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**RESEARCH ARTICLE**

# Preparation, characterization and median lethal dose (LD50) of carboxymethyl chitosan as target Drug Delivery

**H. M. Ibrahim[1], E.M.R. El- Zairy[2] and R. M. Mosaad[3]**

**1.** National Research Center, Textile Research Division, Dokki, Cairo, Egypt
**2.** Faculty of Applied Arts, Printing, Dyeing and Finishing Dept., Helwan Univ., Cairo, Egypt
**3**. College of Women, Ain Shams University, Cairo, Egypt

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***Corresponding Author***

**H. M. Ibrahim**

## *Abstract*

Carboxymethyl chitosan (CMCS) is promising biopolymer for the development of drug delivery systems. CMCS is amphiprotic ether, exhibiting enhanced aqueous solubility, excellent biocompatibility, biodegradability, and biological properties. (CMCS) was synthesized by the reaction of chitosan with monochloroacetic acid (MCAA) in the presence of sodium hydroxide NaOH). CMCS was characterized by FTIR, nitrogen, carboxylic content and degree of substitution (DS). The optimum condition for preparing CMCS has wide range of solubility are 2.5 M MCAA in the Presence of 50% NaOH within 3 hrs. at 60$^{o}$C. CMCS can load hydrophobic drugs and displays strong bioactivity which highlight its suitability and extensive usage for preparing different drug delivery formulations respectively. The median lethal dose (LD50) for CMCS was determined to achieve its ability to be used as poly load for different drugs. In vivo experiments shows that LD 50 for CMCS showed the safety of the use of this compound in drug delivery systems.



## INTRODUCTION

Chitosan is a copolymer of glucosamine and N-acetyl glucosamine units linked by 1, 4-D-glucosidic bonds (1) It has good biocompatibility, biodegradability, non toxic and various biofunctionalities including homeostatic, immunity enhancing, wound healing, antibacterial and antifungal activities with a broad spectra and high killing rate, so that it is widely used in medicine, nutrition, cosmetics, several pharmaceutical and biomedical application fields, textile finishes, artificial skin, ophthalmology, membranes, hollow fibers, drug delivery system, and cell-stimulating materials ….etc(1-4).On the other hand, poor solubility of chitosan in some common solvents, e.g., water, alkali, and organic solvents, limits its applications. To overcome this problem, chemical modification of chitosan is required and lots of derivatives have been synthesized. Among these,carboxymethylchitosan (CMCS), which is a kind of derivative introducing a -CH2COOH group at C-6 position, shows stronger antibacterial activities and better solubility in water than chitosan (6).

Carboxymethyl chitosan is a chitosan derivative of the most intensively investigated due to its water solubility in wider pH range compared with the parent compound, thus extended its use in various applications (5).

CMCS is not only soluble in water, but has unique chemical, physical and biological properties such as high viscosity, large hydrodynamic volume, low toxicity, biocompatibility and film, gel-forming capabilities, all of which make it an attractive option in connection with its use in food products and cosmetics.(7). Carboxymethyl chitosan (CMCS) is an amphiprotic ether derivative, which contains active hydroxyl (–OH), carboxyl (–COOH) and amine (–NH2) groups in the molecule. CMCS is soluble in water at neutral pH (7). It possesses high viscosity, large

hydrodynamic volume, film- and gel-forming capabilities together with the other useful properties, such as biocompatibility, biodegradation, biological activity and low toxicity (5).

In the present study, CMCS have been prepared and characterized. median lethal dose (LD50) of the prepared CMCS evaluated to examine the possibility of CMCS to be used as poly load an anticancer drugs to reduce its cytotoxicity.

## 2. Experimental Work:

**Materials and Methods:**

The polymers used are chitosan (CS), and Carboxymethylchitosan (CMCS).The chitosan was obtained from Aldrich Company with viscosity 1860 cps, deacetylation 78.0%. Carboxymethylchitosan were synthesized as discussed later. Sodium hydroxide (Modern Lab chemicals, Egypt), acrylonitrile (Merck-Schuchardt, Germany) and monochloroacetic (Fluka, Geremany) are used without further purification. Methyl alcohol, ethyl alcohol, acetic acid and isopropyl alcohol (Sisco Research Laboratories, India) and carbon disulfide (Fluka, Germany) and all other chemicals used are analytical grade.

**Preparation of Carboxymethylchitosan (CMCS):**

The carboxymethylation of chitosan was performed based on (8-9), with some modifications. Briefly, chitosan (5g), sodium hydroxide (10-70%), isopropanol (80 ml) and water (20 ml) were added into a three necked flask (250 ml) to swell at room temperature for one hour. The monochloroacetic acid (1-5 M) was dissolved in isopropanol (20 ml), and added into the reaction mixture drop wise for 30 minutes and left to react for proper time (0.5-12 hrs.) at temperature (30-90°C), then the reaction stopped by adding 80% ethyl alcohol. The solid was filtered and rinsed in 70-90% ethyl alcohol to desalt and dewater and dried at room temperature, followed by estimation of nitrogen and carboxylic contents.

**Analysis:**

Nitrogen content was determined using micro- Kehjeldal Procedure (10).Carboxyl Content of O-CMCS was determined according to a reported method (11)The FTIR spectra of the samples were recorded by using an FT-IR spectrophotometer (Nexus 670, Nicolet, USA) in the region of 4000-400cm-1 with spectra resolution of 4 cm-1.

**Evaluation of median lethal dose (LD50):**

**Experimental animals:**

88 adult mice were obtained from the animal house, veterinary division, NRC. Their weights were (20-30 g). The mice were housed in plastic cages, each cage contained eight mice. Animals were kept under controlled temperature of 25±2$^{o}$C and 12 hours light/12 hours dark cycle throughout the experiment. A commercial pelleted diet was used during the experiment. Food and water were available *ad libitum*.

**Treatment and dosage:**

An approximate LD50 was initially determined in pilot study by a so called ***"staircase method"*** using a small number of animals (2 each dose) and increasing doses of carboxymethyl chitosan. Five doses were then chosen for the determination of intraperitoneal LD50 in mice (Table l)and given to ten groups of mice (8 in each). Animals were observed for first 2 hours, 6$^{th}$ and 24$^{th}$ hour for any toxic symptoms. After 24 hours, the number of died animals was counted in each group and transformed to probits and then LD50 determined by the method of Karber(12).

## 3. Results and Discussion

**Carboxymethylation of Chitosan as water soluble chitosan derivative:**

A series of carboxymethylation reactions were performed in order to study the effect of four reaction parameters, namely, the concentration of the monochloroacetic acid (MCAA) (1-5 molar solution), sodium hydroxide (NaOH) (10-70% g/g), the reaction time (0.5–12 h) and reaction temperature (30-90 $^{o}$C) on the degree of substitution (DS). Different ranges of each parameter were chosen based on previous studies (13-16)and these tests listed in Figures (from 2 to 5). Other factors that affect the progress of carboxymethylation reaction, for example, the ratio of $H_2O$/2-isopropyl alcohol/chitosan was set around optimal value according to the previous studies, (14) which

was 2:10:1. The FTIR spectra of all the O-CMCSs prepared in this study were similar, and an example is shown in Figure 13. In IR spectrum, the wide band at 3420 cm-1 corresponds to the axial stretching of the O–H and N–H bonds (17) The peaks at 2927 cm-1 and 1639 cm-1 are attributed to the axial stretching of the C–H bonds and the symmetric stretching vibration of C=O in the –COOH groups, respectively (14, 18). The latter peak, together with the peak at 1420 cm-1, which arose from the asymmetric stretching vibration of the –COO– group, confirm the substitution of carboxymethyl groups onto the chitosan chain.(17, 18) Two bands at 1528 and 1513 cm-1 assigned to NH3+, indicate that the carboxymethylation occurred at OH positions (14, 19). The peaks at 1413 and 1377 cm-1 are related to the symmetric angular deformation of C–H bonds and C– N stretching vibrations (amide III band), respectively(17, 18). The peak at 1377 cm-1 did not increase significantly in the spectra of the O-CMCS, compared to the chitosan spectrum, which indicates that a significant amount of N-carboxymethylation did not take place. (18).The stretching vibration of C–O in the CH2COOH group gives rise to the peak at 1207 cm-1. Peaks located in the range of 1175-878 cm-1 are the result of vibrations of C–O and C–O–C and some other bonds that comprise the polysaccharide chain (17).

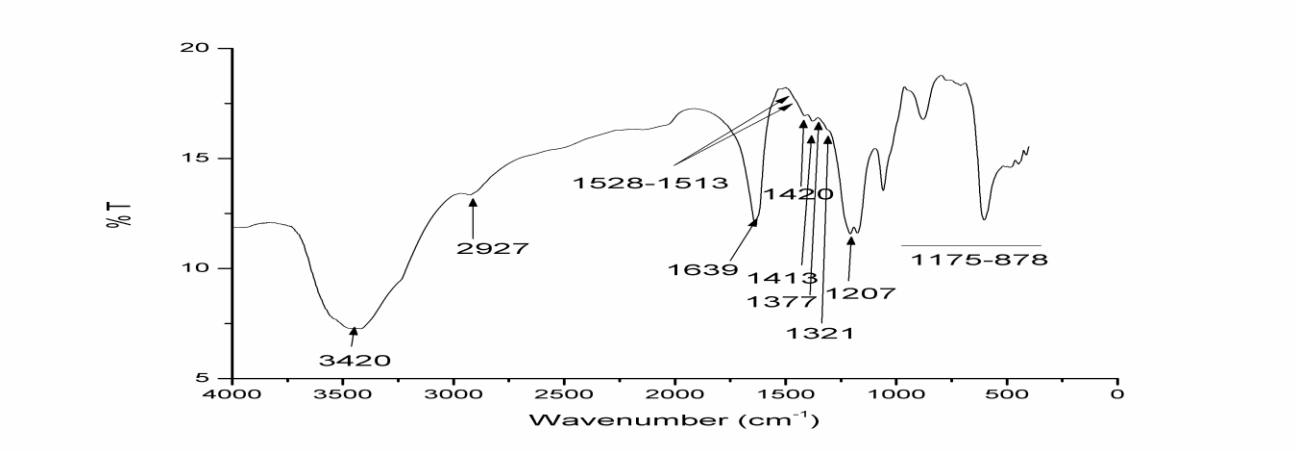


**Figure 1:** FTIR Spectrum of the optimized CMCS Prepared by Reaction of 5 gm Chitosan with 2.5 M MCAA in the Presence of 50% NaOH within 3 hrs. at 60 $^{o}$C

**Optimization of the Reaction Parameters:**

The carboxymethylation of chitosan proceeds by a two-step consecutive reaction and is accompanied by an undesired side reaction. In the main reaction the sodium hydroxide reacts first with the hydroxyl groups of chitosan to give alkali chitosan. The carboxymethyl groups are then formed in a SN2 reaction between the alkali chitosan and monochloroacetic acid (MCAA). The main reaction is given by:

$$\text{CS-OH} + \text{NaOH} \longrightarrow \text{CS-ONa} + \text{H}_2\text{O} \qquad (1)$$

$$\text{CS-ONa} + \text{ClCH}_2\text{COOH} \longrightarrow \text{CS-OCH}_2\text{COOH} + \text{NaCl} \qquad (2)$$

The side reaction takes place and results in the formation of sodium glycolate from MCAA and sodium hydroxide.

$$\text{NaOH} + \text{ClCH}_2\text{COOH} \longrightarrow \text{HOCH}_2\text{COONa} + \text{NaCl} \qquad (3)$$

The optimization of the process of carboxymethylation was performed by varying the process parameters such as concentration of NaOH and MCAA, temperature, and duration of the reaction. Each parameter was varied keeping the others constant.

## 1. Effect of Sodium Hydroxide Concentration

The effect of variation of sodium hydroxide concentration from 10% to 70% (w/v) at 60 $^{o}$C on DS was studied and the results are shown in Figure 2. It was observed that, as sodium hydroxide concentration increases up to 50%, the DS also increases. The increase in DS with increase in NaOH concentration up to 50% suggests that the carboxymethylation reaction shown by Eq. (2) prevails over its competitive reaction shown by Eq. (3). Above 50% concentration of NaOH, the glycolate formation increases (15, 20-23) and, consequently, a lower value of the DS of the O-CMCS sample was obtained. Therefore, the 50% concentration of NaOH constitutes the optimum concentration for carboxymethylation of chitosan

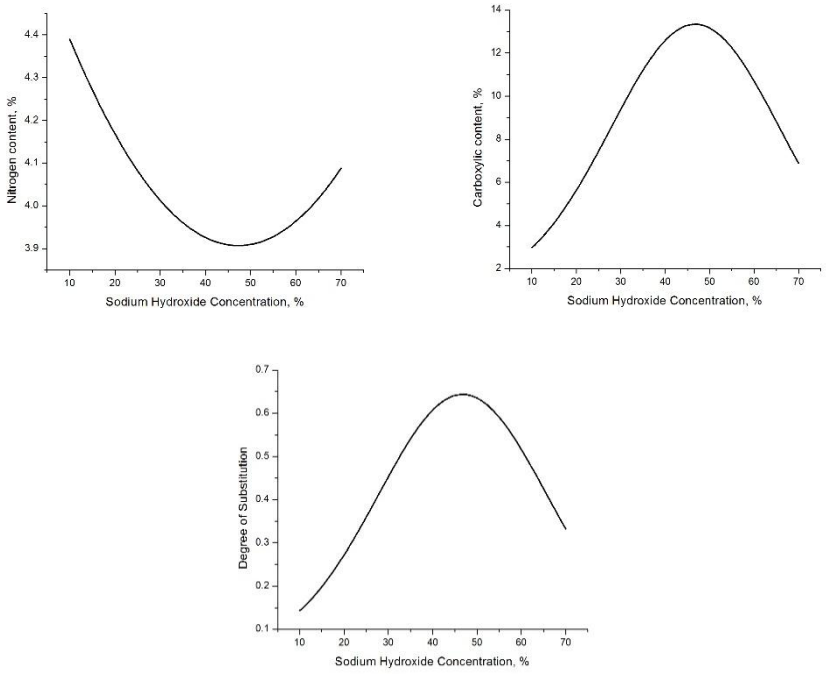


**Figure 2:** Effect of Sodium Hydroxide Concentration on Nitrogen Content percentage; Carboxyl Content percentage and Degree of Substitution (DS) of Carboxymethyl Groups

**Experimental conditions used: [chitosan], 5gm; [MCAA], 2.5 M; reaction temperature, 60$^{o}$C; reaction time: 3hrs; Material to Liquor Ratio (M: LR), 1:20.**

## 2. Effect of Monochloroacetic Acid Concentration

The effect of MCAA concentration on the extent of carboxymethylation reaction of chitosan was studied using the optimum concentration of NaOH (50%). Figure 3 show the effect of monochloroacetic acid concentration on DS of carboxyl groups in carboxymethylation of chitosan. It is clear that the DS of carboxyl groups increase significantly as the concentration of MCAA increases up to 2.5 M, and still increase with increasing the concentration of monochloroacetic acid to 5 M but this increase in substrate concentration is useless for the carboxymethylation reaction due to the formation of glycolate in Eq. (3) which seems to be more pronounced at high concentrations of MCAA and there are some undesirable gels formed in the reaction medium and due to the aggregation of highly acetylated chain segmented or to amide formation subsequent of thermal drying (14).Thus 2.5mol/L of MCAA is the optimum condition for carboxymethylation reaction of chitosan.

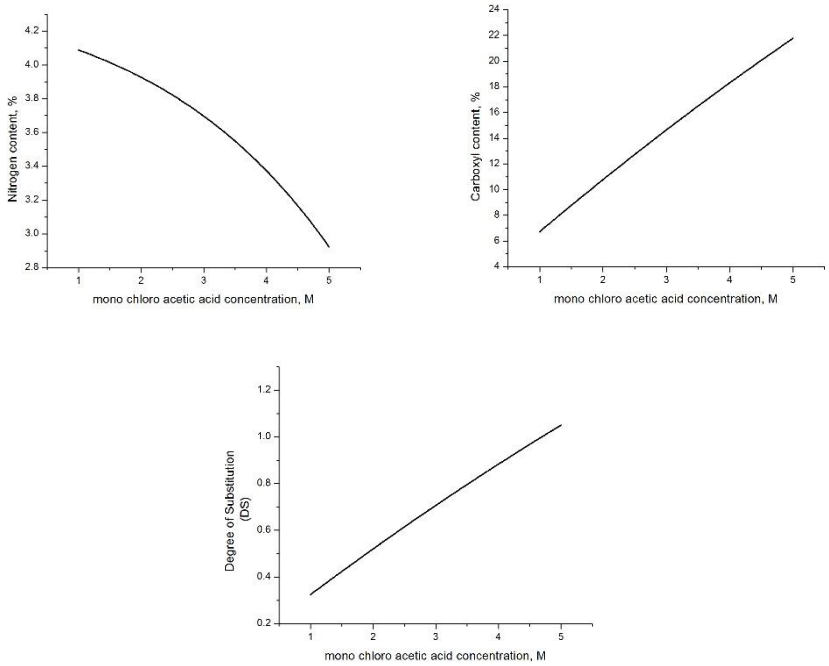


**Figure 3:** Effect of Monochloroacetic Acid Concentration on Nitrogen content, Carboxylic Content and Degree of Substitution (DS)

**Experimental conditions used: [chitosan], 5gm; [NaOH], 50%; reaction temperature, 60oC; reaction time: 3hrs; Material to Liquor Ratio (M: LR), 1:20.**

## 3. Effect of Reaction Time

Figure 4 illustrate the effect of carboxymethylation reaction time on DS of carboxyl groups in O-CMCS. It is clear that the extent of carboxymethylation of chitosan increases by prolonging the duration of reaction. This is rather direct consequence of the favourable effect of time on swellability of chitosan as well as diffusion and adsorption of reactants with the ultimate effect of including better contacts between etherifying agent and chitosan thereby promoting ether formation through improved carboxy-methylation process (20-23).But after 3 hours there are some polymer degradation of O-CMCS and the formation of glycolate which seems to be more pronounced at higher duration and there are some undesirable gels formed in the reaction medium. Thus, 3 hours reaction time is the optimum condition for carboxymethylation reaction of chitosan.

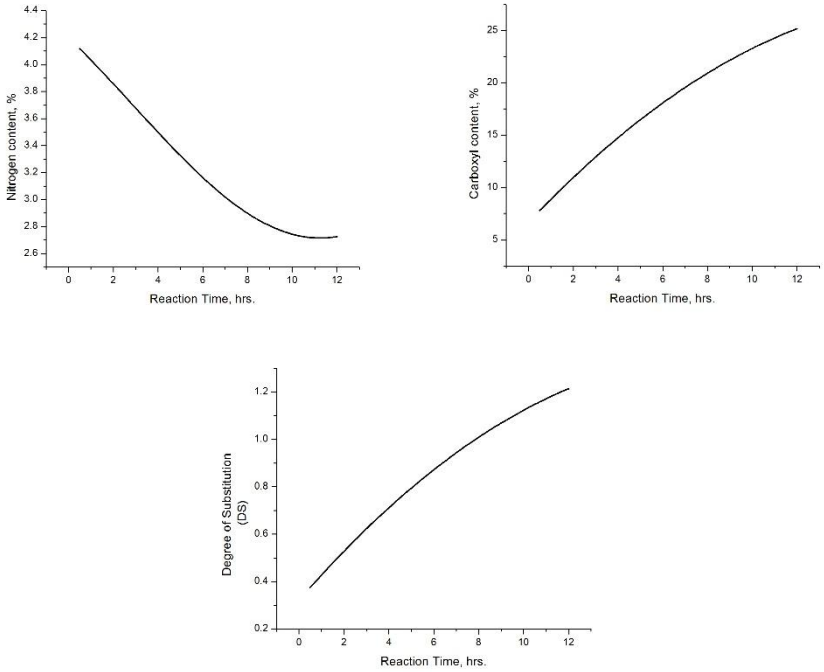


**Figure 4**: Effect of Reaction Time on Nitrogen content; Carboxylic Content and Degree of Substitution (DS)
**Experimental conditions used: [chitosan], 5gm; [NaOH], 50%; [MCAA], 2.5 M; reaction temperature, 60°C; Material to Liquor Ratio (M: LR), 1:20**

### 4. Effect of Reaction Temperature

Carboxymethylation of chitosan was performed at different temperatures (30 – 90 °C). The dependence of DS on reaction temperature is shown in Figure 5. It is observed that DS increases from 0.05 to 0.63 prominently as the reaction temperature increases from 30 to 60 °C and decreases thereafter. It is due to the favorable effect of temperature on the swellability of chitosan as well as the diffusion and adsorption of reactants with the ultimate effect of inducing better contacts between the etherifying agents and chitosan. The value of DS decreases at 90 °C, due to the higher glycolate formation Eq. (3).(19-22) prevailing over the carboxymethylation Eq. (2). Thus 60 °C reaction temperature is the optimum condition for carboxymethylation reaction of chitosan.

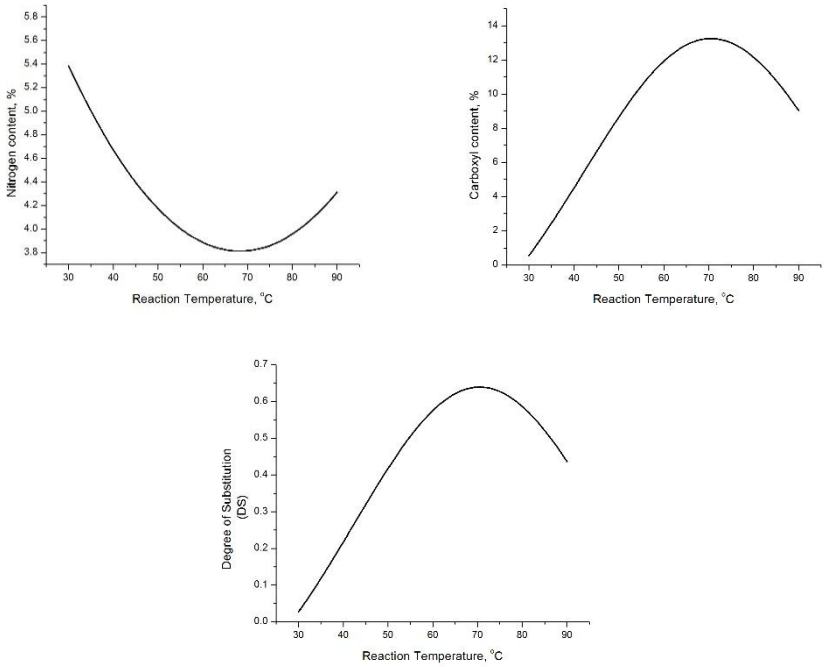


**Figure 5:** Effect of Reaction Temperature on Nitrogen content; Carboxylic content and Degree of Substitution (DS) **Experimental conditions used: [chitosan], 5gm; [NaOH], 50%; [MCAA], 2.5 M; reaction time: 3hrs; Material to Liquor Ratio (M: LR), 1:20.**

**Determination of median lethal dose (LD50)**

**Table 1**: Results of the lethal doses of carboxymethylchitosan for the determination of the LD50 after intraperitoneal injection in mice (n=8)

| group | Dose mg/kg | No. of animal dead | dose difference (a) | mean mortality (b) | probit (a*b) |
|---|---|---|---|---|---|
| 1 | control | | | | |
| 2 | 2 | 0 | 0 | | |
| 3 | 3 | 0 | 1 | 0.5 | 0.5 |
| 4 | 4 | 4 | 1 | 1 | 1 |
| 5 | 5 | 1 | 1 | 1 | 1 |
| 6 | 6 | 8 | 1 | 1 | 1 |

LD50= 4-0.4375= 3.5725g/kg b.wt.

The LD50 of carboxymethylchitosanin adult female mice was 3.5725g/kg b.wt. afterintraperitoneal injection (I.P) (Table 1). The animals receiving I.P.carboxymethylchitosangradually become less responsive and more drowsy before death or recovered.

Chitosan has commercial benefits because of their high nitrogen content and its excellent properties such as biocompatibility, biodegradability, non-toxicity and adsorptive abilities (24, 25). In the present study, LD50 of carboxymethylchitosanwas 3.5725g/kg b.wt. . This result showed the safety of the use of this compound in experimental animals.

***Kumirska et al.*** (26) indicated that, LD50 of chitosan in laboratory mice was 16g/kg b.wt., which is similar to that of sugar and salt. Also, ***Nagpl et al.*** (27), ***Richardson et al.*** (28)found that, LD50 of chitosan was 16g/kg in mice.

## 4. Conclusion:

- Carboxymethyl chitosan (CMCS) is a promising biopolymer for the development of new drug delivery systems.
- CMCS is amphiprotic ether, derived from chitosan, exhibiting enhanced aqueous solubility, excellent biocompatibility, biodegradability, and biological properties.
- Carboxymethyl chitosan (CMCS) synthesized by reaction of chitosan using monochloroacetic acid (MCAA) in the presence of sodium hydroxide NaOH).
- It was characterized by FTIR, nitrogen, carboxylic content and degree of substitution (DS).
- The optimum condition for preparing CMCS has wide range of solubility are 2.5 M MCAA in the Presence of 50% NaOH within 3 hrs. at 60$^{o}$C. CMCS can load hydrophobic drugs and displays strong bioactivity which highlight its suitability and extensive usage for preparing different drug delivery formulations respectively.
- The median lethal dose (LD50) for CMCS was determined to achieve its ability to be used as poly load for different drugs.LD 50 for CMCS showed the safety of the use of this compound in experimental animals (In vivo).

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