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RESEARCH ARTICLE

Genotoxicity Study of Carboxymethyl Chitosan-based Hydrogel for Clinical Use

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ABSTRACT

A carboxymethyl chitosan-based hydrogel used for hemostatic and anti-adhesive on wounds was assessed by a genotoxicity test.

The hydrogel was assessed for its potential to induce bacterial reverse mutation in the histidine auxotroph strains of Salmonella typhimurium and in the tryptophan auxotroph strain of Escherichia coli WP2uvrA. The study was performed with five bacterial test strains at different concentrations of hydrogel, with a negative control, and with five different types of positive controls, both in the presence and absence of a metabolic activation system. In all test strains used, there was no increase in the number of revertant colonies compared to the negative control at any concentration of test item either in the presence of or in the absence of a metabolic activation system. To evaluate additional genotoxicity of carboxymethyl chitosan hydrogel, the chromosome aberration test was performed using a cultured Chinese hamster lung cell line in the absence and presence of a metabolic activation system. The results of the bacterial reverse mutation assay indicated no mutagenic response at the concentration range tested, under the conditions of this study. The chromosome aberration test showed no significant increase of chromosome aberration in cells in metaphase arrest compared to the groups treated with vehicle control. Therefore, the carboxymethyl chitosan-based hydrogel is considered not to induce genotoxicity under the test system utilized.

Introduction

Biocompatible hydrogels are a promising material for use as wound dressing and for hemostasis in the clinic[1-3]. Current development of such hydrogels is focused on adding antibacterial, antimicrobial, antioxidant, and wound debridement functions [4-7]. The advancement of hydrogels will result in more effective treatment of patients with acute traumatic wounds as well as chronic ulcers[8-10]. Before use of new chemically modified hydrogels in the clinic, extensive biological and chemical characterization is required[11-13].

Various types of chitosan hydrogel have been used for wound management because of its desirable binding properties with growth factors, mechanical properties, and compatibility for combination with other gel types such as gelatin, pectin, and polyvinyl alcohol[14-16]. Chitosan is insoluble in water or alcohol but soluble in acid, with strong hydrogen bonding properties making the chemical structure stable[17,18]. Chitosan-based hydrogels are bioabsorbable, biocompatible, possess low toxicity, and can be made bioactive by binding with growth factors[19,20].

A chemically modified chitosan (carboxymethyl chitosan) has been recently used for wound management and hemostasis[21-23]. Carboxymethyl chitosan is biocompatible, bioabsorbable, non-toxic, highly viscous, easy to synthesize and applicable for additional functions[24,25]. Moreover, carboxymethyl chitosan is soluble in water at neutral pH[26]. The similarity of its chemical structure to hyaluronic acid, makes carboxymethyl chitosan useful for visco-supplementation, anti-bacterial therapy, hemostasis, and wound closure[27,28].

Carboxymethyl chitosan, a chemically modified chitosan-based hydrogel, may therefore be a promising material for wound management and hemostasis. The most recent applications of carboxymethyl chitosan derivatives showed effectiveness of antimicrobial, anticancer, antitumor, antioxidant and antifungal biological activities in various areas[22]. Even the clinical trial showed good clinical performance on hemostasis and anti-adhesion after endoscopic surgery[29]. This chemically modified chitosan is not only effective on external wound management but also promising for hemostasis and anti-adhesion during endoscopic and open surgeries.

We recently assessed carboxymethyl chitosan hydrogel for genotoxicity as an initial test of biocompatibility. Genotoxicity refers to the ability of a novel chemical compound to induce alterations at the genetic level. The in vitro and in vivo tests can be designed to evaluate compounds which induce genetic damage directly or indirectly by various mechanisms. These tests should enable a hazard identification with respect to damage to DNA and its fixation. This study therefore addresses the genotoxicity of the chemically modified hydrogel, carboxymethyl chitosan-based hydrogel for potential clinical use in wound management and hemostasis.

Methods

Preparation of carboxymethyl chitosan hydrogel

Chitin (10% w/v) was mixed with 1N HCl for 1 week. After 1 week, the chitin was rinsed with water adjusted to pH 7 and dried at room temperature. The dried chitin was deacetylated by hydrolysis of the acetamide groups with NaOH (40% w/v) at 100°C for 3 hours to form chitosan. To produce carboxymethyl chitosan, chitosan was mixed with NaOH (60% w/v, 25mL), ClCH2COOH (14.5g) and CH3CH(OH)CH3 (100mL) at 50°C for 7 hours (Hemostop/Surgishield, D-med, Seoul, Korea).

Test strains and media for bacterial reverse mutation test

All test strains and media were purchased from Molecular Toxicology Inc. (MOLTOX, Boone, NC).

Base pair substitution types of Salmonella typhimurium such as TA100, TA1535, and Escherichia coli (E. Coli) WP2uvrA, and the frameshift type of Salmonella typhimurium such as TA98 and TA1537 were used as test strains as they are known to be very sensitive to mutagens and widely used in mutagenicity studies with bacteria[30].

Each 15mL of 2.5% Oxoid nutrient broth No.2 was inoculated with test strain from the master plate and incubated in a 37°C shaking incubator at 180 rpm for 10 hours. The minimal glucose agar plates were prepared with 1.5% Difco® Bacto® agar, Vogel-Bonner medium E, and 2% glucose; 25ml dispensed per 100x15 mm petri dish. Top agar was prepared with 0.6% agar and 0.5% NaCl. This agar mix was appropriately supplemented with either 0.5mM of histidine-biotin for the Salmonella strains or 0.5mM tryptophan for E. coli strain. 0.07mL of Dimethyl sulfoxide (DMSO) was added to each 0.8mL of strain after overnight sub-culture and the mixture was stored in a deep freezer until use. Master plates of each strain were made and kept in a refrigerator to be used for each assay[31].

A metabolic activation system I

Rat liver post mitochondrial fraction (S9, Protein contents: 40.4mg/mL) with cofactor-I (S9

mix, Content of 1mL including 0.1mL S9, 8mmol MgCl, 33umol KCl, 5mmol Glucose-6-phosphate, 4mmol NADPH, 4mmol NAHD, and 100mmol pH 7.4 Sodium phosphate) was used for a metabolic activation system (UMU genotoxicity test kit, MOLTOX). S9 fraction was prepared from male rats induced with Aroclor-1254, the xenobiotic metabolizing enzyme.

Treatment of hydrogel extracts I

Carboxymethyl chitosan gel was incubated in saline (4g/mL) at 37° for 72 hours for testing extracts. Extracts of 0.1mLwere tested with 0.5mL of S9 mix (or sodium phosphate buffer, pH 7.4), and 0.1mL of bacterial culture per test tube. The mixture was shaken for 20 minutes using a shaking incubator at 180 rpm, and then dispensed into top agar before being poured onto a minimal glucose agar plate (one tube per plate). Vehicle control groups were treated with vehicle only and each of the appropriate strain-specific positive control groups as well. The testing extracts (0.1mL) and S9 mix (0.1mL), without bacterial culture, were also plated to check sterility. After the top agar was solidified, the plates were inverted, and incubated at 37°C for about 48 hours. Then the revertant colonies were counted. All testing was performed in triplicates per concentration. The results were expressed as the mean numbers of colonies ± standard deviation from triplicate plates per dose. A positive interpretation was a reproducible increase in the number of colonies in a dose-dependent manner, in least in one test strain with or without the metabolic system, clearly exceeding 2 times the colonies in each of control[32].

Test cell line and media for chromosome aberration test

Chinese hamster lung cell line (CHL cells, ATCC, Manassas, VA) was used for the study. The culture media was Minimum Essential Medium (MEM, Gibco, Waltham, MA)) supplemented with 10% fetal bovine serum (FBS, Gibco, Waltham, MA). The CHL cell was subsequently subcloned every 3-4 days[33].

A metabolic activation system and treatment of hydrogel extracts II

S9 mix was made of 0.3mL S9 (Protein contents: 31.9mg/mL), 5mmol MgCl, 33mmol KCl, 5mmol Glucose-6-phosphate, 4mmol HEPES, and 4mmol NADH. The testing extracts were used directly at 0.5mL. $2x10^4$ cells were plated on T-25 flasks and after 4 days, testing extracts (the same as the "hydrogel extracts I") were added in the 4.5mL culture media and incubated for 6 and 24 hours each. The cells were incubated for 6 hours, washed with 9mL PBS and cultured in the fresh culture media for 18 hours. All the cells were cultured for total of 24 hours. Saline was used as a negative control, Vehicle I, Mitomycin C (MMC, Sigma-Aldrich, St. Louis, MO), and Cyclophosphamide monohydrate (CPA, Sigma-Aldrich, St. Louis, MO) were used for each positive control I (S9-) and positive control II (S9+). All positive controls were dissolved in sterilized water and immediately used after preparation for testing. After 24-hour incubation, cells were treated with 100mL Colcemid (Thermo Fisher Scientific, Waltham, MA). After 2 hours, cells were collected and fixed onto a clean glass slide. Each slide was stained with 5% Giemsa solution for the chromosome aberrations. Two slides were prepared from each plate.

The analysis of 100 cells in mitotic and metaphase per plate was classified as follows. The frequencies of aberrations represented the frequencies of cells bearing aberrations. Evaluations were performed blindly using coded slides.

Criteria for observation[31]

- Gap: The unstained region (assumed achromatic region) is located on the vertical axis of the chromatid, the width of which is about the width of the chromatid. The shape of that region should be clearly observed.
- Break: The broken part of the chromosome is not aligned with the vertical axis of the chromatid. If the part is located on the same axis, the achromatic region is two times as wide or wider than the width of the chromatid. An acentric chromosome is recorded only when it is clearly observed.
- Exchange: This is the conjugated form of two or more detached segments on one or more chromosomes, resulting from chromosome breakage. With respect to chromosome type, clearly recognizable examples such as a ring or dicentric chromosome if observed are recorded.
- Other fragmentation: There are many instances of gaps and chromosomal breaks excluding the exchange form.
- Numerical aberration: Aneuploidy is not considered and only polyploidy is documented since the CHL cell line tends to generate chromosome aberrations to some extent. The modal chromosome number of the CHL cell line is 25. Although the modal number of quadploid is 50, polyploidy is

regarded in case the triploid has greater than 37 rods. In addition, as endoreduplication is classified as polyploid, it is important to record in case of a frequent outbreak.

Evaluation of the test results

For the evaluation of the frequencies of structural aberrations and of polyploidy, the following criteria, usually used for chromosomal aberration testing with CHL, were employed.

- Negative (-): less than 5%
- Equivocal (±): from 5% to less than 10%
- Positive (+): 10% or more

In case there is no dose-dependent increase, or the frequency of chromosome aberrations is more than 3% in a negative control group, a re-test is performed.

Results

From bacterial reverse mutation test, growth of bacteria was not observed during the sterility test of the solutions for testing extracts and S9 mix. The test was performed on saline and DMSO extracts, negative and positive control both in the presence (S9+) and absence (S9-) of S9 mix.

In all test strains used, there was no increase in the number of revertant colonies compared to the negative control at any concentration of testing extracts either in the presence or in the absence of a metabolic activation system. The positive controls induced a marked increase in the numbers of revertant colonies (Table 1).

Table 1. Result of bacterial reverse mutation test

Test Strain	Test Sample	Dose	Colonies/plate (mean±SD) [Factor]*	
			Without S9 mix	With S9 mix
TA98	Testing extracts	Saline Sample	26±4 26±6 [1.0]	26±7 26±4 [1.0]
TA100	Testing extracts	Saline Sample	147±9 153±5 [1.0]	138±14 143±7 [1.0]
TA1535	Testing extracts	Saline Sample	19±2 16±2 [0.8]	10±4 10±2 [1.0]
TA1537	Testing extracts	Saline Sample	12±4 13±4 [1.1]	17±3 19±4 [1.1]
WP2uvrA	Testing extracts	Saline Sample	37±5 42±5 [1.1]	35±7 38±6 [1.1]
Positive controls				
TA98 TA100 TA1535 TA1537 WP2uvrA TA98 TA100 TA1535 TA1537 WP2uvrA	AF-2 AF-2 NaN3 9-AA AF-2 2-AA 2-AA 2-AA 2-AA 2-AA	0.1 0.01 0.5 80.0 0.01 0.5 1.0 2.0 2.0 10.0	331±39 [12.7] 577±32 [3.9] 317±24 [16.7] 721±20 [60.1] 274±30 [7.4]	 250±15 [9.6] 614±11 [4.4] 179±17 [17.9] 260±15 [15.3] 226±20 [6.5]

*Factor: No. colonies of treated plate / No. of colonies of negative control plate

From chromosome aberration tests, without a metabolic activation system (S9-), in the 6-hour treatment, chromosome aberration frequencies were 0.5%, 1.0%, and 45.5% at the concentration levels of negative control, test extracts, and positive control, respectively (Table 2). In the 24-hour treatment, chromosome aberration frequencies were 0.5%, 0.5%, and 39.0% at the concentration levels of negative control, test extracts, and positive control, respectively. All the results for the test extracts were less than 5% which was evaluated as negative based on our test criteria (Table 3).

With a metabolic activation system (S9+), chromosome aberration frequencies were 0.0%, 0.5%, and 54.5% at the concentration levels of negative control, test extracts, and positive control, respectively. All the results for the test extracts were less than 5% which was evaluated as negative based on our test criteria (Table 4).

Table 2. Result of Chromosome aberration test – without metabolic activation (S9-, 6 hours)

Treatment	Numbers cells showing polyploid	Numbers of cells showing structural aberration							
		Gap	Chromatid type		Chromosome type		Others	Total	
			Chromatid break	Chromatid exchange	Chromosome break	Chromosome exchange		-Gap	+Gap
Negative control	0	0	1	0	0	0	0	1	1
	0	1	0	0	0	0	0	0	1
	0 (0.0)	1 (0.5)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	2 (1.0)
Test sample	0	0	1	0	0	0	0	1	1
	0	1	1	0	0	0	0	1	2
	0 (0.0)	1 (0.5)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	3 (1.5)
Positive control (MMC)	0	3	19	24	0	0	0	43	46
	1	2	21	27	0	0	0	48	50
	1 (0.5)	5 (2.5)	40 (20.0)	51 (25.5)	0 (0.0)	0 (0.0)	0 (0.0)	91 (45.5)	96 (48.0)

MMC, mitomycin C; ( ), Average

Table 3. Results of Chromosome aberration test – without metabolic activation (S9-, 24 hours)

Treatment	Numbers cells showing polyploid	Numbers of cells showing structural aberration							
		Gap	Chromatid type		Chromosome type		Others	Total	
			Chromatid break	Chromatid exchange	Chromosome break	Chromosome exchange		-Gap	+Gap
Negative control	0	1	0	0	0	0	0	0	1
	0	1	1	0	0	0	0	1	2
	0 (0.0)	2 (1.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	3 (1.5)
Test sample	0	1	1	0	0	0	0	1	2
	0	1	0	0	0	0	0	0	1
	0 (0.0)	2 (1.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	3 (1.5)
Positive control (MMC)	1	4	18	20	0	0	0	38	42
	0	3	16	24	0	0	0	40	43
	1 (0.5)	7 (3.5)	34 (17.0)	44 (22.0)	0 (0.0)	0 (0.0)	0 (0.0)	78 (39.0)	85 (42.5)

MMC, mitomycin C; ( ), Average

Table 4. Result of Chromosome aberration test – with metabolic activation (S9+, 6 hours)

Treatment	Numbers cells showing polyploid	Numbers of cells showing structural aberration							
		Gap	Chromatid type		Chromosome type		Others	Total	
			Chromatid break	Chromatid exchange	Chromosome break	Chromosome exchange		-Gap	+Gap
Negative control	0	0	0	0	0	0	0	0	0
	0	1	0	0	0	0	0	0	1
	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)
Test sample	0	1	1	0	0	0	0	1	2
	1	1	0	0	0	0	0	0	1
	1 (0.5)	2 (1.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	3 (1.5)
Positive control (MMC)	0	3	22	31	0	0	0	53	56
	0	2	21	35	0	0	0	56	58
	0 (0)	5 (2.5)	43 (21.5)	66 (33.0)	0 (0.0)	0 (0.0)	0 (0.0)	109(54.5)	114(57.0)

MMC, mitomycin C; ( ), Average

Discussion

Chitosan is bioabsorbable and biocompatible, and can be bound to growth factors[19,20]. Chitosan has bacteriostatic, antibacterial, hemostatic, wound healing, and scar reduction properties[21,27,34,35]. However complications have been reported because it can induce food allergy and anaphylactic shock [36]. On the other hand, it has been reported that chemically modified carboxymethyl chitosan has properties similar to chitosan but is relatively safe from the side effects of chitosan, making it potentially more suitable for wound management and hemostasis for clinical use[37,38]. The purpose of this study was to assess the genotoxicity of carboxymethyl chitosan-

based hydrogels through bacterial reverse mutation test and chromosomal aberration test.

Chitosan is an abundant polycationic biopolymer derived mainly from chitin of crustaceans by deacetylation of amino groups[39]. The strong hydrogen bonding makes the crystal structure very stable[40]. Chitosan has many useful biological properties leading to applications for wound healing and dressing, drug delivery, blood anticoagulant, antitumor agent, and artificial skin[41]. However, chitosan has some limitations in its use[23,36,39,40,42,43]. First, chitosan has low solubility in water at neutral pH and general organic solvents and is soluble only in acidic solutions with low pH. This acts as a limiting factor for the widespread use of chitosan. Second, certain crustacean sources of chitosan, such as shrimp and crab, are known allergens, which limits the use of chitosan products in patients with crustacean allergies.

Carboxymethylation makes chitosan hydrophilic and soluble at pH 6.0. Carboxymethyl chitosan is well known for its biocompatibility and relative non-toxicity, and for being bio-absorbable, highly viscous, and easily synthesized, making it applicable for the clinical use. Carboxymethyl chitosan is easy to sterilize because it has high temperature resistance[44]. Chitosan has been reported to have side-effects such as anaphylactic shock and food allergy. However, carboxymethyl chitosan is non-toxic and bio-absorbable, and has a molecular structure similar to that of hyaluronic acid making it suitable for joint viscosupplementation, anti-bacterial treatment, hemostasis, wound management, and scar healing[27,28]. Carboxymethyl chitosan has also been used for hydration and as artificial bone, artificial skin, and as a drug carrier[45-51].

In this study, carboxymethyl chitosan hydrogel was evaluated for its potential to induce reverse mutation in four histidine auxotroph strains of Salmonella typhimurium (TA98, TA100, TA1535, Ta1537) and one tryptophan auxotroph strain of Escherichia coli (WP2uvrA). The experiments were performed with the five test strains at concentration levels (each 4g/mL at 37°C for 72 hours) of saline and DMSO extracts, negative and positive control both in the presence (S9+) and absence (S9-) of S9 mix. We did not detect any bacterial contamination from the test item and S9 mix used in the test. We did not find any significant increase in the number of revertant colonies compared to its vehicle control at any dose in any of the strains. In addition, no antibacterial effects such as a decrease in the number of colonies were observed in any of the strains. On the other hand, the positive controls induced a marked increase in the number of revertant colonies, as expected. These evaluations indicate that carboxymethyl chitosan hydrogel does not result in reverse mutations.

To evaluate chromosome aberration of carboxymethyl chitosan hydrogel, the test was performed using Chinese hamster lung cell line cultured in the absence (S9- Mix, 6 and 24 hours) and presence (S9+ Mix, 6 hours) of a metabolic activation system. Under the conditions of our study the carboxymethyl chitosan hydrogel did not induce statistically significant increases in the frequencies of cells with aberration in either the presence (S9+) or the absence (S9-) of a metabolic activation system (S9 Mix). Chromosome aberration frequencies were less than 5%.

Several studies have investigated the safety of carboxymethyl chitosan since Tokura et al.[37] first reported on the low toxicity of carboxymethyl chitosan. Yang et al.[38] did not detect acute in vivo toxicity in the blood system of rats after carboxymethyl chitosan was absorbed in the abdominal cavity and gradually degraded in the blood. It has also been reported that carboxymethyl chitosan is a functional biomaterial without cytotoxicity[21]. Fu et al.[28] reported that carboxymethyl chitosan implanted in the abdominal cavity of rats did not induce any significant differences in coagulation function, anticoagulant performance, fibrinolytic function, and hemorheology of rats, which supports its safety for biomedical application. Although we did not test the hydrogel in vivo, our study is consistent with these previous studies and documents the absence of significant genotoxicity.

Conclusions

Based on the bacterial reverse mutation and chromosome aberration tests, carboxymethyl chitosan hydrogel showed no evidence of mutagenic potential at the concentration range tested in four test strains of Salmonella typhimurium, or in Escherichia coli strain WP2uvrA under the conditions of this study. Carboxymethyl chitosan hydrogel also did not have a clastogenic effect on CHL cells in vitro.

Conflicts of Interest Statement

The authors have no conflicts of interest to declare.

References

1. Lee KI, Koo TH, Chen P, D'Lima DD. Subcutaneous toxicity of a dual ionically cross-linked atelocollagen and sodium hyaluronate gel: Rat in vivo study for biological safety evaluation of the injectable hydrogel. *Toxicol Rep*. 2021;8:1651-1656. doi:10.1016/j.toxrep.2021.09.001

2. Ho TC, Chang CC, Chan HP, et al. Hydrogels: Properties and Applications in Biomedicine. *Molecules*. May 2 2022;27(9)doi:10.3390/molecules27092902

3. Zhang L, Liu M, Zhang Y, Pei R. Recent Progress of Highly Adhesive Hydrogels as Wound Dressings. *Biomacromolecules*. Oct 12 2020;21(10):3966-3983. doi:10.1021/acs.biomac.0c01069

4. Li D, Chen J, Wang X, Zhang M, Li C, Zhou J. Recent Advances on Synthetic and Polysaccharide Adhesives for Biological Hemostatic Applications. *Front Bioeng Biotechnol*. 2020;8:926. doi:10.3389/fbioe.2020.00926

5. Cao Z, Luo Y, Li Z, et al. Antibacterial Hybrid Hydrogels. *Macromol Biosci*. Jan 2021;21(1):e2000252. doi:10.1002/mabi.202000252

6. Pourshahrestani S, Zeimaran E, Kadri NA, Mutlu N, Boccaccini AR. Polymeric Hydrogel Systems as Emerging Biomaterial Platforms to Enable Hemostasis and Wound Healing. *Adv Healthc Mater*. Oct 2020;9(20):e2000905. doi:10.1002/adhm.202000905

7. Peers S, Montembault A, Ladavière C. Chitosan hydrogels for sustained drug delivery. *J Control Release*. Oct 10 2020;326:150-163. doi:10.1016/j.jconrel.2020.06.012

8. Gao D, Zhang Y, Bowers DT, Liu W, Ma M. Functional hydrogels for diabetic wound management. *APL Bioeng*. Sep 2021;5(3):031503. doi:10.1063/5.0046682

9. Ribeiro CT, Dias FA, Fregonezi GA. Hydrogel dressings for venous leg ulcers. *Cochrane Database Syst Rev*. Aug 5 2022;8(8):Cd010738. doi:10.1002/14651858.CD010738.pub2

10. Ji JY, Ren DY, Weng YZ. Efficiency of Multifunctional Antibacterial Hydrogels for Chronic Wound Healing in Diabetes: A Comprehensive Review. *Int J Nanomedicine*. 2022;17:3163-3176. doi:10.2147/ijn.S363827

11. Ebhodaghe SO. A short review on chitosan and gelatin-based hydrogel composite polymers for wound healing. *J Biomater Sci Polym Ed*. Aug 2022;33(12):1595-1622. doi:10.1080/09205063.2022.2068941

12. Kharaziha M, Baidya A, Annabi N. Rational Design of Immunomodulatory Hydrogels for Chronic Wound Healing. *Adv Mater*. Oct 2021;33(39):e2100176. doi:10.1002/adma.202100176

13. Paul P, Kolesinska B, Sujka W. Chitosan and Its Derivatives - Biomaterials with Diverse Biological Activity for Manifold Applications. *Mini Rev Med Chem*. 2019;19(9):737-750. doi:10.2174/1389557519666190112142735

14. Alven S, Aderibigbe BA. Chitosan and Cellulose-Based Hydrogels for Wound Management. *Int J Mol Sci*. Dec 18 2020;21(24)doi:10.3390/ijms21249656

15. Valentine R, Athanasiadis T, Moratti S, Hanton L, Robinson S, Wormald PJ. The efficacy of a novel chitosan gel on hemostasis and wound healing after endoscopic sinus surgery. *Am J Rhinol Allergy*. Jan-Feb 2010;24(1):70-5. doi:10.2500/ajra.2010.24.3422

16. Patrulea V, Ostafe V, Borchard G, Jordan O. Chitosan as a starting material for wound healing applications. *Eur J Pharm Biopharm*. Nov 2015;97(Pt B):417-26. doi:10.1016/j.ejpb.2015.08.004

17. Zhu Y, Zhang Y, Zhou Y. Application Progress of Modified Chitosan and Its Composite Biomaterials for Bone Tissue Engineering. *Int J Mol Sci*. Jun 12 2022;23(12)doi:10.3390/ijms23126574

18. Wang W, Meng Q, Li Q, et al. Chitosan Derivatives and Their Application in Biomedicine. *Int J Mol Sci*. Jan 12 2020;21(2)doi:10.3390/ijms21020487

19. Tian B, Hua S, Tian Y, Liu J. Chemical and physical chitosan hydrogels as prospective carriers for drug delivery: a review. *J Mater Chem B*. Nov 18 2020;8(44):10050-10064. doi:10.1039/d0tb01869d

20. Hao Y, Zhao W, Zhang H, Zheng W, Zhou Q. Carboxymethyl chitosan-based hydrogels containing fibroblast growth factors for triggering diabetic wound healing. *Carbohydr Polym*. Jul 1 2022;287:119336. doi:10.1016/j.carbpol.2022.119336

21. Chen XG, Wang Z, Liu WS, Park HJ. The effect of carboxymethyl-chitosan on proliferation and collagen secretion of normal and keloid skin fibroblasts. *Biomaterials*. Dec 2002;23(23):4609-14. doi:10.1016/s0142-9612(02)00207-7

22. Shariatinia Z. Carboxymethyl chitosan: Properties and biomedical applications. *Int J Biol Macromol*. Dec 2018;120(Pt B):1406-1419. doi:10.1016/j.ijbiomac.2018.09.131

23. Upadhyaya L, Singh J, Agarwal V, Tewari RP. Biomedical applications of carboxymethyl chitosans. *Carbohydr Polym*. Jan 2 2013;91(1):452-66. doi:10.1016/j.carbpol.2012.07.076

24. Lei M, Huang W, Jin Z, Sun J, Zhang M, Zhao S. Effect of molecular structure and ionization state on aggregation of carboxymethyl chitosan: A molecular dynamics study. *Carbohydr Polym*. Dec 1 2022;297:119993. doi:10.1016/j.carbpol.2022.119993

25. Khalil AM, Abdel-Monem RA, Darwesh OM, Hashim AI, Nada AA, Rabie ST. Synthesis, Characterization, and Evaluation of Antimicrobial Activities of Chitosan and Carboxymethyl Chitosan Schiff-Base/Silver Nanoparticles. *Journal of Chemistry*. 2017/03/15 2017;2017:1434320. doi:10.1155/2017/1434320

26. Fonseca-Santos B, Chorilli M. An overview of carboxymethyl derivatives of chitosan: Their use as biomaterials and drug delivery systems. *Mater Sci Eng C Mater Biol Appl*. Aug 1 2017;77:1349-1362. doi:10.1016/j.msec.2017.03.198

27. Wang D, Zhang N, Meng G, He J, Wu F. The effect of form of carboxymethyl-chitosan dressings on biological properties in wound healing. *Colloids Surf B Biointerfaces*. Oct 2020;194:111191. doi:10.1016/j.colsurfb.2020.111191

28. Fu D, Han B, Dong W, Yang Z, Lv Y, Liu W. Effects of carboxymethyl chitosan on the blood system of rats. *Biochem Biophys Res Commun*. Apr 29 2011;408(1):110-4. doi:10.1016/j.bbrc.2011.03.130

29. Chung YJ, An SY, Yeon JY, Shim WS, Mo JH. Effect of a Chitosan Gel on Hemostasis and Prevention of Adhesion After Endoscopic Sinus Surgery. *Clin Exp Otorhinolaryngol*. Jun 2016;9(2):143-9. doi:10.21053/ceo.2015.00591

30. Bringezu F, Simon S. Salmonella typhimurium TA100 and TA1535 and E. coli WP2 uvrA are highly sensitive to detect the mutagenicity of short Alkyl-N-Nitrosamines in the Bacterial Reverse Mutation Test. *Toxicol Rep*. 2022;9:250-255. doi:10.1016/j.toxrep.2022.02.005

31. Kumaravel TS, Sathya TN, Balaje R, et al. Genotoxicity evaluation of medical devices: A regulatory perspective. *Mutat Res Rev Mutat Res*. Jan-Jun 2022;789:108407. doi:10.1016/j.mrrev.2021.108407

32. Gatehouse D. Bacterial mutagenicity assays: test methods. *Methods Mol Biol*. 2012;817:21-34. doi:10.1007/978-1-61779-421-6_2

33. Abbondandolo A. Chromosome-aberration tests. *Food Addit Contam*. Apr-Jun 1984;1(2):165-72. doi:10.1080/02652038409385839

34. Chien HF, Chen CP, Chen YC, Chang PH, Tsai T, Chen CT. The use of Chitosan to enhance photodynamic inactivation against Candida albicans and its drug-resistant clinical isolates. *Int J Mol Sci*. Apr 3 2013;14(4):7445-56. doi:10.3390/ijms14047445

35. Pusateri AE, McCarthy SJ, Gregory KW, et al. Effect of a chitosan-based hemostatic dressing on blood loss and survival in a model of severe venous hemorrhage and hepatic injury in swine. *J Trauma*. Jan 2003;54(1):177-82. doi:10.1097/00005373-200301000-00023

36. Ylitalo R, Lehtinen S, Wuolijoki E, Ylitalo P, Lehtimäki T. Cholesterol-lowering properties and safety of chitosan. *Arzneimittelforschung*. 2002;52(1):1-7. doi:10.1055/s-0031-1299848

37. Tokura S, Nishimura S-I, Sakairi N, Nishi N. Biological activities of biodegradable polysaccharide. *Macromolecular Symposia*. 1996;101(1):389-396. doi:https://doi.org/10.1002/masy.19961010144

38. Yang Z, Han B, Fu D, Liu W. Acute toxicity of high dosage carboxymethyl chitosan and its effect on the blood parameters in rats. *J Mater Sci Mater Med*. Feb 2012;23(2):457-62. doi:10.1007/s10856-011-4467-4

39. Kalliola S, Repo E, Srivastava V, et al. Carboxymethyl Chitosan and Its Hydrophobically Modified Derivative as pH-Switchable Emulsifiers. *Langmuir*. Feb 27 2018;34(8):2800-2806. doi:10.1021/acs.langmuir.7b03959

40. Mourya VK, Inamdara N, Ashutosh Tiwari N. Carboxymethyl Chitosan And Its Applications. *Advanced Materials Letters*. 2010;1(1):11-33. doi:10.5185/amlett.2010.3108

41. Jayakumar R, Nagahama H, Furuike T, Tamura H. Synthesis of phosphorylated chitosan by novel method and its characterization. *Int J Biol Macromol*. May 1 2008;42(4):335-9. doi:10.1016/j.ijbiomac.2007.12.011

42. Desislava Tzaneva AS, Nadezhda Petkova, Ventzislav Nenov, Albena Stoyanova, Panteley Denev. *Synthesis of Carboxymethyl Chitosan and its Rheological Behaviour in Pharmaceutical and Cosmetic Emulsions*. vol Volume: 7. ssue: 10; 2017:070-078.

43. Lim JW, Lim JH, Kim CZ, Lee SU, Lee SJ. Intraocular Inflammation after Use of Anti-adhesion Agents. *J Retin*. 2022;7(1):16-20. doi:10.21561/jor.2022.7.1.16

44. Katugampola P, Winstead C, Adeleke A. Thermal stability of carboxymethyl chitosan varying the degree of substitution. 2014:

45. Souza M, Carvalho Gd, Stamford TCM, Tenório PP, Sampaio FC, Pessoa J. Chitosan as an oral antimicrobial agent. 2011:

46. Müller WE, Neufurth M, Wang S, Tolba E, Schröder HC, Wang X. Morphogenetically active scaffold for osteochondral repair (polyphosphate/alginate/N,O-carboxymethyl

chitosan). *Eur Cell Mater*. Feb 22 2016;31:174-90. doi:10.22203/ecm.v031a12

47. Zhang XY, Chen YP, Han J, et al. Biocompatiable silk fibroin/carboxymethyl chitosan/strontium substituted hydroxyapatite/ cellulose nanocrystal composite scaffolds for bone tissue engineering. *Int J Biol Macromol*. Sep 1 2019;136:1247-1257. doi:10.1016/j.ijbiomac.2019.06.172

48. Rao KM, Sudhakar K, Suneetha M, Won SY, Han SS. Fungal-derived carboxymethyl chitosan blended with polyvinyl alcohol as membranes for wound dressings. *Int J Biol Macromol*. Nov 1 2021;190:792-800. doi:10.1016/j.ijbiomac.2021.09.034

49. Shi Y, Xiong Z, Lu X, Yan X, Cai X, Xue W. Novel carboxymethyl chitosan-graphene oxide hybrid particles for drug delivery. *J Mater Sci Mater Med*. Nov 2016;27(11):169. doi:10.1007/s10856-016-5774-6

50. Yan Y, Wu Q, Ren P, et al. Zinc ions coordinated carboxymethyl chitosan-hyaluronic acid microgel for pulmonary drug delivery. *Int J Biol Macromol*. Dec 15 2021;193(Pt B):1043-1049. doi:10.1016/j.ijbiomac.2021.11.088

51. Liu X, He Z, Chen Y, et al. Dual drug delivery system of photothermal-sensitive carboxymethyl chitosan nanosphere for photothermal-chemotherapy. *Int J Biol Macromol*. Nov 15 2020;163:156-166. doi:10.1016/j.ijbiomac.2020.06.202