different amount of KCl crosslinker. The samples were designated as KC/MB $_{(0.08)}$ (0), KC/MB $_{(0.08)}$ (1), KC/MB $_{(0.08)}$ (3) and KC/MB $_{(0.08)}$ (5) respectively. Here, number in parenthesis is the % concentration of crosslinker KCl used to crosslinked the films. The MB loaded films were immersed in definite volume of PBS buffer solutions and the quantity of MB released at different time intervals, was monitored Spectrophotometrically [16].

3. RESULT AND DISCUSSION

3.1. Molar Mass of Kappa-Carrageenan

The molar mass of a polysaccharides is usually determined by viscosity measurement due to fact that this method does not require any expensive instrumental setup and it is more convenient as compared to other methods.

The basic well-known equation, given by Mark-Houwink is known as below.

$$[\eta] = KM^{\alpha} \quad \text{... (2)}$$

Where, $[\eta]$ is Intrinsic Viscosity, **M** is Molar Mass and **K** and **α** are characteristic constant for a particular polymer solvent system and are usually obtained from previous literature available. The Intrinsic Viscosity is usually given as Reduced Viscosity when the concentration of polymer solution approaches towards zero. In addition, the Reduced Viscosity is calculated as:

$$\text{Reduced Viscosity} = \frac{\text{Specific Viscosity}}{\text{Concentration}} \quad \text{… (3)}$$

'Or'

$$\text{Reduced Viscosity} = 1/\text{Concentration}\ (t - t_o/\ t_o) \quad \text{... (4)}$$

Where, t_o and **t** are flow times recorded with pure solvent and polymer solutions respectively. The data related with Reduced Viscosity and corresponding % concentration is given in (Table 1) and (Figure 2). It is clear that the linear plot, when extended towards 0 concentration gives value of Intrinsic Viscosity. In this study, value of Intrinsic Viscosity was found to 67.34. Finally, the viscosity average molar mass was found to be 19953 Da.

Table 1. Data showing values of Reduced Viscosity and % concentrations for molar mass determination of Kappa-Carrageenan.

% Concentrations of Kappa-Carrageenan solutions (w/v)	Values of Reduced Viscosity for Kappa-Carrageenan solutions (w/v)
0.016	81.25
0.032	93.75
0.048	109.16
0.064	121.56

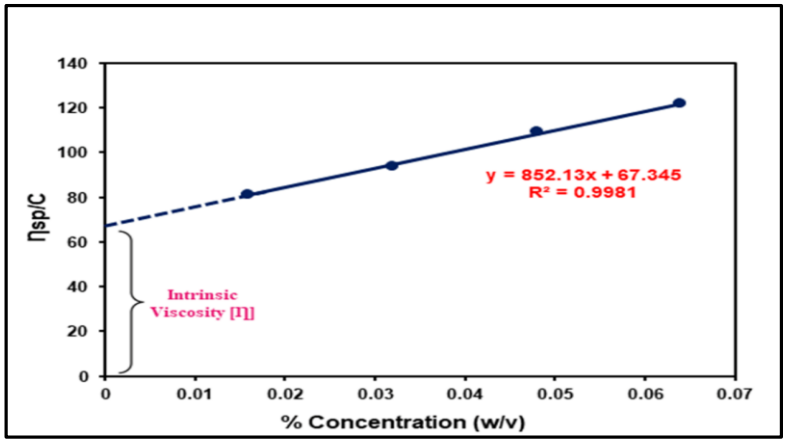


Figure 2. Linear plot between ηsp/C and % concentration for Kappa-Carrageenan solutions at room temperature.

3.2. Moisture Content Determination of Polymer

The results of moisture content study is illustrated in (Figure 3). It can be seen that as the time increases more and more water vapor leave the Carrageenan powder and therefore the weight of powder continues to decrease and attains a constant value after 4 h. The total moisture content of Carrageenan as determined using the Equation 1. was found to be 17%. The relatively higher moisture content of KC may probably be due to the fact that it contains a number of polar groups such as -OH and therefore possessive great affinity for water vapor.

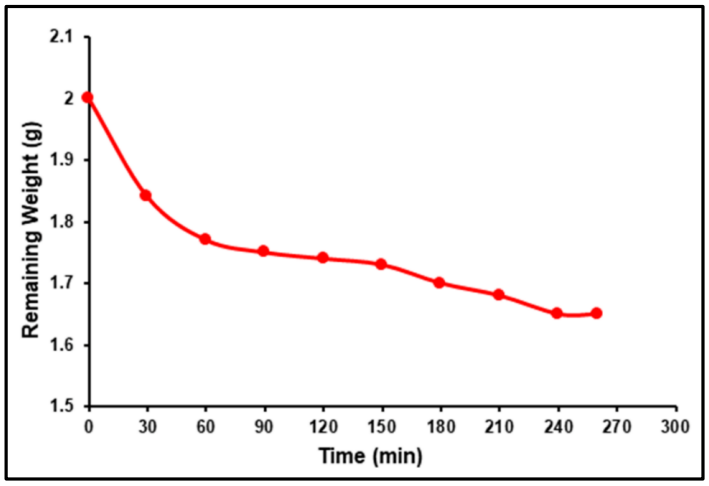


Figure 3. Kinetic plot for remaining weight of KC powder at 60°C.

3.3. Preparation of Methylene Blue-loaded KC Film

In this study, the MB loaded Carrageenan hydrogel were prepared by the method of direct addition of Methylene Blue (MB) into pre-polymerization solution of Carrageenan. The reason was that, this method enables as to know the exact quantity of MB present in the hydrogel. The overall mechanistic aspect of gelation mechanism consists of preparation of hot aqueous mixture that contains dissolved molecules of Carrageenan and Methylene Blue, followed by its gelation and cooling down to room temperature. During the cooling process, the Carrageenan polymeric chains undergo gelation owing to the formation of helix. The Methylene Blue molecules are just entrapped physically within the hydrogel. The optical images of plain and MB loaded hydrogel are shown in (Figure 4).

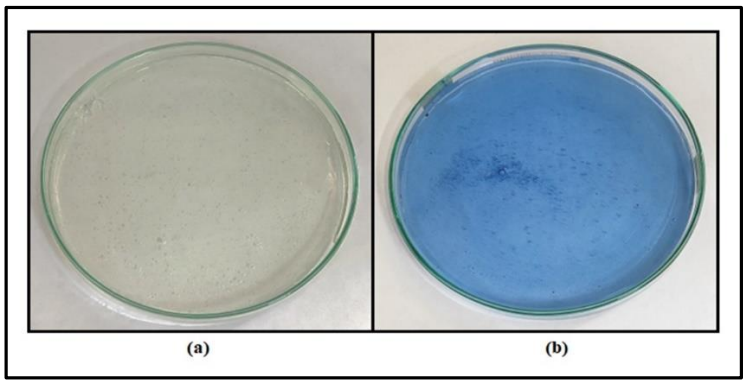


Figure 4. (a) The optical images of plain Carrageenan polymeric film and **(b)** Methylene Blue photosensitizer loaded composite film.

3.4. Characterization

3.4.1 Fourier Infrared Spectroscopy Analysis

As the present study involves use of Methylene Blue loaded hydrogel for Photo Dynamic Therapy, the FTIR spectra of native MB powder and representative sample KC/MB $_{(0.08)}$ were recorded and are given in (Figure 5). On the comparison with the standard data available it was found that most of the peaks were in fair agreement with the reported data available in the literature [17], [18]. The is given in (Table 2) below.

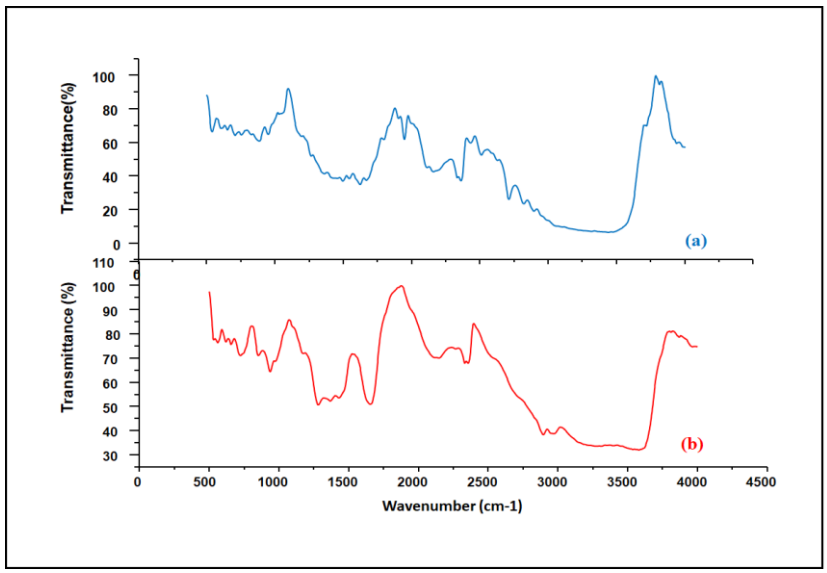


Figure 5. FTIR spectra of (a) Methylene Blue powder and (b) Methylene Blue loaded Carrageenan composite film (KC/MB $_{(0.08)}$) (CIF, Savitribai Phule Pune University (SPPU).

Table 2. The data for IR spectrum of Methylene Blue

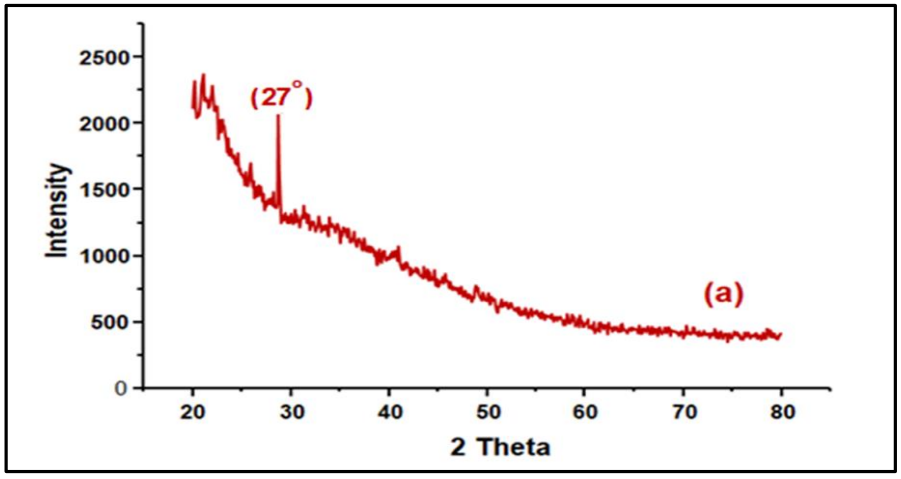
Vibrations	**Wavenumber (cm^{-1})**
Broad absorption of -OH	3234 – 3480
Asymmetric stretching of C=O	1710
Aromatic compound	1550
$-NH_2$	1456
C=O	1078
-C-C-	880

3.4.2 XRD Analysis

The XRD pattern of Kappa-Carrageenan (KC), native Methylene Blue (MB) and KC/MB$_{(0.08)}$ composite film are given in the (Figure 6 a, b and c) respectively. It is clear that Carrageenan powder is purely amorphous in nature, and possess scattered board Bumb. Around 2θ value of 27^o which is a characteristic of polysaccharides similar results are also reported elsewhere [19].

The XRD pattern of native MB powder shows crystalline in nature a sharp peak at 25^o corresponds to reflection by (200) plane, in addition a relatively smaller peak is around 35^o corresponds to reflection at (202) plane. However, other peaks were not show prominent similar results are reported by some other workers [20].

The KC/MB$_{(0.08)}$ composite film shows a prominence of amorphous nature this may probably be due to fact that as MB is present in very small quantity in the KC based film, the amorphous nature of later dominates.



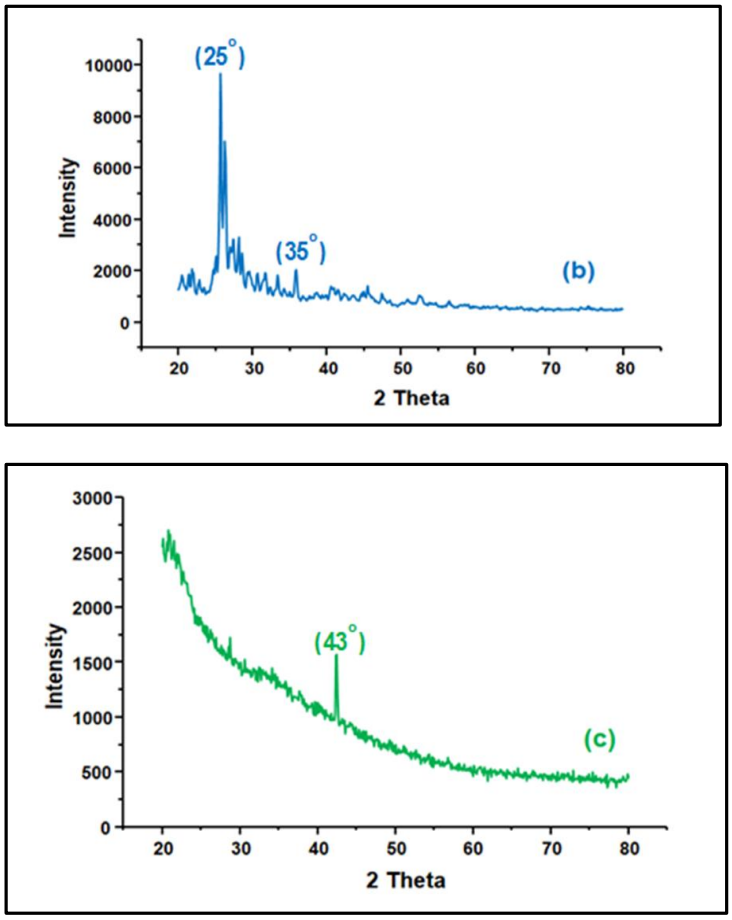


Figure 6. X-Ray Diffractogram of (a) Plain Kappa-Carrageenan film ($KC/MB_{(0)}$), (b) Pure Methylene Blue powder and (c) Methylene Blue loaded composite ($KC/MB_{(0.08)}$) hydrogel film (CIF, Savitribai Phule Pune University (SPPU).

3.4.3 SEM Analysis of Film Samples

The surface texture of plain Carrageenan film and MB loaded composite film are shown in Figure 7 and Figure 8 respectively. The SEM images of plain KC film were recorded at 30,000, 10,000, 6,000 and 3,000X magnification as shown in (Figure 7 a, b, c and d) respectively. It can be seen that texture of the film was almost smooth with same unevenness at some places, which could be due to agglomeration at molecular level during the drying process. However, the SEM images of MB loaded composite film with magnification of 30,000, 10,000, 6,000 and 3,000X (Figure 8 a, b, c and d) show much more smooth texture throughout the film, this could be due to fair solubility of MB which is well mixed with the Carrageenan chains during the pre-polymerization step and occupy the all-available space with in the Carrageenan network after gelation.

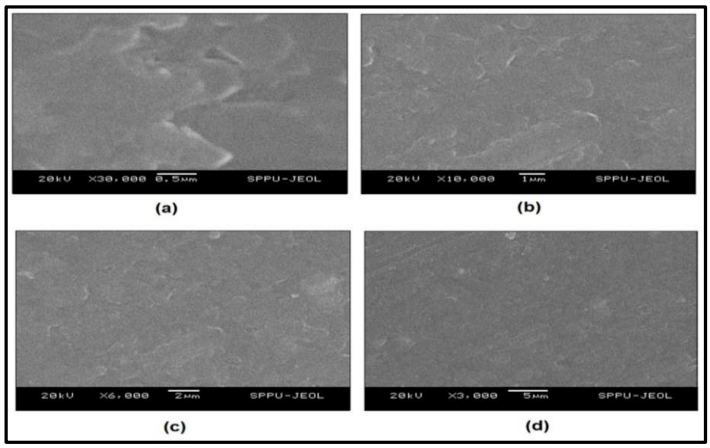


Figure 7. SEM images of plain Kappa-Carrageenan film ($KC/MB_{(0)}$) with different magnifications (CIF, Savitribai Phule Pune University (SPPU).

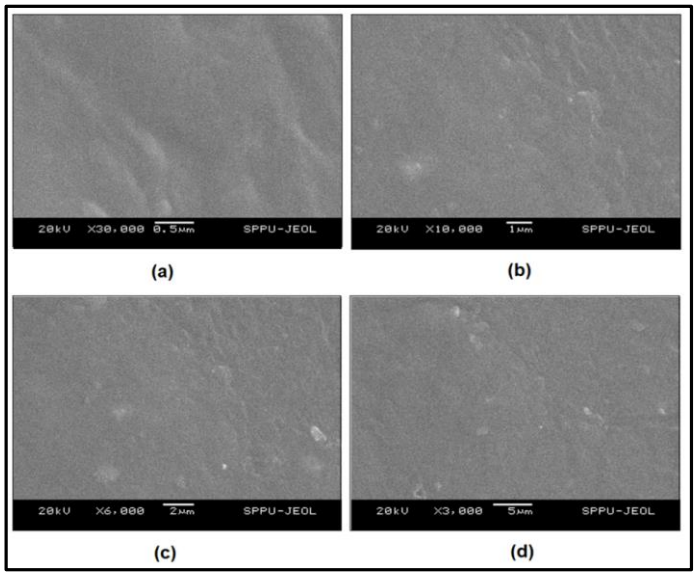


Figure 8. SEM images of MB loaded Kappa-Carrageenan ($KC/MB_{(0.08)}$) film with different magnifications (CIF, Savitribai Phule Pune University (SPPU).

3.5. λ_{max} Measurement of Methylene Blue

The spectrum of Methylene Blue solution with a concentration of 0.001% was recorded in the wavelength range 400 to 800nm. The result was as shown in (Figure 9). clearly indicate that a λ_{max} of 664 nm is obtained. All the measurments of concentrations of Methylene Blue solutions were made at 664 nm throughout the experiments.

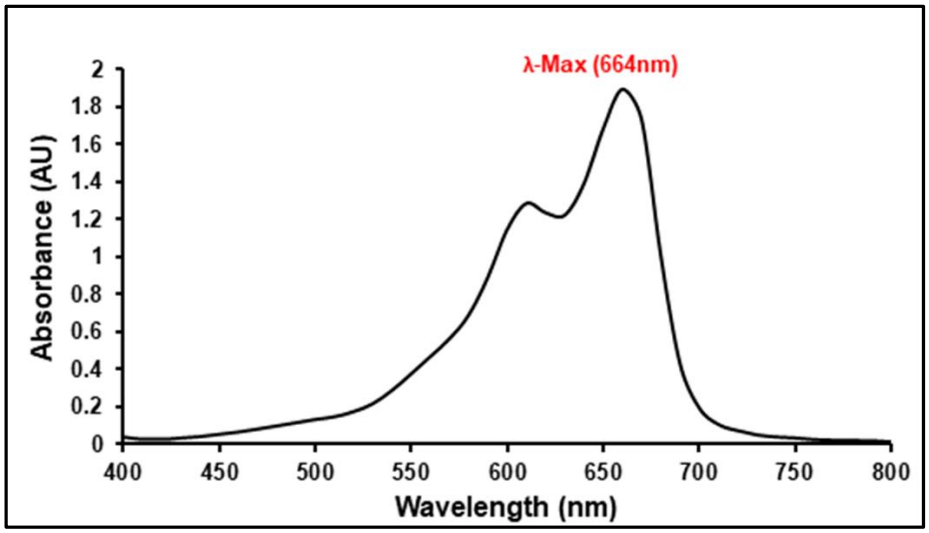


Figure 9. UV-Visible spectrum of Methylene Blue solution at room temperature.

3.6. Beer-Lambert Polt for Methylene Blue Solutions

The results of Beer-Lambert law experiment as linear plot is shown in (Figure 10). It can be seen that a straight line with fairly high regression value of 0.99 is obtained which indicates that the solution of Methylene Blue follows the Beer-Lambert law, to a great extent in the concentrations range studied. This plot was used to determine concentration of Methylene Blue in unknown solutions.

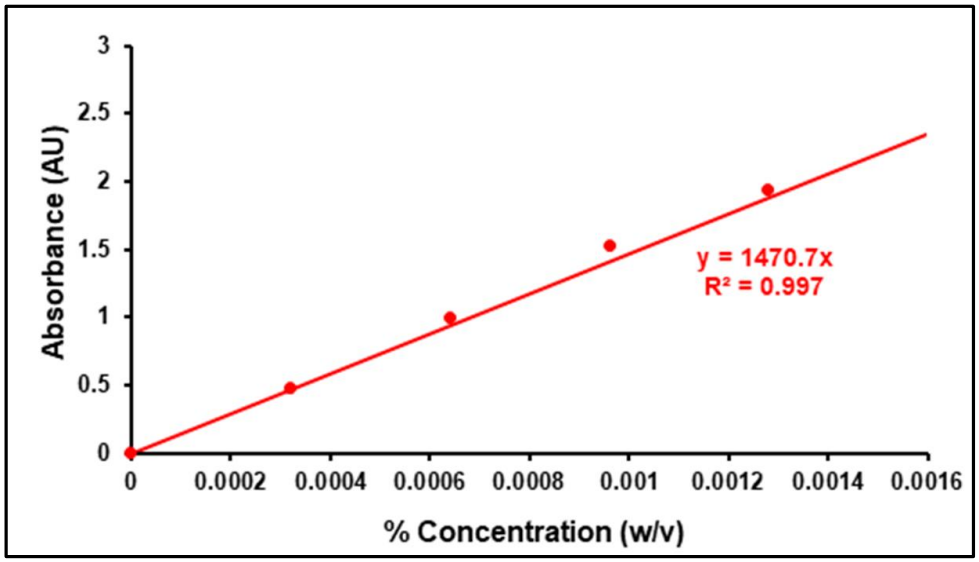


Figure 10. Beer-Lambert plot for Methylene Blue solutions at room temperature.

3.7. Effect of Cross-linking on Methylene Blue Release

The dynamic release of Methylene Blue from the hydrogel samples KC/MB$_{(0.08)}$ (0), KC/MB$_{(0.08)}$ (1), KC/MB$_{(0.08)}$ (3) and KC/MB$_{(0.08)}$ (5) is given in the (Table 3) and (Figure 11). It can be seen that at a definite time, amount of MB released varies with the degree of crosslinking. In other words, as the amount of KCl used to crosslinked the film increases, MB release decreases. Therefore, it can be concluded that quantity of MB released from the film can be controlled by using appropriate amount of crosslinker. Hence the KCl crosslinked KC/MB$_{(0.08)}$ composite film has a benefit that it can control the quantity of MB that is to be used for Photo Dynamic Therapy as shown in (Figure 12).

Table 3. The data showing amounts of Methylene Blue released (µmol/g wet gel) at different time intervals from hydrogel samples crosslinked with different KCl concentrations at 37ºC.

Time (min)	**Amounts of Methylene Blue released (µmol/g wet gel) from samples**			
	KC/MB$_{(0.08)}$ (0)	**KC/MB$_{(0.08)}$ (1)**	**KC/MB$_{(0.08)}$ (3)**	**KC/MB$_{(0.08)}$ (5)**
20	0.028	0.024	0.021	0.013
40	0.032	0.027	0.025	0.016
60	0.040	0.036	0.028	0.018
80	0.045	0.042	0.032	0.020
100	0.051	0.047	0.034	0.022
120	0.059	0.052	0.036	0.023
140	0.065	0.054	0.039	0.025
160	0.074	0.056	0.041	0.028
180	0.079	0.059	0.043	0.031

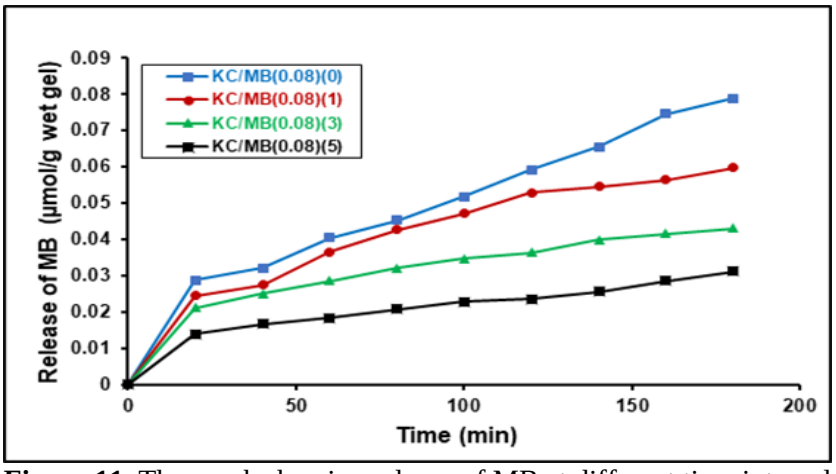


Figure 11. The graph showing release of MB at different time intervals at 37ºC.

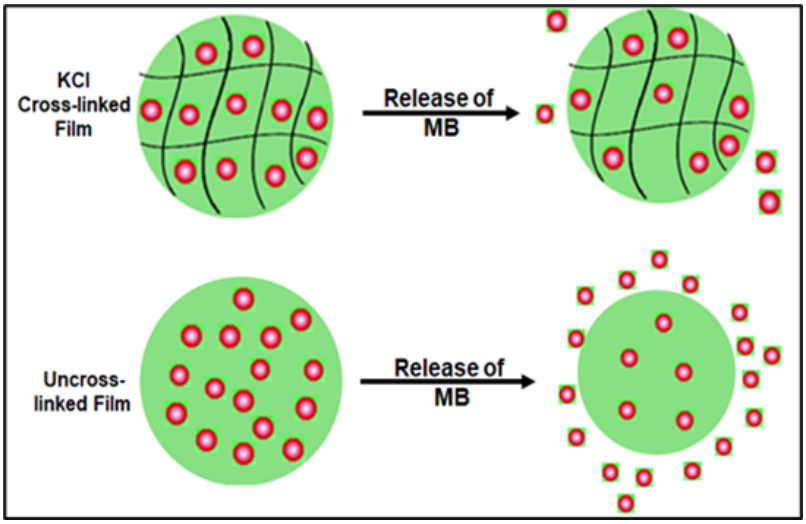


Figure 12. Schematic diagram of release of Methylene Blue from the KCl crosslinked and uncross-linked MB loaded film samples.

4. CONCLUSION

It can be concluded from the above study that Methylene Blue loaded polymeric film can be employed in Photo Dynamic Therapy of wounds and controls the release of Methylene Blue photosensitizer by variation in degree of ionic crosslinking with K^+ ions.

5. CONFLICT OF INTEREST

We declare that we do not have any conflict of interest.

6. REFERENCES

1. Misba, L., Zaidi, S., Khan A. U. 2018. Efficacy of photodynamic therapy against Streptococcus mutans biofilm: Role of singlet oxygen. J Photochem Photobiol B., 183: 16-21. doi:10.1016/j.jphotobiol.2018.04.024.
2. Percival, S. L., Emanuel, C., Cutting, K. F., Williams, D. W. 2012. Microbiology of the skin and the role of biofilms in infection. Int. Wound J., 9(1): 14-32. doi: 10.1111/j.1742-481X.2011.00836.x.
3. Dougherty, T. J., Gomer, C. J., Henderson, B. W., Jori, G., Kessel, D., Korbelik, M. et al.1998. Photodynamic therapy. J. Natl Cancer Inst., 90(12): 889-905. DOI: 10.1093/jnci/90.12.889.
4. Dolmans, D. E., Fukumura, D. Jain, R. K. 2003. Photodynamic therapy for cancer. Nat. Rev Cancer, 3(5): 380-387. DOI: 10.1038/nrc1071.
5. De Rosa, F. S., Bentley, M. V. 2000. Light-sensitive lipid-based nanoparticles for drug delivery. Pharm Res.,27(7), 364-381. doi.org/10.3109/09687688.2010.507788.
6. Moan, J., Berg, K. 1991. Photochem Photobiol., The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen. 53(4): 549-553. doi: 10.1039/c6tb03367a.
7. Dougherty, T. J., Grindey, G. B., Fiel, R., Weishaupt, K. R., Boyle, D. G. 1975. Photoradiation therapy II. Cure of animal tumour with hematoporphyrin and light. J Nat. Cancer Inst., 55(1): 115-121. doi: 10.1039/c6tb03367a
8. Unsgaard, B., and O'Toole, C. 1975. The influence of tumour burden and therapy on cellular cytotoxicity responses in patients with ocular and skin melanoma. Br J Cancer, 31(3): 301–316. https://doi.org/10.1038/bjc.1975.
9. Chin, L., Tam, A., Pomerantz, J., Wong, M., Holash, J., Bardeesy, N. et al. 1999. Essential role for oncogenic Ras in tumour maintenance. Nature, 400(6743): 468-472. doi: 10.1039/c6tb03367a.
10. Felsher, D. W., Bishop, J. M. 1999. Reversible tumorigenesis by MYC in hematopoietic lineages. Mol. Cell, 4(2): 199-207. doi: 10.1039/c6tb03367a.

11. Jain, M., Arvanitis, C., Chu, K., Dewey, W., Leonhardt, E., Trinh, M., et al. 2002. Sustained loss of a neoplastic phenotype by brief inactivation of MYC. Science, 297(5578): 102-104. doi: 10.1039/c6tb03367a.
12. Boccalini, G., Conti, L., Montis, C., Bani, D., Bencini, A., Berti, D., Giorgi, C., Mengoni, A. 2017. Methylene blue-containing liposomes as new photodynamic anti-bacterial agents. J. Mater Chem B., 5(15): 2788-2797. doi: 10.1039/c6tb03367a.
13. Rosenau T., and Potthast, A. 2015. Overview of Methods for the Direct Molar Mass Determination of Cellulose. Molecule, 20(6): 10313-10341. doi.org/10.3390/molecules200610313
14. Tian, Y., Kuzimenkova, M. V., Halle, J., Wojdyr, M., et al. 2015. Molecular Weight Determination by Counting Molecules. J. Phys. Chem. Lett., 6(6): 923-927. doi.org/10.1021/acs.jpclett.5b00296
15. Selvaraj, V., Mahboub, H, Ganapathi, U., Chandran, S. 2022. Enhanced photodegradation of methylene blue from aqueous solution using Al-doped ZnS nanoparticles. *Environ Sci* Pollut. Res Int., 29(48): 73528-73541.doi: 10.1016/j.heliyon.2023.e15605
16. Liew, J. W. Y., Loh, K. S., Ahmad, A., Lim, K. L., Wan Daud, W. R. 2017. Synthesis and characterization of modified κ-carrageenan for enhanced proton conductivity as polymer electrolyte membrane. PLoS ONE, 12(9): 0185313. doi.org/10.1371/journal.pone.0185313
17. Ainane, T., Khammour, F., Talbi, M. 2014. A Novel Bio-adsorbent of Mint Waste for Dyes Remediation in Aqueous Environments: Study and Modelling of Isotherms for Removal of Methylene Blue. Oriental journal of chemistry., 30(3): 1183-1189. doi:10.13005/ojc/300332
18. Sangeetha, P., Selvakumari, T.M., Selvasekarapandian, S., et al. 2020. Preparation and characterization of biopolymer K-carrageenan with MgCl2 and its application to electrochemical devices. Ionics, 26(5): 233-244. doi.org/10.1007/s11581-019-03193-0
19. Joshi, J. H., Kalainathan, S., Kanchan, D. K., Chaudhari, K., Joshi, M. J., Parikh, K. D. 2023. Crystal growth, structural, optical, photoluminescence, electrical and third-order nonlinear optical studies of pure and methylene blue dye-doped ammonium dihydrogen phosphate crystals. Journal of Materials Science: Materials in Electronics., 34(1), doi:10.1007/s10854-022-09447-4
20. Cesco, C., Valente, A.S., Paulino, A. 2021. Methylene Blue Release from Chitosan/Pectin and Chitosan/DNA Blend Hydrogels. Pharmaceutic., 13(6): 842. doi.org/10.3390/pharmaceutics13060842