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Posted Date: 22 July 2024

doi: 10.20944/preprints202407.1733.v1

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Article

Oral Administration of Berberine Hydrochloride Based on Chitosan/Carboxymethyl- β -Cyclodextrin Hydrogel

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Abstract: In this study, a novel oral formulation of berberine hydrochloride (BBH) hydrogel was successfully synthesized through physical cross-linking using chitosan (CS) and carboxymethyl- β -cyclodextrin (CMCD). The characterization results confirmed the successful synthesis of CS/CMCD hydrogel and the subsequent loading of BBH into this composite (CS/CMCD/BBH) was effectively accomplished. BBH was used as a model drug and the resulting hydrogel demonstrated a sustained drug release profile. In addition to its improved solubility and sustained release characteristics, the hydrogel exhibited excellent antibacterial activity against common pathogens such as *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), and *Candida albicans* (*C. albicans*). Additionally, in vitro studies indicated that the hydrogel was not cytotoxic to NIH3T3 and HaCaT cells, suggesting its safety for biomedical applications. This lack of cytotoxic effects, combined with the mechanical strength, solubility improvements, and antibacterial properties of the hydrogel, positions the CS/CMCD/BBH hydrogel as a promising candidate for effective oral delivery of BBH. By addressing the solubility and delivery challenges of BBH, this hydrogel offers a viable solution for the oral administration of berberine hydrochloride, with potential applications in various biomedical fields.

Keywords: berberine hydrochloride; Chitosan/Carboxymethyl- β -Cyclodextrin; hydrogel formulation; Solubility enhancement; oral drug delivery

1. Introduction

Berberine hydrochloride is a natural alkaloid with a wide range of therapeutic applications, including antimicrobial [1–4], anti-inflammatory [5], anticancer [6–9], and antidiabetic effects [10]. Despite its potential, the clinical use of BBH has been severely limited by its low solubility and stability, particularly when administered orally [11–13]. These challenges require the development of innovative drug delivery systems to improve their bioavailability and therapeutic efficacy [14,15]. Hydrogels have emerged as a promising solution for drug delivery, capable of improving the solubility and stability of hydrophobic drugs [16,17]. Among the different hydrogel forming materials, chitosan and carboxymethyl- β -cyclodextrin deserve special attention ([18]). Chitosan is a biocompatible polymer with excellent film forming properties, while CMCD is known for its ability to form inclusion complexes with hydrophobic molecules, thereby improving their solubility [19]. Chitosan cross-linked with β -cyclodextrin forms a chitosan hydrogel, making it a good carrier for poorly water-soluble drugs [20]. β -Cyclodextrin is a cyclic oligosaccharide composed of seven glucose units [21,22]. It has a hydrophobic internal cavity and a hydrophilic external surface, allowing it to form inclusion complexes with hydrophobic drugs, such as BBH [23,24]. β -Cyclodextrin can improve the solubility, stability and bioavailability of poorly soluble drugs by encapsulating them in its cavity [25,26]. By combining the advantages of chitosan and β -cyclodextrin, a physically cross-linked CS/CMCD hydrogel can be developed as an effective carrier for BBH [27]. The physical cross-linking between CS and CMCD allows easy fabrication of hydrogels without the need for chemical

cross-linkers, thereby preserving the biocompatibility and bioactivity of the encapsulated drug [28]. The hydrogel matrix can provide sustained release of BBH, ensuring a prolonged therapeutic effect and minimizing the frequency of administration [29]. Several studies have explored the application of CS/ β -cyclodextrin hydrogels as drug carriers, demonstrating their potential to improve drug delivery [30,31]. For example, Hui et al. developed a BBH-loaded chitosan/ β -cyclodextrin hydrogel that showed improved solubility, sustained release, and enhanced antibacterial activity [31]. Another study by Lin et al. investigated a nanocellulose/ β -cyclodextrin hydrogel for ocular delivery of BBH, which exhibited sustained drug release and improved therapeutic efficacy [32]. Tsai et al. investigated chitosan/ β -cyclodextrin hydrogels for transdermal delivery of BBH and showed that the hydrogel formulation effectively improved therapeutic outcomes in a mouse model of psoriasis, indicating its potential as a delivery system. transdermal administration for BBH [30,33]. Therefore, physically cross-linked hydrogels are more favorable for biomedical applications, including drug delivery systems [34,35]. In this study, we developed an oral, physically cross-linked hydrogel formulation of BBH by loading it onto a CS/CMCD hydrogel matrix. Our results showed that the physical cross-linking interaction between CS and CMCD leads to the formation of stable complexes. This approach exploits the solubility-enhancing properties of CMCD as well as the biocompatibility and stability offered by chitosan. The resulting hydrogel formulation not only significantly increases the solubility of BBH, but also ensures its sustained release and improved bioavailability, thereby addressing the major limitations of free BBH in oral administration. Our results demonstrate the superior performance of this innovative formulation compared to free BBH.

2. Results

2.1. Wavelength Study and Solubility

The wavelength study confirmed the characteristic absorption peaks of BBH in the ultraviolet-visible (UV-Vis) spectrum. BBH is known to exhibit strong absorption at approximately 250, 350 and 430 nm in the UV-visible region of the electromagnetic spectrum [36]. The absorption spectrum of BBH typically shows two distinct peaks. The first peak occurs in the UV range (approximately 260-280 nm), while the second peak occurs in the visible range (approximately 350-400 nm). In the wavelength range of 200-400 nm, the absorption at 263 nm is stronger than that at 345 nm, so 263 nm is used as the detection wavelength. The scan result is shown in Figure A1 in the Appendix A. This result is consistent with the work of Li et al [8].

The solubilization effect of BBH was evaluated by the solubility phase method [36]. The phase solubility diagram is shown in Figure A2 in the Appendix A. The graph was created with concentration ($\mu\text{g/mL}$) on the x-axis and absorbance on the y-axis. The absorbance values obtained from the standard solutions at their corresponding concentrations are shown in Table A1 in the appendix A. Regression analysis was performed on the plotted data to determine the best-fit line that represents the relationship between concentration and absorbance. The most common regression method for this purpose is linear regression. The equation of the line was calculated as $y = mx + c$, where y is the absorbance, x is the concentration, m is the slope, and c is the y-intercept. Once the standard curve was established, we used it to determine the concentration of an unknown sample of BBH by measuring its absorbance and applying the equation of the best-fit line obtained from the standard curve.

2.2. Synthesis of CS/CMCD and Synthesis of CS/CMCD Loaded BBH Hydrogel

The synthesis of the CS/CMCD complex hydrogel involved the combination of CS and CMCD through physical cross-linking, followed by a freeze-drying process to form a stable network. The resulting hydrogel exhibits a three-dimensional network structure, with CMCD molecules trapped within the chitosan matrix (Figure A3 in Appendix A and Video S1). This structure gives the hydrogel properties such as controlled drug release, high water absorption capacity (as shown in Table 1). The formation of hydrogels from highly concentrated and less concentrated solutions indicates strong physical cross-linking interactions between CS and CMCD. The carboxymethyl groups of CMCD

interact with the amino groups of chitosan via hydrogen bonds and electrostatic interactions, leading to the formation of physical cross-links between the polymer chains. The concentrations of CS and CMCD in solution impact the gelation behavior and properties of the resulting hydrogel. Higher concentrations of both components generally result in the formation of stronger and stiffer hydrogels. The development and properties of the CS/CMCD hydrogel highlight its potential as an effective oral delivery system for BBH. The synthesis of CS/CMCD hydrogel involved the cross-linking of CS and CMCD. BBH was subsequently loaded into the hydrogel matrix during the synthesis process. The synthesis was confirmed by various characterization techniques, which provided detailed insights into the structure and composition of the hydrogel and its BBH loaded counterpart.

2.3. Characterization of CS/CMCD and CS/CMCD/BBH Hydrogels

2.3.1. Analysis of Water Content

Water content determination (WCD) analysis is a process used to measure the amount of water present in a substance or sample [37]. The method used here was loss on drying (LOD); this method involves weighing a sample before and after drying until a constant weight is reached. The weight loss corresponds to the water content of the sample. The weight of the product was recorded for each set of samples. Analysis of the water content of CS/CMCD hydrogels showed a slight increase in water retention in samples with high concentration of CMCD, as shown in Table 1. This increase is attributed to the hydrophilic nature of CMCD, which improves the water retention capacity of the hydrogel matrix. The higher water content is beneficial for the sustained release of BBH.

Table 1. Water Content determination of CS/CMCD hydrogels.

Sample (g)	First weighing (g)	Second weighing (g)	Water content (g)	WCD (%)
CS/CMCD(1:10)	3.972±0.005	1.211±0.005	2.760±0.001	69.5
CS/CMCD(1:8)	3.567±0.0005	1.198±0.005	2.369±0.001	66.4
CS/CMCD(1:6)	3.248±0.005	1.192±0.005	2.056±0.001	63.3
CS/CMCD(1:4)	2.989±0.005	1.172±0.005	1.817±0	60.1
CS/CMCD(1:2)	0.937±0.005	0.394±0.005	0.539±0.001	57.5
CS/CMCD(1:1)	0.195±0.005	0.112±0.005	0.083±0	42.4

2.3.2. Proton NMR

¹HNMR spectra of CS, CMCD, CS/CMCD hydrogel are shows in Figure A4 in the Appendix A, ¹HNMR spectra of BBH, and CS/CMCD/BBH hydrogel are shown in Figure 1. In the CS and CMCD spectra, multiple proton signals at 4.08-3.80 ppm are attributed to the H protons of the CS and CMCD. A new signal at 3.35-4.09 ppm is caused by the H proton of the CMCD, and the peak at 5.21 ppm as reported by Hui et al. [31,38,39], 2024 corresponds to the proton peak of glucosamine in the CS. Furthermore, the peak at 3.2-4.0 ppm is attributed to the glucopyranose ring of CS. These observations confirm that the predicted CS/CMCD product was synthesized efficiently. In the ¹HNMR spectrum of the CS/CMCD/BBH hydrogel, a peak at 3.35 or 5 ppm corresponds to the chemical shifts of the composite CS/CMCD/BBH H proton. The peaks at 9.905 ppm and 7.095 ppm are attributed to the H protons of the BBH, while the peak at 3.353 ppm is attributed to the C-H2 proton of the BBH. The presence of these characteristic peaks of BBH in the CS/CMCD/BBH spectra confirms the successful incorporation of BBH into the hydrogel matrix and, supporting the enhanced solubility and stability of BBH in the oral formulation.

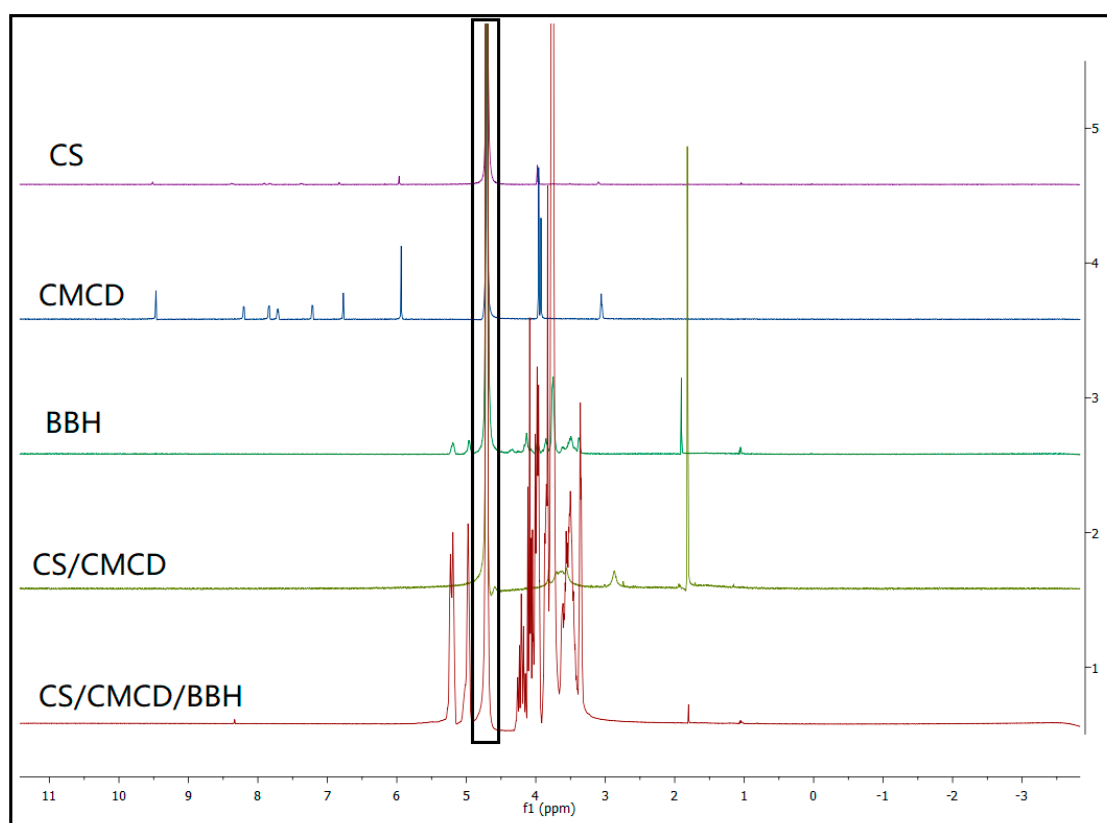


Figure 5. ^1H NMR spectra of the BBH and CS/CMCD/BBH (1%CS_6%CMCD_1%BBH) hydrogel.

2.3.3. FTIR

The FTIR spectra of CS, CMCD, and CS/CMCD hydrogel with different CMCD formulations are shown in Figure A5 in Appendix A. In the infrared spectrum of CS, the peak at 3367 cm^{-1} corresponds to an amine symmetry vibration, while the peak at 2927 cm^{-1} is indicative of a stretching vibration of CH. The peaks at 890 cm^{-1} , 902 cm^{-1} and 1155 cm^{-1} are associated with the sugar structure of chitosan. Furthermore, the broad peak at 1080 cm^{-1} corresponds to the stretching vibration of CO. Other notable peaks include those at 1600 cm^{-1} , 1320 cm^{-1} , 896 cm^{-1} and 500 cm^{-1} , which are assigned to the amino proton group and the intermediate group, respectively. In the infrared spectrum of CMCD, broad absorption peaks at 2928 cm^{-1} and 1419 cm^{-1} are assigned to the -OH and -C-O groups. The broad absorption peak at 3000 cm^{-1} is due to the hydroxyl group. The spectrum of CS/CMCD hydrogel shows the α -pyranose vibration of CMCD at 1030 cm^{-1} and the secondary amide band of chitosan at 1600 cm^{-1} [40]. These results confirm the successful synthesis of the CS/CMCD hydrogel. The FTIR spectra of BBH and CS/CMCD/BBH hydrogel is displays in Figure 2. The BBH spectrum shows characteristic peaks at 1616 , 1579 , 1178 , 1141 and 1233 cm^{-1} . The peak at 1616 cm^{-1} is identified as the quaternary ammonium ion C=N. The peaks at 1178 cm^{-1} and 1141 cm^{-1} are due to the vibration of the C-O bond in BBH [41,42]. Comparing the spectra of BBH with the CS/CMCD/BBH hydrogel, a notable shift of protons from the C=N peak from 1616 cm^{-1} to 1670 cm^{-1} is observed. In addition, the C-O vibration peak disappeared and new peaks appeared in the range of $500\text{--}600\text{ cm}^{-1}$ due to C skeletal vibrations. Moreover, the peak intensity between 2000 and 3000 cm^{-1} decreased, indicating a change in the IR range within the complex. These spectral changes suggest strong interactions between BBH and the hydrogel matrix [43]. Therefore, FTIR analysis provides strong evidence for the successful synthesis of CS/CMCD hydrogel and efficient encapsulation of BBH in this matrix. The observed spectral shifts and changes indicate significant interactions between BBH and hydrogel components, which are crucial for improving the solubility and stability of BBH for oral administration.

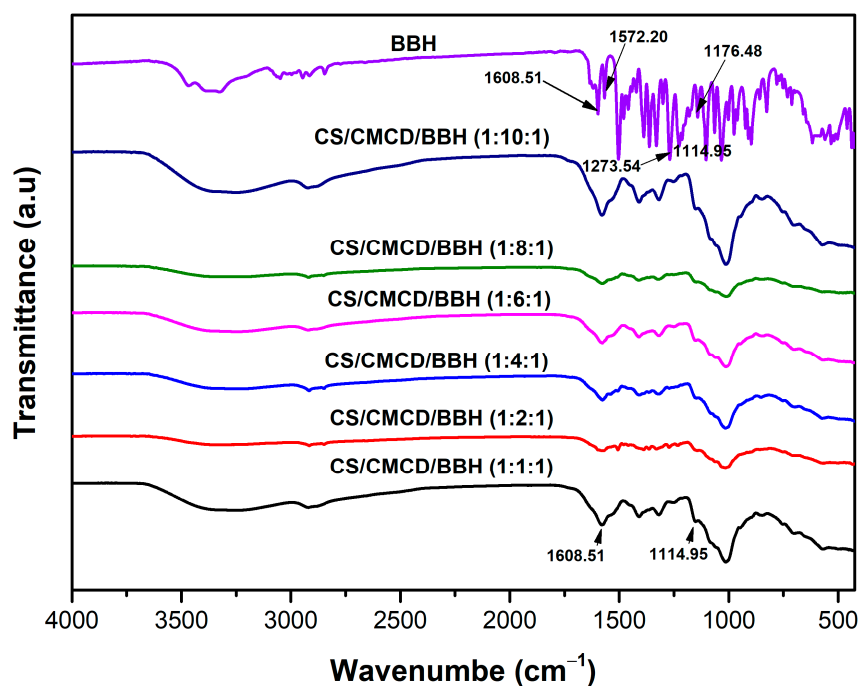


Figure 2. FTIR images of the BBH and CS/CMCD/BBH hydrogel with different formulations of CMCD.

2.3.4. XRD

The XRD patterns of CS, CMCD, CS/CMCD hydrogel with different CMCD formulations are shown in Figure A6 in the Appendix A. The XRD patterns of BBH and CS/CMCD/BBH hydrogel are shown in Figure 3. The XRD profile of the CS showed distinct crystal peaks at approximately $2\theta = 10^\circ$ - 11° , attributed to the presence of amine I (-N-CO-CH₃), and at 19° - 22° , attributed to the amine II (-NH₂) [44]. These peaks are indicative of the crystalline nature of chitosan. The XRD pattern of the CMCD showed characteristic intense peaks in the range of 15° to 30° [45]. This pattern reflects the crystal structure of CMCD. In the case of CS/CMCD hydrogel, the XRD pattern exhibited a more amorphous nature, as indicated by the broadened peaks around $2\theta = 7.90^\circ$ - 10° and $2\theta = 21.0^\circ$. The results demonstrated that the interaction between CS and CMCD led to the formation of a hydrogel with increased amorphous characteristics. The appearance or disappearance of specific diffraction peaks and the change in crystallinity confirmed the successful incorporation of CMCD into the CS backbone, resulting in the formation of the CS/CMCD hydrogel. The XRD pattern of BBH showed common crystal peaks at $2\theta = 8.6^\circ$, 9.1° , 12.9° , 16.2° , 20.9° , 25.4° , 30.5° and 45.3° [25,46]. These peaks illustrate the crystalline nature of the BBH [47]. When BBH was incorporated into the CS/CMCD hydrogel, the resulting XRD pattern showed a more amorphous structure. This amorphization indicates that the BBH is well dispersed in the hydrogel matrix. The enhanced amorphous nature of the CS/CMCD/BBH hydrogel is significant because it contributes to the increased solubility of BBH, an essential factor for its effectiveness in oral administration.

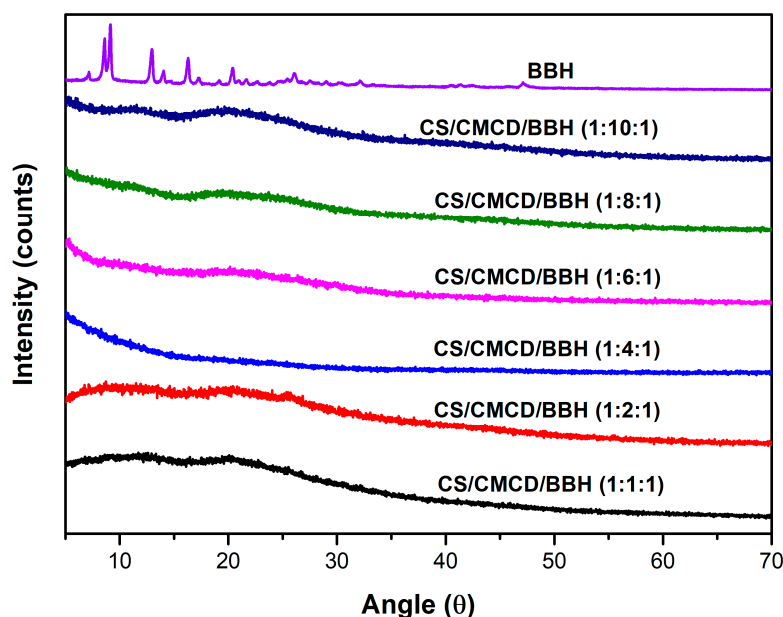


Figure 3. XRD images of the BBH and CS/CMCD/BBH with different formulaions of CMCD.

2.3.5. TGA

The thermal stability of CS, CMCD and CS/CMCD are shown in figure A7 in the Appendix A. The TGA of BBH and CS/CMCD/BBH are shown in Figure 4. TGA of CS typically exhibits weight loss in two stages. The TGA curve of CS shows a weight first stage at about 46-125 °C with weight loss of 10.2% due to loss of residual or physically adsorbed water on membranes surfaces. Then the weight loss starts at 295 °C and that continues up to 545 °C during which there was 41.5% weight loss due to the degradation of CS. TGA Curve of CMCD shows a weight loss in three stages. The first stage starts under 100 °C with a 14.8% mass loss can be attributed to the evaporation of superficial water associated with the cyclodextrin. The second stage, at around 110 °C with a 10% mass loss, can be attributed to the evaporation of internal water [48]. Then, the third stage at around 307 °C can be related to the degradation of the β -CD [49]. At this point, 65.7% of the mass is reduced. TGA Curve of CS/CMCD shows a weight loss in two stages. The first stage starts with weight loss at the ranges from 25 to 258 °C with 8% of the adsorbed and bound water weight loss on membranes surfaces. The second stage weight loss start from 275 to 742 °C during which there is 37.5% of weight loss due to the degradation of chitosan at. There is 29.5% weight loss observed in the ranges from 750 to 995 °C that contributes to the decomposition of CMCD at CS/CMCD interaction indicated a high degree of thermal stability of the composite. The CS/CMCD might improve the thermal stability through hydrogen bonding and electrostatic attraction interactions. The TGA curve for BBH exhibits weight loss in four stages. The initial weight loss of BBH start at 109°C with 8.3% of weight loss is attributed to the evaporation of moisture or which corresponded to the loss of moisture [50]. The second stage start between 109 and 188°C, with 1.3% of weight loss, signified the melting temperature of the BBH. the third step showed 20.6% of weight loss at 250°C, revealing decomposition of the BBH [35]. The fourth step in the 250-790°C range was ascribed to the destruction of the BBH skeleton structure. The TGA curve for CS/CMCD/BBH, the maximum weight loss occurred at about 265°C with 18.3% of weight loss reached approximately 500°C, and disintegration was finished at 549°C, showing that CS/CMCD/BBH displays great intensity obstruction. However, The TGA curves demonstrated that the CS/CMCD/BBH hydrogel had a slightly lower decomposition temperature compared to the CS/CMCD hydrogel, likely due to the presence of BBH. Additionally, the curves showed that the thermal stability of the BBH-based polymer was not impacted by the temperature during the process. The TGA curves demonstrated that the CS/CMCD/BBH hydrogel had a slightly lower decomposition temperature compared to the CS/CMCD hydrogel, likely due to the presence of BBH. However, the thermal stability remained adequate for potential oral administration applications.

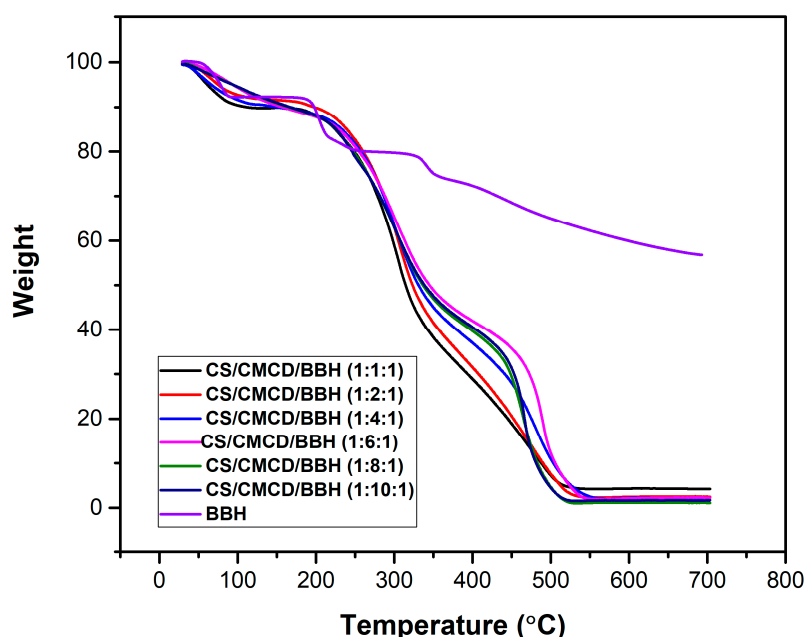


Figure 4. TGA of BBH and CS/CMCD/BBH hydrogel with different formulations of CMCD.

2.3.6. Mechanical Properties

Tensile tests were also carried out to evaluate the mechanical properties of the hydrogels, as shown in Figure A8 in Appendix A. The hydrogels demonstrated exceptionally high compressive strength and toughness. Notably, no fracture was observed even at a strain of 100% for any of the hydrogels tested. When the concentration of CMCD in CS/CMCD hydrogels increased from 2% to 10%, the compressive stress increased accordingly from 20 MPa to 180 MPa. This improvement is attributed to the increased chemical cross-linking and hydrogen bonds due to the higher CMCD content, significantly improving the toughness of the hydrogels. For the CS hydrogels, although the strain value increased significantly beyond 100%, the strain did not show a corresponding significant increase. The addition of CMCD was found to be crucial in increasing the degree of chemical cross-linking and hydrogen bonding, leading to a notable improvement in toughness and cross-linking density. Nevertheless, the mechanical properties of the CS/CMCD hydrogel remained within acceptable ranges for oral formulations, ensuring that the hydrogel maintains its structural integrity while effectively delivering the drug.

2.3.7. SEM

The SEM images of CS/CMCD hydrogels are shown in Figure A9 (Appendix A) and Figure 5 shows the SEM images of the CS/CMCD/BBH hydrogel. Compared with the CS/CMCD/BBH hydrogel shown in Figure 9, the gel structure of CS/CMCD/BBH becomes looser, the wall thickness increases, and the pore size becomes more uneven. The layered gel structure is more pronounced, forming a dense, uniform and orderly hydrogel network. However, there was no significant difference between the microstructures. As shown in Figure A9a (Appendix A), the pore size of the prepared hydrogels increases with the concentration of CMCD, but all hydrogels maintain an interconnected network structure. Figure A9b (Appendix A) purely describes the construction of the CS/CMCD structure interaction, revealing interconnected pores and a network of connected pores throughout the hydrogel matrix. This further confirms the interaction or cross-linking reaction [51,52]. Furthermore, the hydrogels prepared in this study demonstrate improved performance, essential for efficient drug loading and release, compared to hydrogels prepared in similar studies in which cross-linkers are added and still exhibit poor mechanical properties and signs of toxicity [53,54]. This improvement is crucial for their potential application in drug delivery systems, providing a more reliable and efficient method for the oral administration of BBH [55].

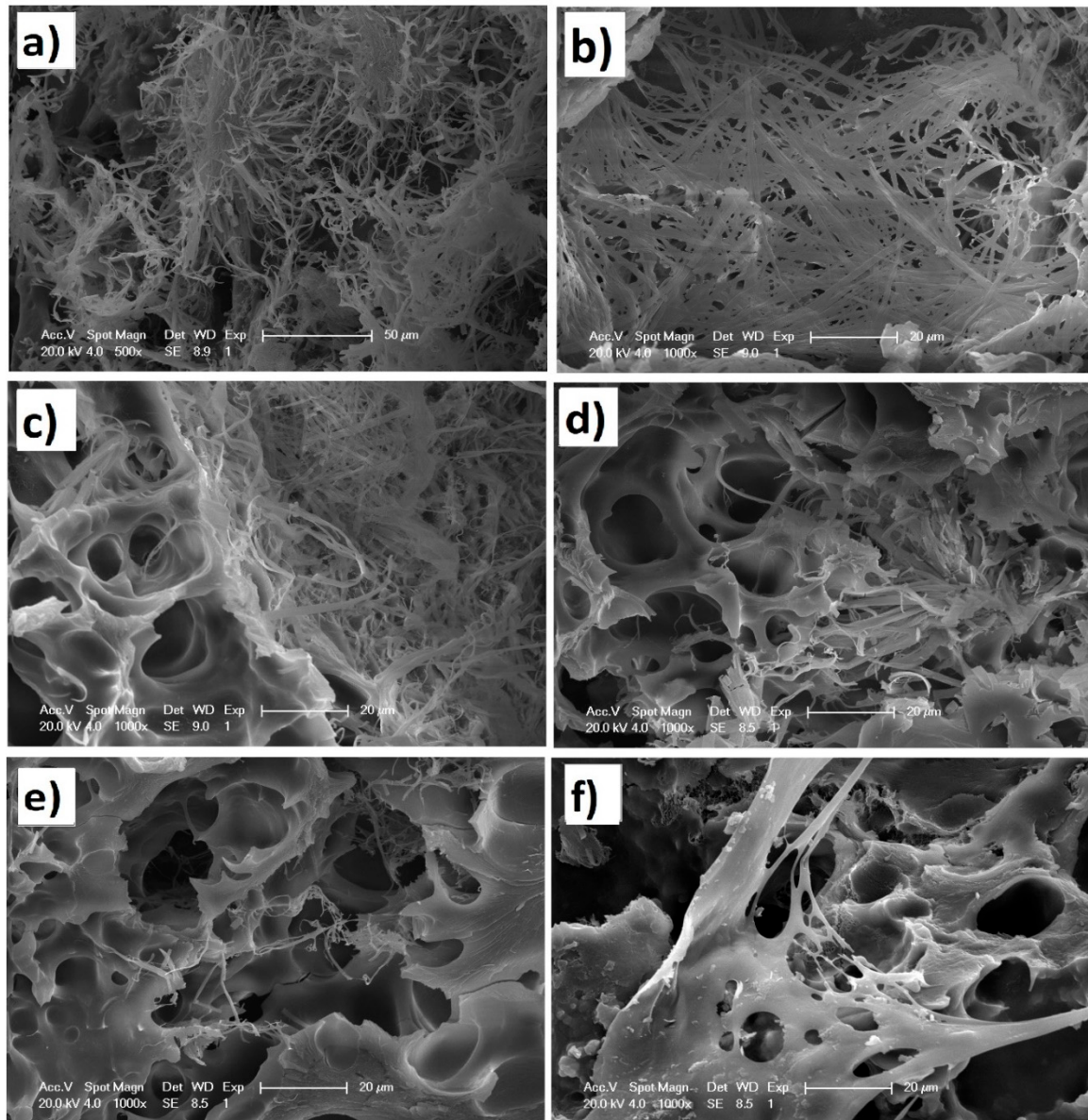


Figure 5. SEM image of CS/CMCD/BBH with different formulation of CMCD, scale bars equal to 20 μm and 200 μm . a) 1% CS_1%CMCD_1%BBH, b) 1% CS_2%CMCD_1%BBH, c) 1% CS_4%CMCD_1%BBH, d) 1% CS_6%CMCD_1%BBH, e) 1% CS_8%CMCD_1%BBH, 1% CS_10%CMCD_1%BBH.

2.4. Drug Release and Loading System

2.4.1. Loading Capacity and Encapsulation Efficiency

The loading capacity (LC) and encapsulation efficiency (EE) of BBH in the CS/CMCD hydrogel were calculated based on the experimental data obtained from the analytical methods described below. The results demonstrate the ability of the hydrogel to effectively entrap and deliver BBH, as shown in Table 2. The loaded weight of CS/CMCD was 10 times greater than the initial weight, and the LC capacity and EE capacity of the composite were approximately 92% and 85%, respectively. A higher LC and EE indicate a greater drug-loading capability and better drug retention within the hydrogel matrix. The determination of the LC and EE of BBH in the CS/CMCD composite provides essential quantitative data for evaluating the efficacy of the hydrogel as a drug delivery system. The results contribute to the development of an efficient formulation for enhancing the solubility, stability, and controlled release of BBH, thus improving its therapeutic potential.

Table 2. Determination of the LC and EE of BBH-loaded CS/CMCD.

Hydrogel weight (mg)	Loaded weight (mg)	Drug weight (mg)	LC (%)	EE (%)
18.6±2.57±1.656	260.9±4.17	242.3±1.656	92.3±0.86	85.7±5.15

2.4.2. Drug Release Profile

Drug release studies are conducted to study how a drug is released from a particular delivery system over time [56–58]. In this study, the *in vitro* release of BBH from the CS/CMCD hydrogel was evaluated using the basket method by simulating body fluids and performing periodic sampling to measure the concentration of the drug released [59]. The environments used were simulated gastric fluid (SGF) with a pH of 2.1, simulated intestinal fluid (SIF) with a pH of 6.8, simulated colon fluid (SCF) with a pH of 7.2, and the simulated basic liquid (SBF) with a pH of 12. As shown in Figure 6, the drug release patterns in all simulated fluids exhibited two distinct stages. An initial abrupt release followed by a sustained release or extended diffusion controlled release. The initial abrupt release can be attributed to the presence of the drug on the hydrogel surface and the high concentration gradient of the drug, which acts as a driving force to release the drug from the hydrogel matrix. Following this burst release, the drug was released steadily over time, likely due to the formation of inclusion complexes between the drug and CMCD and the structure of the hydrogel network that hindered diffusion. drug molecules. The hydrogel in SIF showed lower release efficiency, with only 10% in 24 h. In the SCF, approximately 15% of the BBH was released within 24 h, while in the SBF the release was approximately 35%. SGF posted a 50% release in the same period. The release rate of BBH was fastest in SGF, followed by SBF, then SIF, and finally SCF. The faster release of SGF could be due to the protonation of CS in the acidic medium, which increases the swelling of the hydrogel composite matrix, making its structure looser and allowing faster drug release. Conversely, the slower release in SCF could be due to the formation of inclusion complexes between CMCD and drug molecules, resulting in a slower drug release rate. Therefore, the sustained release of BBH from the CS/CMCD hydrogel was effectively modulated, thereby improving its bioavailability and therapeutic efficacy. This controlled release profile highlights the potential of the CS/CMCD/BBH hydrogel as a viable drug delivery system for oral administration, addressing the solubility and delivery challenges associated with BBH.

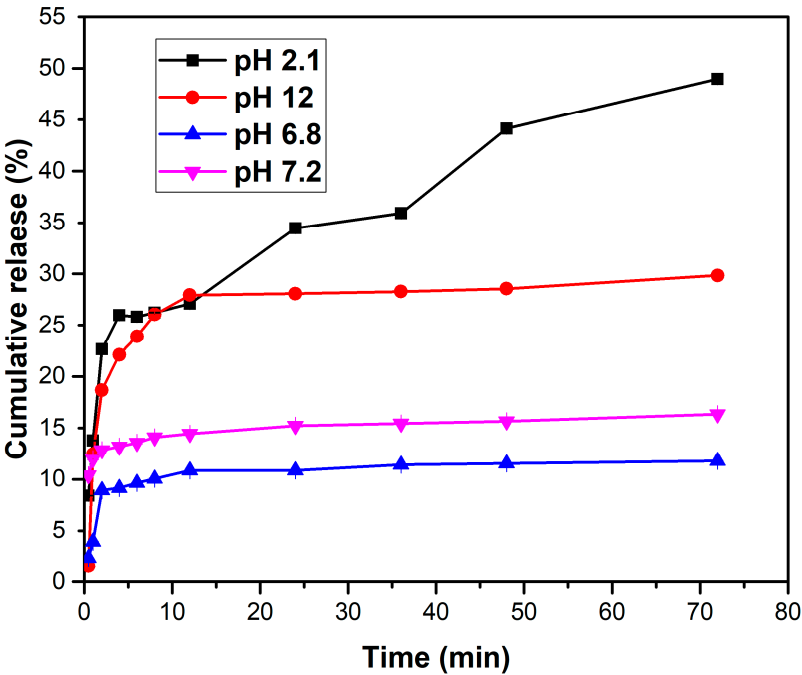


Figure 6. The release profile of BBH solution at different pH values at room temperature.

2.5. Anti-Bacterial Effect of CS/CMCD and CS/CMCD/BBH Hydrogels

The antibacterial activities of CS/CMCD and CS/CMCD/BBH hydrogels against *E. coli*, *S. aureus*, and *C. albicans* were evaluated using the agar diffusion method. As shown in Figure 7a,b, the CS/CMCD hydrogel demonstrated less antibacterial activity against *E. coli*, *S. aureus*, and *C. albicans* compared to CS alone. However, the CS/CMCD/BBH hydrogel exhibited significantly better antibacterial activity against these pathogens. This increased activity is likely due to the antibacterial properties of BBH, which disrupt the structure and function of microbial cells, interfere with their DNA, and inhibit various enzymes [2]. The inhibition zone areas for *E. coli*, *S. aureus*, and *C. albicans* were significantly larger for the CS/CMCD/BBH hydrogel compared to CS/CMCD. The sustained release of BBH from the hydrogel into the solid medium contributed to this effect. As shown in Figure 7b, the CS/CMCD/BBH hydrogel maintained good antibacterial activity against *E. coli* for 24 hours, with inhibition zone diameters greater than 10 mm, and the inhibition persisted for 48 and 72 hours. Similarly, the hydrogels showed more pronounced inhibitory regions against *S. aureus* and *C. albicans*, with inhibition diameters greater than 15 mm on the first day and lasting antibacterial effects for up to three days. Even after three days, the hydrogel still demonstrated significant inhibition compared to CS alone. The data suggest that BBH is well permeated into the hydrogel, resulting in a longer lasting antibacterial effect. Therefore, CS/CMCD/BBH hydrogel exhibited sustained antibacterial action for three days against *E. coli*, *S. aureus* and *C. albicans* on agar plates. This sustained antibacterial activity is attributable to the inherent properties of BBH, making the formulation suitable for the treatment of infections requiring prolonged antibacterial action.

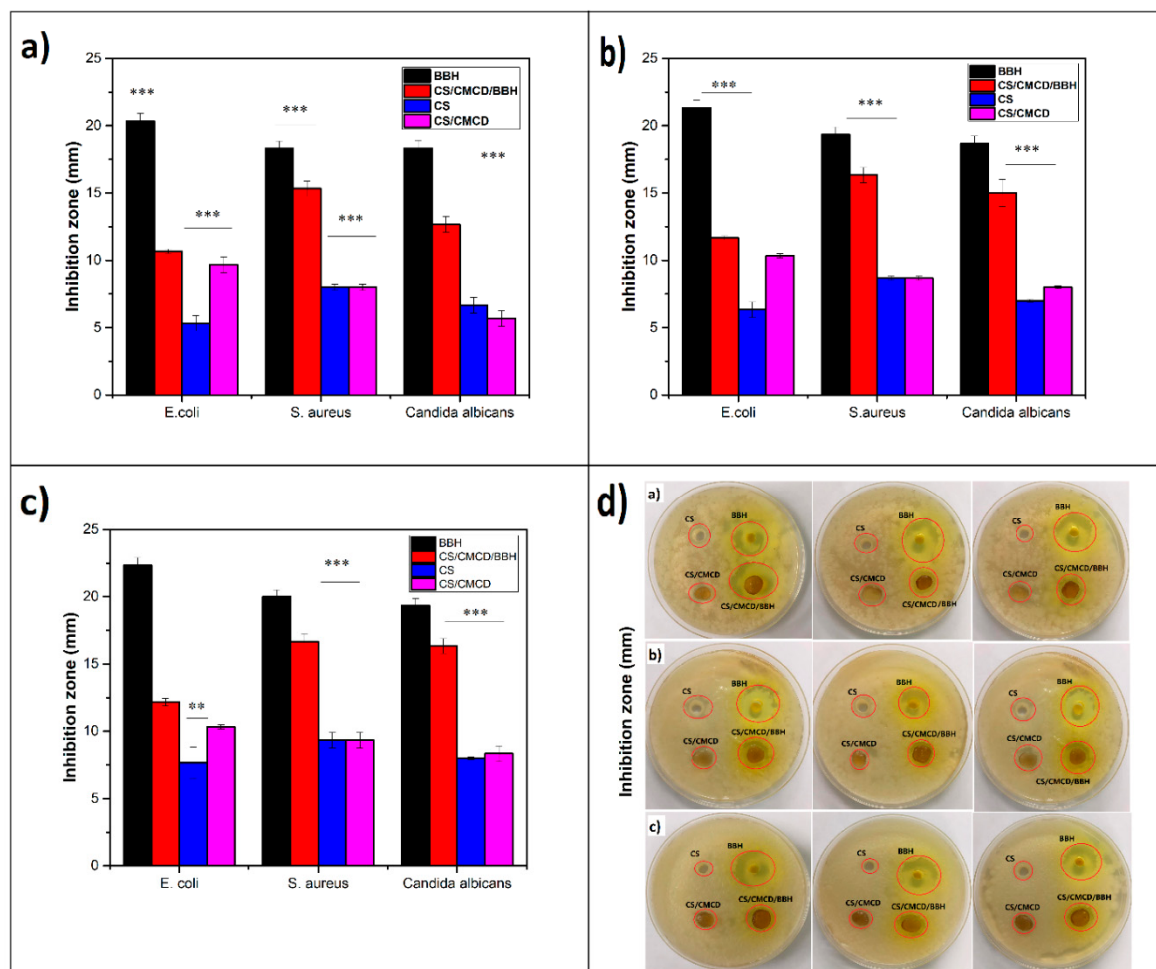


Figure 7. Anti-bacterial effect of a) Twenty-four hour, b) fourty-eight hour, c) senven-two hour invasion zones of the samples: d) *E. coli*, *S. aureus*, and *C. albicans* after 24 h incubation. Mean $\pm$ sd (n=3).

2.6. Cytotoxicity Effect of CS/CMCD and CS/CMCD/BBH Hydrogels

The cellular toxicity of CS/CMCD hydrogels was studied to evaluate their biocompatibility. Fibroblast cells (NIH/3T3) and keratinocyte cells (HaCaT) were cultured on scaffolds, and their cellular cytotoxicity was assessed using live/dead assays and quantification of cell proliferation via CCK-8 trials. Cells were directly cultured on the samples and on extraction media derived from the samples. The CCK-8 assay is commonly used to quantify the cell viability of cells growing on or inside bioengineered scaffolds [60]. To check the initial cell viability of CS/CMCD/BBH, CS/CMCD and BBH, live/dead assays were performed (Figure 8). As shown in Figure 8a, live NIH3T3 cells exhibited green fluorescence when treated with calcein AM. The CS/CMCD/BBH hydrogel showed almost no dead cells. After 24 hours of seeding on CS/CMCD/BBH, the cells were both alive and well attached to the samples. Furthermore, cells adhering to CS/CMCD hydrogels exhibited a stretched morphology after 24 hours, indicating high cellular affinity of CS/CMCD hydrogels. Cell proliferation on each sample was confirmed after 3 to 5 days of culture under direct contact and extract conditions. As shown in Figure 8b and Figure A10 in the Appendix A, the cell proliferation rate was compared to the 1-day control group (free cells) for quantification. The CS/CMCD/BBH hydrogel showed a significant difference in proliferation rates compared to the control group on day 1 of culture, with a 30% higher rate in solvent extraction culture than in contact culture direct. There was no significant difference in proliferation rates between the CS/CMCD/BBH group and the control group in direct contact culture, with only a difference of 22%. This trend continued for 2 days. However, on day 3, there was a significant difference in proliferation rates between the CS/CMCD/BBH group and the control group, with the CS/CMCD/BBH group showing an increase of more than 100%. For the CS/CMCD hydrogel as shown in Figure A9 in the Appendix A, there was no significant difference in proliferation rates compared to the control group on day 1, with only a 11% difference. This trend continued through 5 days, except for a significant difference on day 3, where the CS/CMCD group showed a 25% increase compared to the control group. No statistical differences were found between the two groups for the remainder of the testing period, indicating that although CS/CMCD modification may slightly alter its physicochemical properties, it does not induce cytotoxic effects. These results demonstrate that the CS/CMCD/BBH hydrogel is biocompatible and safe for use at therapeutic concentrations, highlighting its potential for safe oral administration.

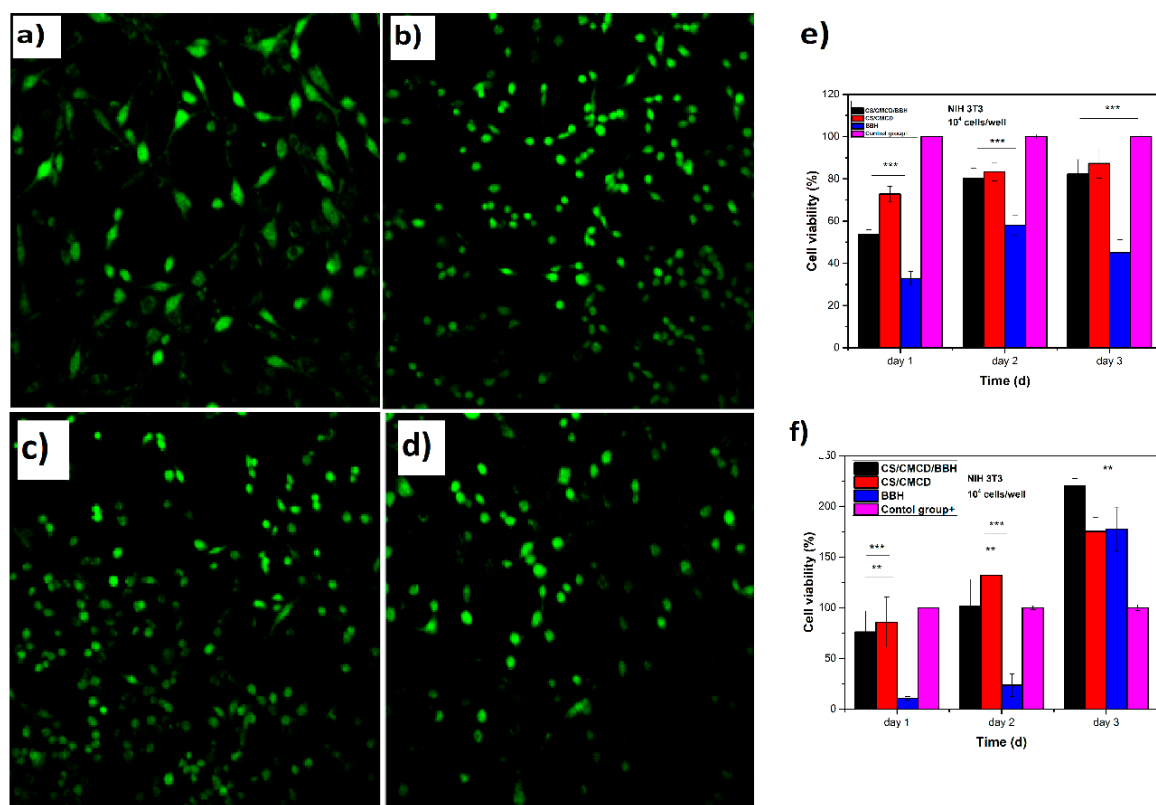


Figure 8. Cytotoxicity effect of of CS/CMCD/BBH on NHI3T3 and HacaT cells: a) live and dead staining for samples: a) CS, b) CS/CMCD, c) BBH and d) CS/CMCD/BBH at twenty-four hours incubation, e) cell viability through the solvent extraction of the samples, f) cells viability direct contact with the samples.

3. Discussion and Conclusion

In this study, an innovative hydrogel of chitosan (CS) and carboxymethyl- β -cyclodextrin (CMCD) was developed by physical cross-linking to serve as an oral formulation for berberine hydrochloride (BBH). This formulation improves the solubility of BBH and addresses the limitations of its suitability for oral administration. The results demonstrated that the CS/CMCD composite was successfully prepared. An increase in the amount of negative protons in H¹H NMR improved the quality of the composite. FTIR analysis indicated intermolecular interactions within the hydrogel and SEM images revealed a three-dimensional porous structure. XRD and TGA characterizations showed that the synthesized CS/CMCD significantly improved the solubility of BBH. Loading capacity (LDC) studies showed that BBH was well loaded into the CS/CMCD matrix up to 90%, and encapsulation efficiency (EE) indicated that 82% of the drug was encapsulated in the hydrogel matrix.

The hydrogel also showed a good swelling rate in water of up to 50%. The prepared hydrogel demonstrated slow and sustained release of BBH, with the drug release profile varying under different pH values. Additionally, the CS/CMCD/BBH hydrogel exhibited good biocompatibility, with no significant impact on cell viability during the initial five-day period. After 24 hours, analyses on the living dead revealed signs of cell proliferation. CS/CMCD/BBH hydrogels exhibited good antibacterial activity against *Staphylococcus aureus*, with a maximum inhibitory diameter of 28 mm and sustained inhibition for three days in solid media. The hydrogel also had a significant antibacterial effect on *Escherichia coli*, with a maximum inhibitory diameter of 10 mm and an antibacterial effect lasting 48 hours in a solid medium. These results suggest that the prepared hydrogel can serve as a novel controlled drug release carrier and a promising material for drug delivery systems, including antibacterial wound dressings.

The study provides valuable evidence for the application of hydrogels in biomedical materials and their potential in the development of multifunctional biomedical materials. However, further

research and development is required to optimize formulation parameters, study long-term stability, and evaluate the safety profile of CS/CMCD hydrogels for BBH delivery. Additionally, real-time *in vivo* studies of hydrogels could provide valuable information for their future clinical application.

4. Materials and Methods

4.1. Chemicals, Microbial Strains, and Cell Strains

CS was obtained from Aladdin Industrial Biotechnology Co., Ltd. (Shanghai), China; CMCD was purchased from Shandong Binzhou Zhiyuan Biotechnology Co., Ltd.; 50 kDa cut-off dialysis membrane and acetic acid were purchased from Biosharp, USA; and BBH was obtained from Chemical Reagent Co., Ltd. *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*, fibroblasts or NIH3T3 cells and epithelial cells or HaCaT cells were obtained from Tongji Medical College of Huazhong University of Science and Technology.

4.2. Selection of Detection Wavelength

BBH was prepared according to the method of Huang et al. [61] with slight modifications. First, the wavelength was selected by accurately weighing 0.05000 g of BBH with an analytical balance, dissolving it in a 500 ml beaker, and then diluting it with a 1 L volumetric flask to obtain a certain concentration of BBH solution. A Thermo Fisher Scientific Multiscan-GO full-wavelength microplate reader was used to scan the spectrum of the BBH solution, and water was used as a blank control.

4.2. Determination of the Solubility

To determine the solubility of BBH using a standard curve or a single spectrum, we prepared a series of BBH standard solutions with known concentrations. The concentrations covered a range of values that allowed accurate determination of the concentration of unknown samples. We prepared standard solutions with concentrations ranging from 1.0 µg/mL to 0.1 µg/mL. Thermo Fisher Scientific Multiscan-GO spectrophotometry was used to determine the absorption (OD) at 263 nm, as described in Table 1 in the appendix.

4.3. Synthesis of CS/CMCD and Synthesis of BBH-Loaded CS/CMCD

A CS/CMCD physical crosslinking hydrogel was obtained by magnetic stirring to form a physical bond cross-linking reaction. Typically, CS was dissolved in a 1% acetic acid solution, and CMCD at different ratios (10%, 8%, 6%, 4%, 2%, and 1%) was dissolved in deionized water. After rigorous mixing, CMCD solution was added to the CS solution, which was stirred at room temperature and allowed to react for a few minutes. Then, a dialysis membrane bag (MW: 3500, 36 MM, Biosharp, USA. Atomic cut-off: 2 kDa) was used for dialysis in distilled water for 5 days to expel unreacted reagents, as demonstrated in Figure 1. The dialysate was replaced with deionized water every 4 hours. Finally, the reactants were removed from the solution and freeze-dried to obtain a white flocculated CS/CMCD composite hydrogel. The CS/CMCD hydrogel composite loaded with solid BBH was prepared by the lyophilization method at a molar ratio based on the results of the CS/CMCD synthesis studies. Briefly, 1% BBH was dissolved in water at 45°C for 2 minutes and rapidly filtered through 0.45 µm pore size syringe filters to remove free BBH. The solution was added to the CS/CMCD solution, and the resulting loaded composite hydrogel was stirred continuously for 1 h before slowly cooling to room temperature. The prepared CS/CMCD/BBH complex hydrogel was transferred to a dialysis bag, dialyzed against deionized water for 72 hours and replaced every 4 hours to remove any unbound berberine hydrochloride.

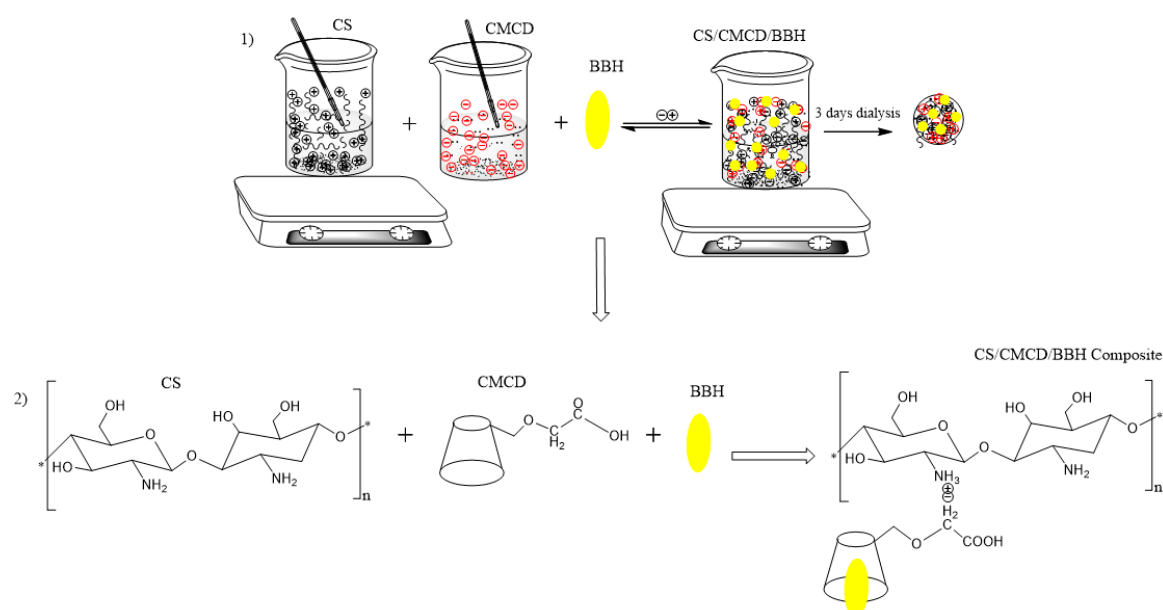


Figure 1. The CS/CMCD/BBH hydrogel composite formulation.

4.4. Characterization of the CS/CMCD and CS/CMCD/BBH Composite Biomaterials

Fourier transform infrared spectroscopy (FTIR) of the CS/CMCD and CS/CMCD/BBH composites was performed with a Fourier transform infrared spectrometer (Bruker VERTEX 70, Germany). The samples (CS, CMCD, CS/CMCD/BBH, and CS/CMCD/BBH) were prepared using the dry KBr disk method. The experimental data were recorded in the range of 400 to 4000 cm^{-1} . Furthermore, proton nuclear magnetic resonance analysis of the composite biomaterials was performed by a nuclear magnetic resonance spectrometer (AV300 NMR, Bruker, Switzerland) using a 1% solution of deuterium acetic acid (D_2O) for dissolution, while the X-ray diffraction of the composite biomaterial dry powders was recorded by using an X-ray diffractometer (40 kV, 100 mA). In addition, thermogravimetric analysis of the samples was performed using a TGA spectrometer (TGA800, Perkin, USA). The tensile properties of the CS/CMCD were tested using a mechanical performance testing machine (i-Strentek 1510 10 N (2.20 lbf)), with a diameter of 30 mm and a height of 1.28 mm, and the tensile properties were measured at 25°C at 5 mm compression at a rate of 5 mm/minute, and the software automatically recorded the curve of force versus deformation. Finally, the morphology of the samples was observed using an FEI Sirion 200 scanning electron microscope. Determination of the water content (WDC) of the CS/CMCD was performed using the loss on drying method. The initial weight of the composite compound was measured, and the sample was then completely freeze-dried. The WDC was calculated as follows:

$$\text{WCD}(\%) = \left(\frac{iw}{dw} \right) \times 100 \quad (1)$$

The (iw) is equivalent to the first weighing, and the (dw) is equivalent to the second

4.5. Drug Loading and Drug Delivery System

4.5.1. Determination of the Loading Capacity and Encapsulation Efficiency

The loading capacity (LC) of BBH in the chitosan/ β -cyclodextrin hydrogel was determined by quantifying the amount of BBH entrapped in the hydrogel matrix. The encapsulation efficiency (EE) represents the percentage of BBH effectively encapsulated within the hydrogel matrix. Briefly, the CS/CMCD was weighed and placed in BBH at 45°C for 24 hours. Then, it was removed and placed under vacuum for 5 min to remove the access BBH, weighed, and then dried overnight. After freeze-drying, the sample was weighed again. The loading capacity was calculated by dividing the amount of drug loaded into the hydrogel by the weight of the hydrogel itself. The encapsulation efficiency

was calculated by dividing the amount of drug loaded into the hydrogel by the total amount of drug initially added and then multiplying by 100 to express it as a percentage (Massella et al 2018). The EE and LC of BBH were calculated according to the following formulas:

$$EE\% = \frac{w_l - w_0}{w_f} \times 100 \quad (2)$$

$$LC\% \equiv \frac{w_l - w_0}{w_l} \times 100 \quad (3)$$

where w_0 , w_f , and w_l are the amount of blank composite material, the weight of the initial drug, and the amount of remaining drug, respectively.

4.5.2. In Vitro Drug Loading and Drug Release

First, BBH was prepared according to the method of Huang et al. (2013) with slight modifications. Briefly, 1 mg/ml BBH was dissolved in water, mixed, and slightly heated. The CS/CMCD composite was added to the BBH solution and heated for 1 min at 45°C. Then, the samples were frozen and dried for 40 h to obtain a loaded or charged CS/CMCD/BBH complex, as shown in Figure 5 in appendix A. The dosage of BBH was calculated with a calibration curve of 0.1 µg/ml⁻¹ - 10 mg/ml⁻¹. The corresponding regression equation was (µg/ml⁻¹) = -0.007 + 0.2929x, R² = 0.9994 (where x is the absorbance of the drug, y is the drug concentration, and R² is the drug loading concentration), as described in Figure 2. Second, the drug release curve of BBH from CS/CMCD was obtained using the rotating basket method at a speed of 100 revolutions per minute in the automatic dissolution apparatus, and the in vitro release curve at 37°C was drawn. The oral drug delivery system is simulated. An acidic solution with a pH of 2.1 in the culture medium stimulates the gastric environment, a basic solution with a pH of 6.8 simulates the intestinal environment, and a neutral solution with a pH of 7.4 simulates the colon environment. For the rigor of the experiment, we also discussed the release behavior in alkaline media. The dissolution medium of 10 mg of drug was 9 ml of SGA:HCL solution, SIE:HCL solution, and SCE:phosphate buffer or NaOH solution (i.e., pH 2.1, 6.8, 7.4, and 12), and the temperature was maintained at 37°C ± 0.5°C. The sample (1 ml) was removed after 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 36 h, and 48 h and immediately changed to 10 ml of the new preheating medium after 72 h. The maximum absorption wavelength of the drug was obtained by scanning the whole waveband.

4.6. Antimicrobial Assessment

Antibacterial tests were carried out on CS, BBH, CS/CMCD/BBH, and CS/CMCD composites by the uniform diffusion method. In this study, three bacteria and yeasts were selected for antibacterial testing: the gram-negative bacteria *Escherichia coli* and the gram-positive bacteria *Staphylococcus aureus* and *Candida albicans*. Microbial culture media were prepared with MHB and YPDA media at 37°C. The inoculum density (105c.F. of broth culture solution) was evaluated by an MC-Farland 0.5 standard solution, spectrophotometer and 1:10 second dilution method. The surface of the agar plate was inoculated to distribute the microorganisms. The hydrogel was cut into small discs with a diameter of 1 cm and placed on agar. The agar plates containing the samples were labelled separately, placed upside down and incubated at 37°C for 24 hours. After 24 hours, the samples were removed and observed. The diameter of the zones of inhibition was recorded at 24, 48, and 72 hours.

4.7. Cell Viability Cytotoxicity Assay

The cytotoxicity of the CS/CMCD composite was evaluated by the CCK-8 method (CK04 cell counting detection kit) in fibroblast and epithelial cell lines. The cells were seeded at a concentration of 1×10⁴ in a 96-well plate and incubated overnight. Then, different concentrations of the CS/CMCD hydrogel were added to each well. After incubating for 48 hours, 10 µl of CCK-8 solution was added to each well. After incubating for 30 minutes in a humid environment containing 5% CO₂ at 37°C, the

absorbance was measured at 450 nm using a microplate reader (Multiscan GO Thermo Fisher USA). Cell viability was calculated using the following formula.

$$\text{Cell viability} = \frac{\text{OD1} - \text{OD2}}{\text{OD3} - \text{OD2}} \times 100 \quad (4)$$

4.8. Drug Efficacy Assay

The cytotoxicity of the BBH-loaded CS/CMCD hydrogel was evaluated in 3T3 cells and HatCat cells. The cells were seeded in 96-well plates at 1×10^4 cells per well and incubated at 37°C overnight. The cells were added to different formulations of the sample composite biomaterials and incubated for several minutes, after which 10 µl of CCK-8 solution was added to each well after incubation for 24, 48 or 72 h. After 30 min of incubation at 37°C in a humidified atmosphere containing 5% CO₂, the absorbance was measured at 450 nm using a microplate reader (Thermo Fisher, USA). The cell viability was calculated using the following formula.

$$\text{Cell viability} = \frac{\text{OD1} - \text{OD2}}{\text{OD3} - \text{OD2}} \times 100 \quad (5)$$

4.9. Confocal or Staining Assay

To confirm the cytotoxicity of the drug, the cellular uptake of CS/CMCD/BBH into the NIH 3T3 cell line was studied. NIH 3T3 cells (5×10^4 /well) were seeded on 8-chamber slides (Lab Tek II, Thermo Fisher) and incubated overnight. Then, the CS/CMCD/BBH (containing 5 µg/mL BBH) was diluted in culture medium and incubated with the cells for 1 h at 37°C. After the medium containing the CS/CMCD/BBH was removed, the cells were washed with PBS three times, stained with DAPI solution for 15 min, and eventually washed three times with PBS. The sample was imaged with a confocal laser scanning microscope (Olympus, FV1000) using an excitation wavelength of 405 nm and blue emission at 425 nm to observe the DAPI nuclei, while an excitation wavelength of 535 nm and red emission at 595 nm were used to observe the intracellular contents of BBH. The cellular uptake of the CS/CMCD hydrogel composite and free BBH was investigated as controls.

4.10. Statistical Analysis

All the statistical analyses and measurements were performed using Origin PROLAB. The data obtained were analysed using analysis of variance (ANOVA) in triplicate, and the results are expressed as the mean ± standard deviation.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Video S1: CS/CMCD hydrogel.

Author Contributions: Project administration, Lin Xiao; Supervision, Guang Yang; Writing – original draft, Bukatuka Clemence.

Funding: This research received no external funding.

Acknowledgments: We acknowledge support from the Chinese and Congolese authorities, as well as Sino-Congolese cooperation.

Conflicts of Interest: We have no conflicts of interest to declare.

Appendix A

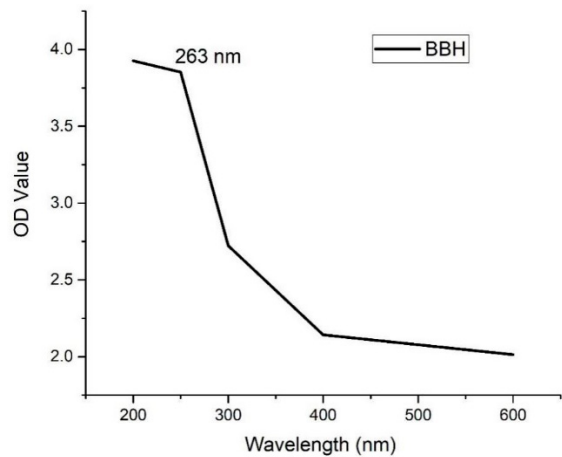


Figure A1. Selection of detection wavelength of BBH.

Table A1. Absorption spectrum of BBH from 0.1 to 10 $\mu\text{g/ml}$ -1.

Conc. (mmol/L)	OD value	Absorbance value	Absorbance value
0.1		3.1123	0.0537
0.2		3.1581	0.0995
0.4		3.2017	0.1431
0.6		3.2441	0.1735
0.8		3.2725	0.2139
1.0		3.3204	0.2618
10		6.0000	2.9414

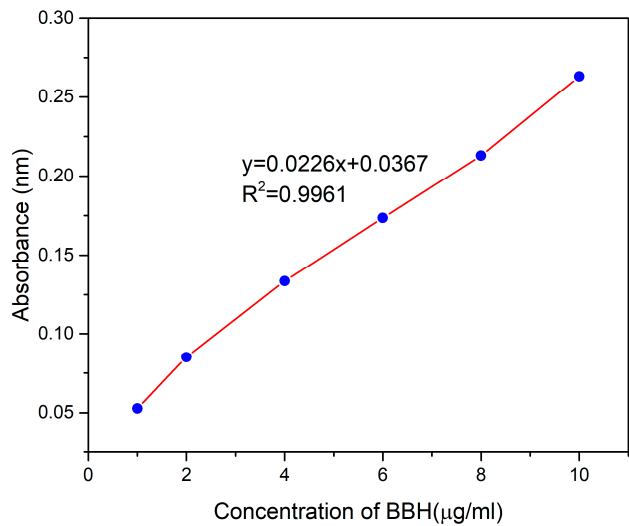


Figure A2. Phase solubilty curve of BBH in aqueous solution.

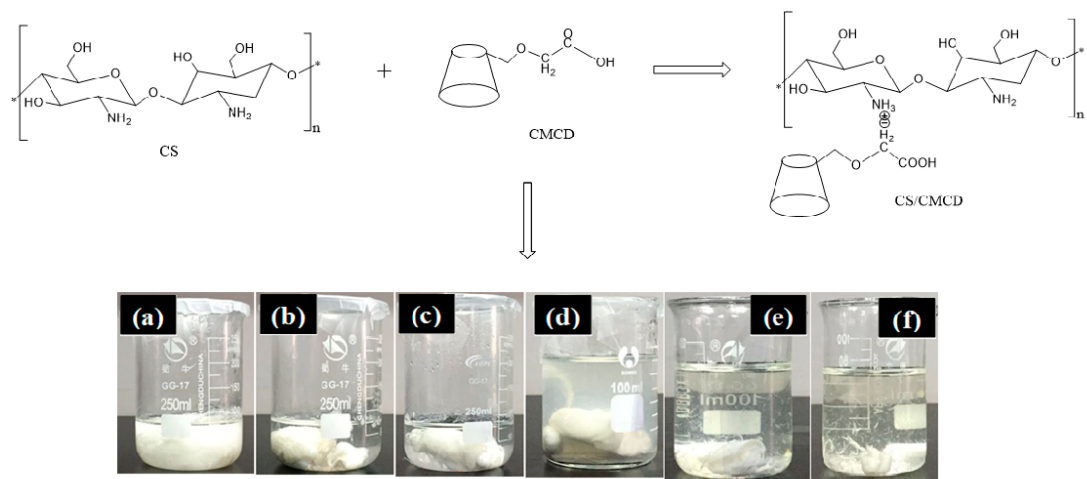


Figure A3. CS/CMCD composite hydrogel with different formations of CMCD: a) 1% CS_1%CMCD, b) 1% CS_2%CMCD, c) 1% CS_4%CMCD, d) 1% CS_6%CMCD, e) 1% CS_8%CMCD, f) 1% CS_10%CMCD respectively.

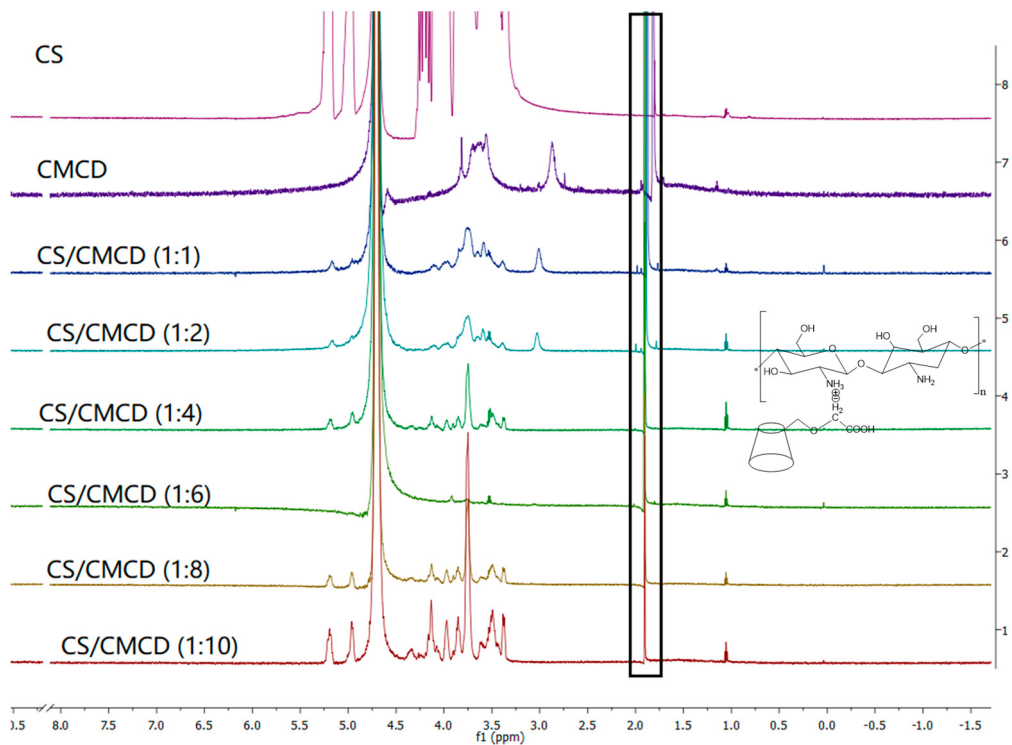


Figure A4. ¹H NMR spectra of the BBH and CS/CMCD/BBH (1%CS_6%CMCD_1%BBH) hydrogel.

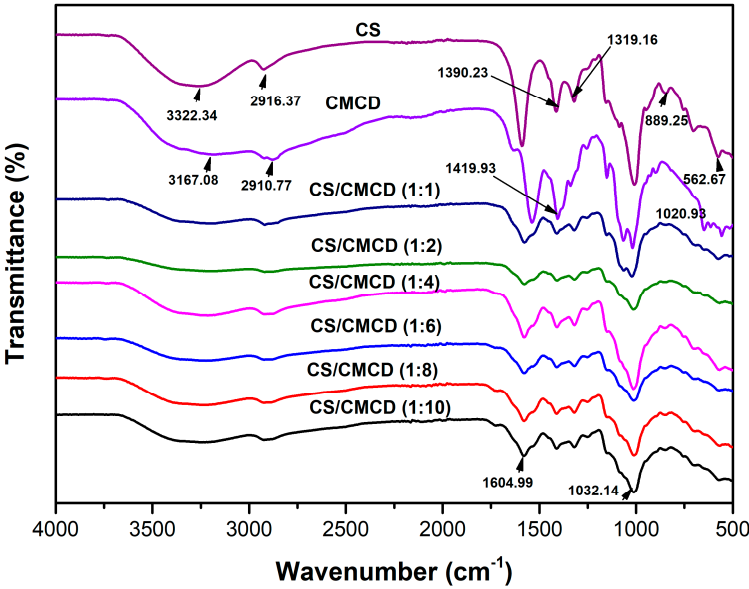


Figure A5. FTIR image of CS, CMCD and CS/CMCD hydrogel with different fromulations of CS/CMCD.

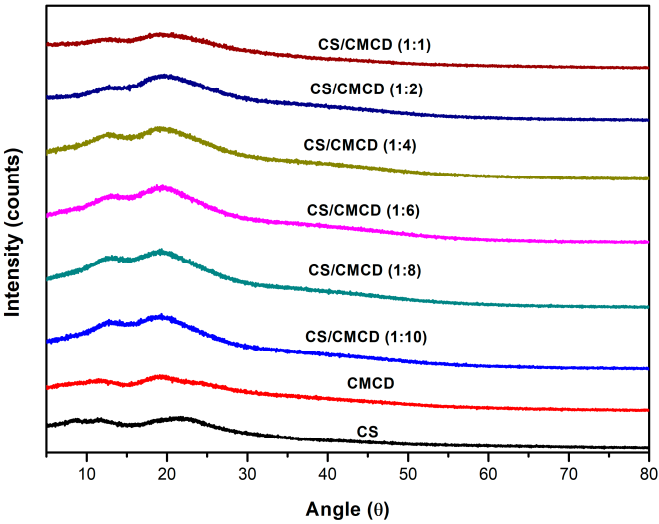


Figure A6. XRD image of CS, CMCD and CS/CMCD hydrogel with different formulations of CMCD.

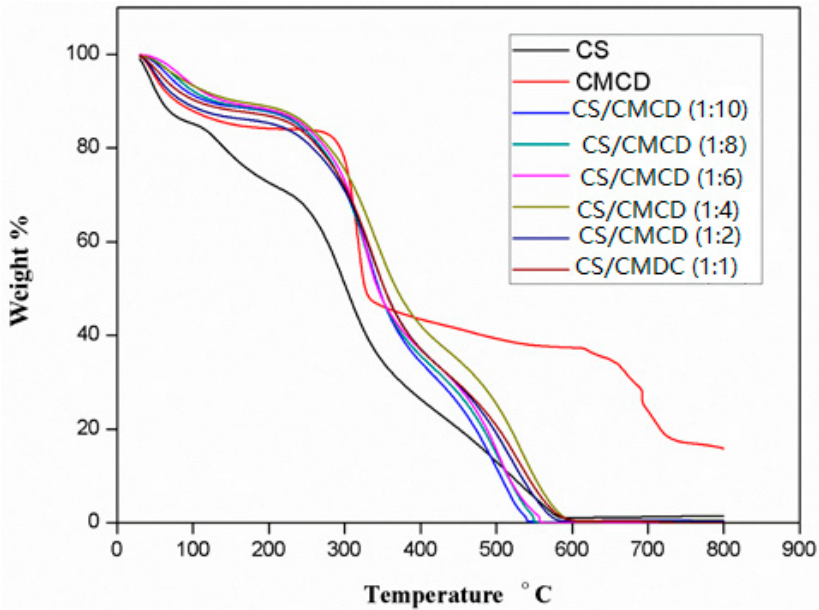


Figure A7. TGA image of CS, CMCD and CS/CMCD with different formulation of CS/CMCD.

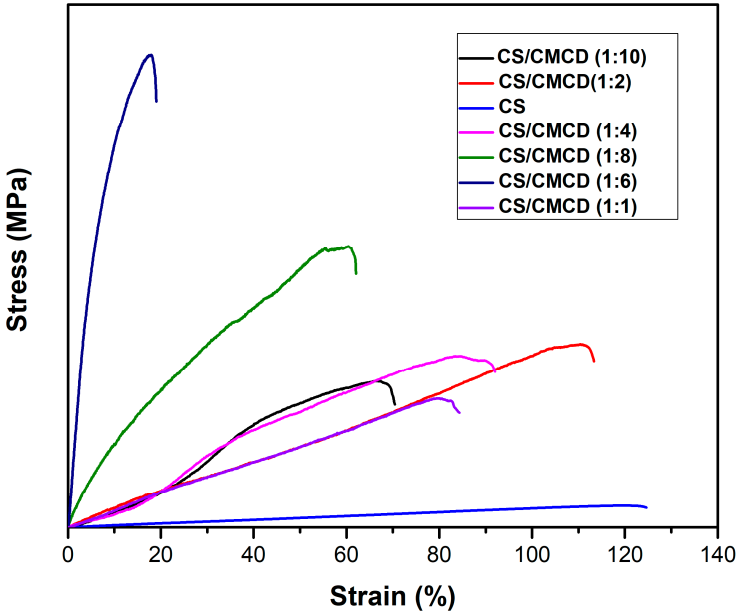


Figure A8. Tensile image of CS, CMCD and CS/CMCD hydrogel with different formulations of CMCD.

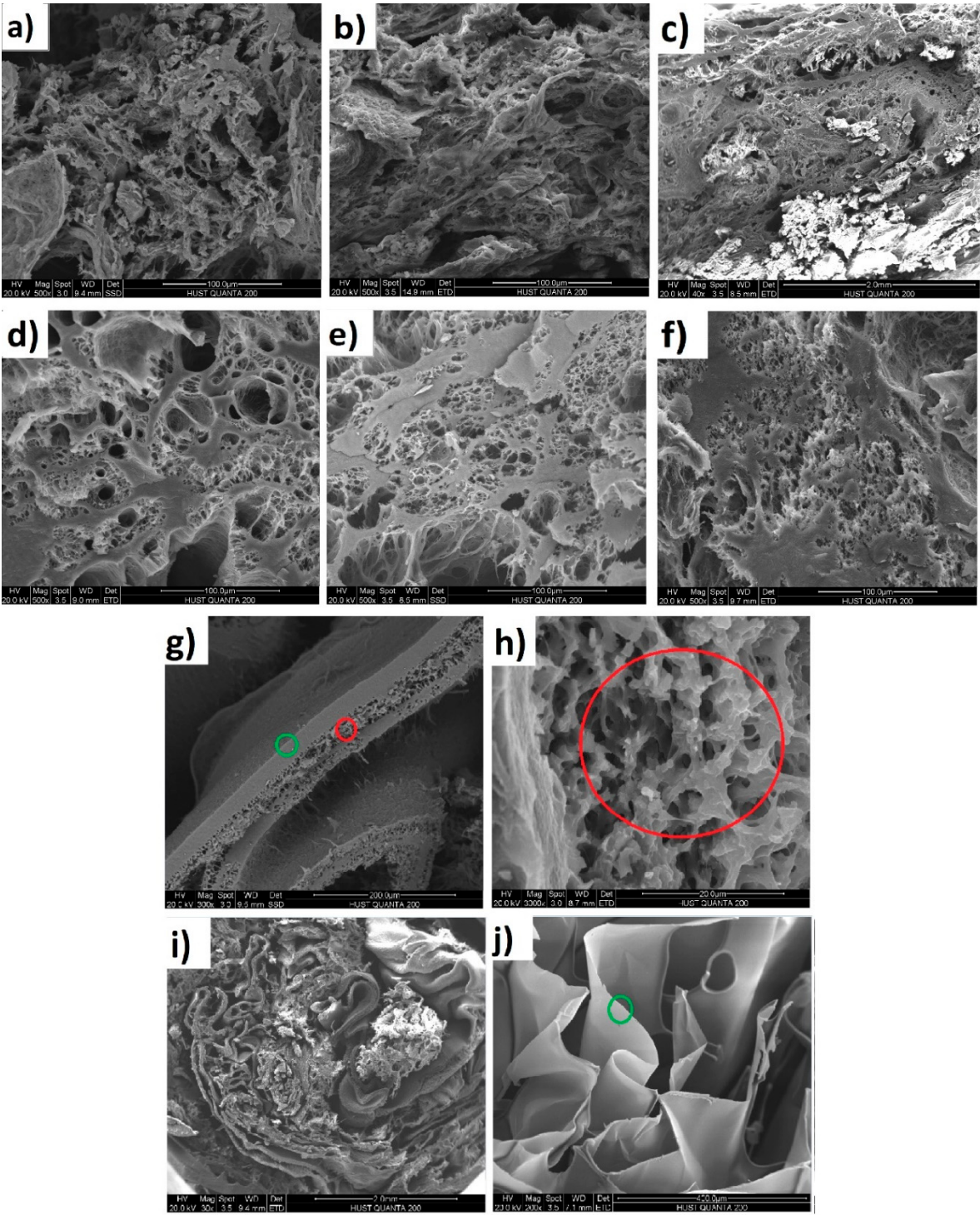


Figure A9. SEM image of of CS/CMCD with different formulation of CMCD: a) 1% CS_1%CMCD, b) 1% CS_2%CMCD, c) 1% CS_4%CMCD, d) 1% CS_6%CMCD, e) 1% CS_8%CMCD, f) 1% CS_10%CMCD, g, h, i and j image illustrating the croslliking reaction of CS/CMCd interaction, scale bare equal to 100μm, 50μm and 400μm.

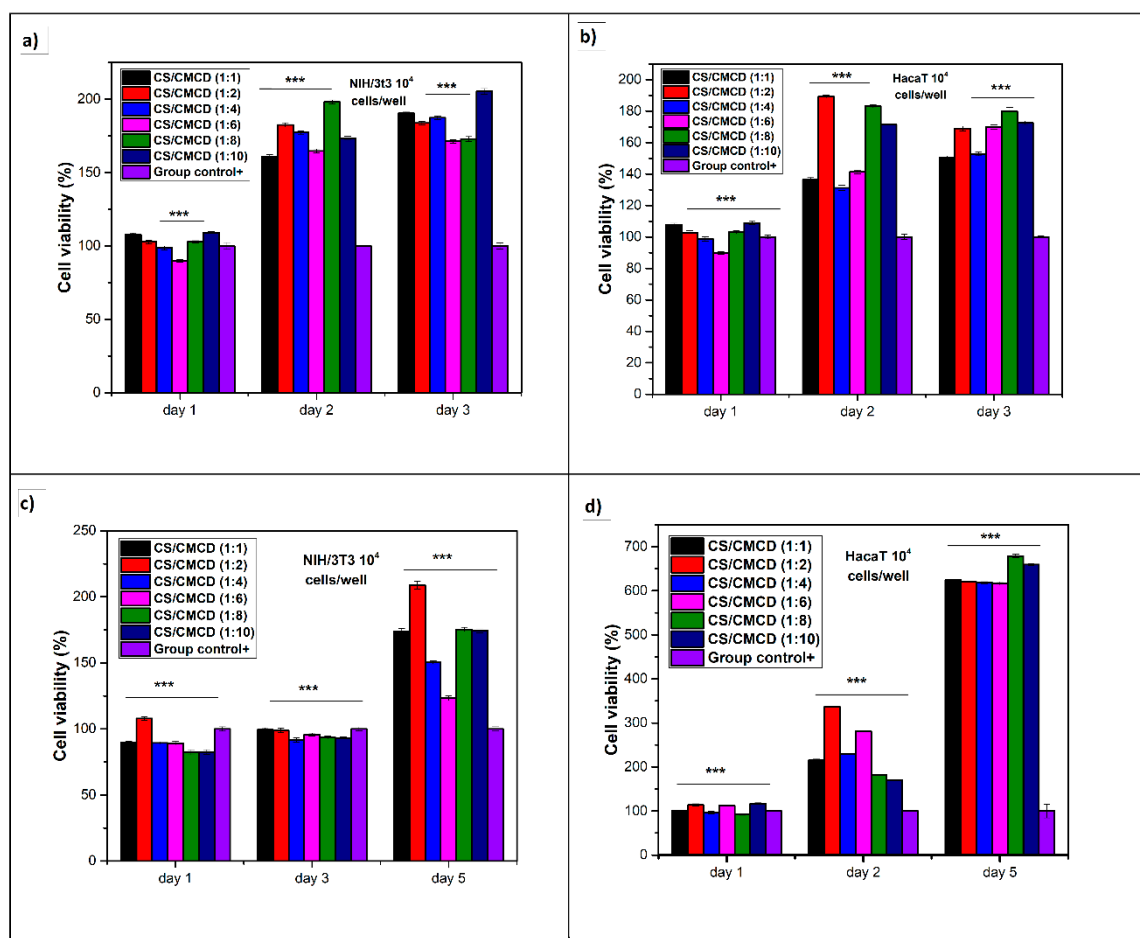


Figure A10. Cytotoxicity CS/CMCD samples performing on NIH3T3 and HacaT cell a,b) through solvent extraction method, c,d) direct contact with samples.

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