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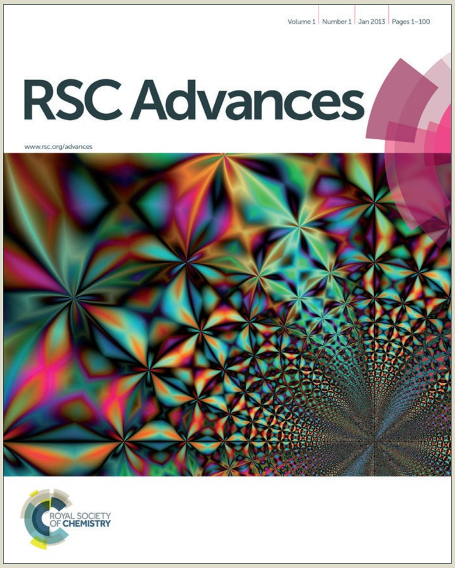

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ARTICLE TYPE

# In Situ Crosslinkable Hydrogels Formed from Modified Starch and O-Carboxymethyl Chitosan

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Abstract

In situ hydrogel based on oxidation cholesterol starch (OCS) and O-carboxymethyl chitosan (CMCT), which completely devoid of potentially cytotoxic small molecule cross-linkers and that do not require complex maneuvers or catalysis, have been formulated and characterized. The network structure was created by Schiff base formations. The mechanical properties, internal morphology and swelling of injectable hydrogel were examined. Rheological measurements demonstrated that increasing the concentration of the monomer improved the storage modulus. SEM showed the hydrogel possessed a well-defined porous structure. And the Schiff base reaction was acid sensitive. At acid conditions, the hydrogel could hydrolysis quickly compared with the high pH conditions. Doxorubicin (DOX) was used as model drug to investigate the control and release property of hydrogel. The cytotoxicity potential of the hydrogel was determined by an in vitro viability assay using L929 cell as a model and the results revealed that the hydrogel was noncytotoxic.

## Introduction

Cancers, as a group account for approximately 13% of all deaths each year had attracted more and more attention[1]. The death rates have been increasing primarily due to an aging population and lifestyle changes in the developing world[2]. The common pharmaceutical to curing the tumor were Doxorubicin[3-5], Dactinomycin[6-8] and Daunorubicin[9-11]. However, due to the toxicity and side effects of these drugs for normal tissue and cells[12-15], develop suitable transport to transmit it to the right place was necessary. So many kinds of drug-carriers were arising to fit the requirement[16-18]. The typical carriers for the anti-tumour drug were nanoparticles[19, 20], nanogel[21, 22], micelles[23, 24] and hydrogels[25, 26]. Although those transports contains well performance, the low loading capacity of the nano-base particles and the loss in biological cycle process limited its application. Meanwhile, the three dimensional structure of the hydrogels made it non-avoidable carry out a surgery for the people with solid tumour. The appearance of the in situ hydrogels opens a new way to solve that problems[27].

Injectable hydrogels is based on the ideal that certain biomaterial can be injected as a liquid, and then in situ form a solid gel[28]. Injectable hydrogels are of particular interest because the drugs, proteins, and cells can be easily incorporated into polymer solutions prior to administration and the hydrogels formed in the situation of wounded tissues[29]. The drugs, proteins or cells can be easily diffuse around tumor. Therefore, injectable hydrogels have vast application prospect in drug/cell delivery and tissue engineering[30]. In consideration of the biomedical application of injectable hydrogel, the natural polymers are favour material to construct such hydrogel compared with the synthetic polymer due to their biocompatibility and biodegradability. Those polymers included starch, chitosan, hyaluronic acid, cellulose, xanthan gum, gelatin and so on. Among them, starch, the cheapest and entirely biodegradable biopolymer, has been used to fabricate hydrogel materials for biomedical applications[31]. Chitosan is another biopolymers which is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). The amino group in chitosan has a $pK_a$ value of ~6.5, which leads to a protonation in acidic to neutral solution with a charge density dependent on pH and the degree of deacetylated. It has been widely utilized in gene therapy[32], cell regulation[33], and tissue regeneration[34].

Generally, in situ hydrogels can be synthesized via physical or chemical approach. Compare to physical pattern, the chemical approach may produce hydrogel with stable covalent bond. The typical in situ chemical approach includes Schiff base reactions[35], Michael addition reactions[36], ionic interactions[37] or photo-crosslinking reactions[38]. Among those approaches, Schiff bases are imines formed by the condensation of aldehyedes or ketones with primary amines. It is degradable via hydrolysis actions, and the stability of these bonds decreases as the pH decreasing[39]. The reversibility dynamic nature of the Schiff base makes it utilized in a wide range of preparing in situ hydrogels with integrated properties including pH-sensitivity[40], self-healing[41], biocompatibility and degradation[42].

Recently, we have reported a novel kind of injectable hydrogels which were synthesized by core-shell starch nanoparticles with aldehyde and poly(vinyl amine) (PVAM) with primary amines via a Schiff base reaction. The starch nanoparticels were prepared with addition of cholesterol group and aldehyde groups which would assemble to form core-shell structures[43]. Compared to linear starch with aldehyde groups, the introduction of the hydrophobic cholesterol group offered the hydrogel enhanced mechanical properties. However, the high-cost and non-natural property of the PVAM may be a drawback of such hydrogels as applied in the biomedical fields. Herein, new injectable hydrogels composed of O-carboxymethyl chitosan and core-shell starch nanoparticles were also synthesized via Schiff base reaction. The hydrogels' mechanical properties, swell properties are investigated in details. Moreover, Doxorubicin hydrochloride, as a model drug, was used to evaluate the controlled-release properties of hydrogel. In addition, the 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay was also conducted to investigate its cytotoxic activity against L929 cell lines. The combination of in situ gelation, feasible mechanical properties, good water uptake ability, well controlled release behavior and excellent biocompatibility make the polysaccharide based hydrogels apply in various biomedical fields.

## 2. Experiment

### 2.1. Materials

Cholesterol modified oxidation starch (OCS) was obtained according to a previously published method[43] (The degree of the cholesterol substitution was 0.01; the degree of oxidation was 46%). Chitosan (with a degree of deacetylation of 93%) and chloroacetic acid (98%) were purchased from Aladdin Chemistry Co, Ltd. Doxorubicin hydrochloride (DOX) was purchased from Beijing Zhongshuo Pharmaceutical Technology Development Co, Ltd. All the other chemicals were obtained from Beijing Chemical Factory and used as received.

### 2.2. Synthesis of O-carboxymethyl chitosan (CMCT)

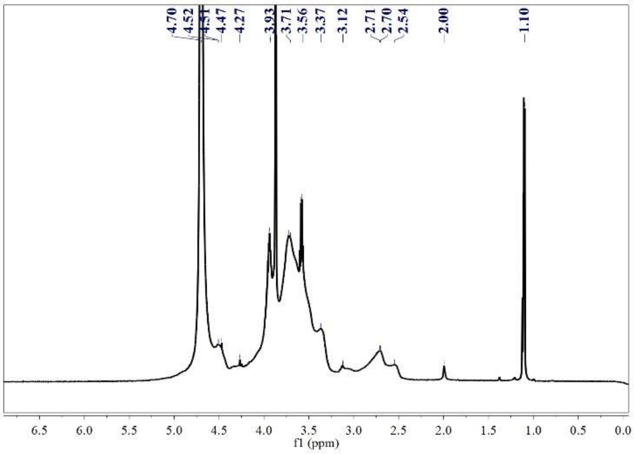


**Fig.1** The $^1$H-NMR of the CMCT in $D_2O$.

CMCT was synthesized as per a procedure described in the literature[44]. Chitosan powder (10 g) was suspended in 100 ml of isopropyl alcohol and the resulting slurry was stirred in a 500-ml flask at room temperature. A 25 ml of 10 N aqueous NaOH solutions, divided into five equal portions, was then added to the stirred slurry over a period of 25 min. The alkaline slurry was stirred for additional 30 min. Subsequently, chloroacetic acid (60 g) was added in five equal portions, at 1 min intervals. Heat was then applied to bring the reaction mixture to a temperature of 60℃ and stirring at this temperature was continued for 3 h. Afterward, the reaction mixture was filtered and the filtered solid product (CMCT) was thoroughly rinsed with methanol. The resultant CMCT was dried in an oven at 45℃.

The degree of substitution of carboxymethyl groups on 6-O- and 3-O- can be determined by the ratio of peak areas of the carbonxymethyl group (($O$-$CH_2COOD$) 4.40-4.05ppm) and the -$CH_3$ group (($CH_3CO$-) 2.0ppm) from the $^1$H-NMR spectra in Fig. 1 and it was 0.60.

### 2.3. Preparation of hydrogel.

CMCT (50mg) and OCS (50mg) were firstly dissolved in water (1ml) to form 5% (w/w) solutions respectively (for CMCT solution, it was necessary to control the concentration to 5%. If >5%, the viscosity of the solution was high. It was not easy to inject for syringe) and stored at 5℃. The hydrogel was prepared by mixing of CMCT and OCS solutions with equal volume ratio using syringe with a "T" structure. Finally, the mixture was kept at 37℃ for completely gelation.

### 2.4. Determination of Gelation Time

The gelation times of all the hydrogels were estimated by a vialtilting method. The concentration of the CMCT was constant with 5%(w/w), and the concentrations of the OCS were 2.5%(w/w), 5.0%(w/w), 7.5%(w/w) and 10%(w/w) respectively. All the solutions were keeping in 37℃ for 1h sealed with parafilm before the test. The testing was started immediately after mixing two components until no flow was observed for at least 30s when a vial containing the hydrogel was inverted at 37℃[45].

### 2.5. Characterization

$^1$H-NMR spectra were recorded using a Bruker AV400 spectrometer (Germany) operating at room temperature. And the sample was dissolved in $D_2O$. FTIR characterization was performed on Perkin-Elmer Spectrum 100 spectrometer (USA), and the data were collected *via* 32 scans with a resolution of 2 $cm^{-1}$ at room temperature. The lyophilized hydrogel samples were characterized by scanning electron microscopy (SEM) with the accelerating voltage 5.0kv using a Model XL 30 ESEM (Philips).

### 2.6. Rheological Measurements

To investigate the mechanical property of the resultant hydrogels, the dynamic frequency sweep tests were conducted on a US 302 rheometer (Anton Paar) in linear viscoelastic region. The frequency applied to the hydrogel sample increased from 0.1 to 100 rad/s. The data were collected under a controlled strain $\gamma$ 1%. $G'$ is an elastic component of the complex modulus for measure of the gel-like behavior of a system, whereas $G''$ is a viscous component of the complex modulus and is a measure of the sol-like behavior of the system.

### 2.7. Swelling experiments

The hydrogel samples were immersed into various PBS solutions and distill water at 37 ℃. The swelling degree (SW) was determined by the equation as follow;

$$SW = \frac{M_t - M_0}{M_0} \times 100\%$$

Where $M_0$ represents the weight of the dry hydrogel, $M_t$ represents the weights of the hydrogel at equilibration time. All the experiments were carried out with three samples and the reported data were average values.

### 2.8. Release of DOX from hydrogel

For DOX release experiment, DOX (2.0, 1.0 and 0.5 mg/ml) were dissolved in OCS solution in a phosphate-buffered saline (PBS, pH = 7.4, 5.0 and 3.0) and then reacted with CMCT to form DOX-loaded hydrogel. Freeze-dried hydrogel samples were immersed into PBS solution, sealed in a dialysis bag (*Mw* cutoff: 3.5 kDa) and incubated in the release medium (25 mL) at 37℃ under oscillation at 45 rpm. At selected time intervals, buffer solution outside the dialysis bag was removed for UV-Vis analysis and then replaced with fresh buffer solution. The released amount of DOX was determined from the absorbance at 480 nm with the help of a calibration curve of DOX in the same buffer. Then the accumulative weight and relative percentage of the released DOX were calculated as a function of incubation time.

### 2.9. Cell lines

L929 (mouse fibroblasts cells) was chosen for cell tests. L929 was supplied by the Medical Department of Jilin University, China. L929 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplied with 10% heat-inactivated fetal bovine serum (FBS, GIBCO), 2mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma), and the culture medium was replaced once very day.

### 2.10. In vitro Cytotoxicity of Supramolecular Hydrogel

L929 cells were used to evaluate the biocompatibility of the hydrogel by MTT assay. The cells were seeded in 96-well plate at a density of 10,000 cells/well in 100μL of DMEM. Then CMCT, OCS and CMCT/OCS solutions were added to the wells with mixing. Three parallel wells for each sample were used at a specific concentration. After co-incubation with L929 cells for 24h, 48h and 72h, 20 μL of MTT solution in PBS (5 mg/mL) was added to each well and the plate was incubated for another 4 h at 37 ℃. After that, the medium containing MTT was removed and 150 μL of DMSO was added to each well to dissolve MTT formazan crystals. Finally, the plates were shaken for 10 min, and the absorbance of formazan product was measured at 492 nm by a microplate reader.

## 3. Results

### 3.1. Synthesis of CMCT/OCS hydrogel

In our process, the carboxymethyl groups were introduced into the backbone of the chitosan to improve the hydrophilic property in physiological conditions. The O-carboxymethyl chitosan (CMCT) was synthesized successfully through the etherification between chitosan and chloroacetic acid. The degree of substitution of carboxymethyl groups was 0.60 determined by $^1$H-NMR spectra.

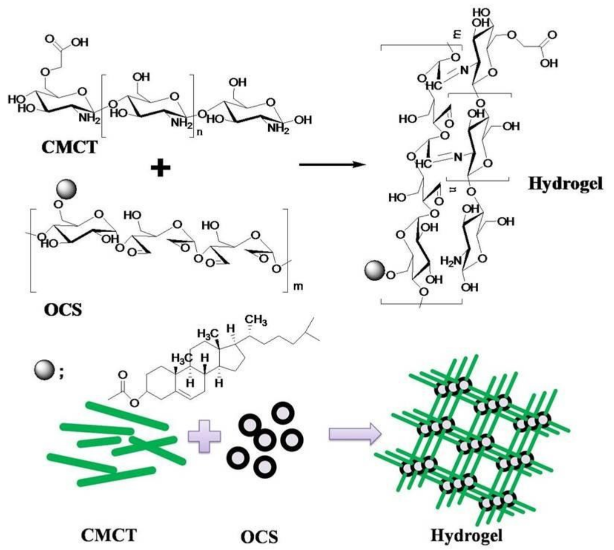


**Fig. 2** The schematic description of hydrogel formation process.

**Table 1** the characteristics of hydrogels formed with different weight ratio between CMCT solutions and OCS solutions at 37℃. The gel represents hydrogel.

| Samples | CMCT/OCS (w%/w%) | $t_{gels}$(s) | Products |
|---|---|---|---|
| 1 | 5.0/2.5 | 500 | gel |
| 2 | 5.0/5.0 | 210 | gel |
| 3 | 5.0/7.5 | 90 | gel |
| 4 | 5.0/10 | 30 | gel |

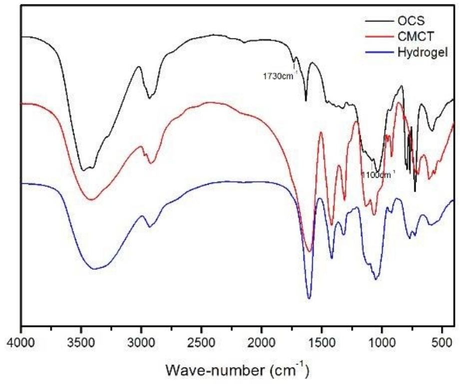


**Fig. 3** The FTIR of the samples for OCS, CMCT and hydrogel.

Meanwhile, starch was successively decorated with cholesterol group and aldehyde groups as described previously. This modification sustains a certain hydrophilic–hydrophobic balance for starch macromolecules so that nanoparticles with well-defined core–shell structure were obtained as shown in Fig. 2. The core-shell structure offered the starch hydrogel to enhance mechanical properties and water absorbability. The degree of substitution of cholesterol groups (DS, i.e. the number of cholesterol groups per glucopyranose units) used in this study was 0.01. The degree of oxidation of the starch was 46%.

After injecting the CMCT and OCS solution out of the syringe,

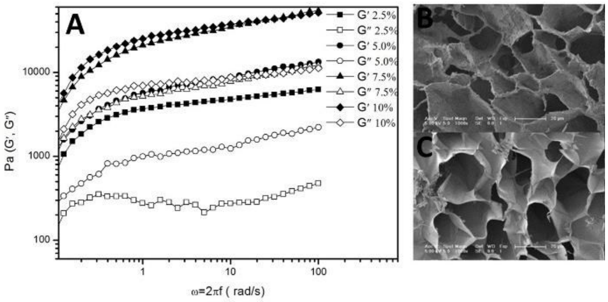


**Fig. 4** A: The modulus of CMCT/OCS based hydrogel. The concentration of the CMCT solution before mixed was 5% (w/w) in all the samples. The concentration of the OCS solution were varies. B: The morphology of the hydrogel with 5/5 % (w/w) before swelling and C: after swelling.

the hydrogel could be formed in situ cross-linked via Schiff base reactions within mins (Table 1). The ability of pH response for the Schiff base reaction offered the hydrogels with reversibility and degradability that makes it a suitable material to utilize in the field of control and release[46].

The structure of hydrogels was confirmed by FTIR characterization. As shown in Fig. 3, the peak of aldehyde groups in the wave-number 1730$cm^{-1}$ was disappear after reaction. At the same time, the intensity of the hemiacetals peak in the wave-number 1100$cm^{-1}$ was decreased and become a shoulder after reactions. Those demonstrated that the consumption of aldehydes most likely by a reaction with amines. However, the peak of the imines (1665$cm^{-1}$) wasn't detectable which might have been masked by the multiple absorbance peaks at this wave-number[47]. Indeed, the $–NH_2$ groups of CMCT and water adsorbed on the polysaccharide in this area show broad and strong peaks at this wave-number.

### 3.2. Rheological analysis

For the in situ formed hydrogel, the rheological analysis was a powerful and more accurate method to investigate the viscoelastic property of the hydrogels[48]. Frequency sweep experiments were performed after 24h when the gel formed with the protection of parafilm in 37℃.As shown in Fig. 4A, with the concentration of the OCS solution increasing, the storage modulus (G′) of the hydrogel was increasing. Meanwhile, when the concentration of the OCS reached 7.5% (w/w), the modulus got the maximum. After that, there was no significant change for the storage modulus if the content of the OCS was increasing from 7.5% (w/w) to 10% (w/w). It was worthy noted that as had been calculated form the experiment parts, the oxidation degree of the OCS was about 46% and the degree of the substitution of the carboxymethyl groups in CMCT was 0.6. So the aldehyde groups and the amine groups in this mass ratio didn't show equal molar ratio. But the data demonstrated the hydrogel at this mass ratio exhibited the biggest storage modulus. This phenomenon could be explained as follows: there was exist a balance between the aldehyde groups and the hemiacetals groups. When the Schiff base reaction was occurring, not all of the hemiacetals groups were reacted with amine groups while the aldehyde groups was all consumed as had been confirmed by the FTIR in Fig. 3.

Next, we take the sample 2 to analyse the relation between rheology performance and its network structure with considering the anti-infection property of the chitosan. For the sample 2 in Fig. 4A, a typical hydrogel character was exhibiting. Both the storage modulus and the loss modulus were increasing slightly with the frequency increasing. It means the hydrogel form a well-defined pore structure[49]. In order to confirm the hypothesis, SEM was adopted to observe the morphology of the dried hydrogel. As had been show in Fig. 4B, the dried hydrogel show regular pore dispersion with a 20μm pore diameter. In order to well understand the inter structure of the hydrogel and eliminate the interference of residual monomers, the morphology of the hydrogel after swelling in distilled water was also observed. As had been show in Fig. 4C, the pore diameter was became large and the pore dispersion was still well-defined. That demonstrated a well-defined cross-linking network was formed in the hydrogel[50].

### 3.3. Swelling analysis

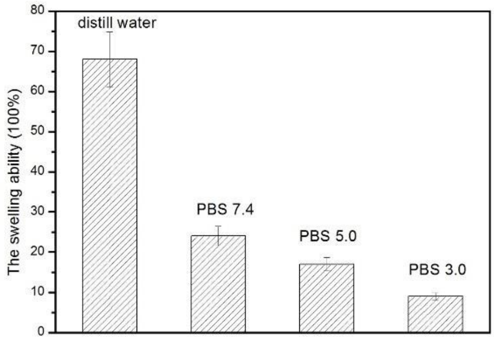


**Fig. 5** Water absorption capacity of the hydrogel (sample 2) in various PBS solutions and distilled water.

The swelling behavior of hydrogel played an important role for its practice application. Fig. 5 present the swelling property of CMCT/OCS hydrogel in distill water and various PBS solutions. With the pH increasing from 3.0 to 7.4, the swelling degree was increasing from 8.5 to 23 accordingly. In this process, the osmotic pressure played an important role to affect the swelling degree of the hydrogel. The volume of the hydrogel would be changed if the concentration gradient exist, and the bigger the concentration gradient, the more obvious the volume change. So the hydrogel in water exhibited the highest swelling degree compared with it in PBS solutions. Moreover, due to the electrostatic interaction among the chains, the pH value exhibited significant role to regulate the change of the hydrogels' volume[43]. At low pH value, the gel which containing amounts of unreacted amino groups got shrank in the presence of a large number of cations along with declining the water capability. When the pH value increasing, the gel got swelling due to the reduce of the cations in solutions. It is also worthy noted that the hydrolysis of the imine groups in the hydrogel might affect the swelling degree in the acid environment which lead to the decline of the swelling degree.

### 3.4. In vitro release of the DOX

Hydrogel, which was very similar to the biological tissue, was a

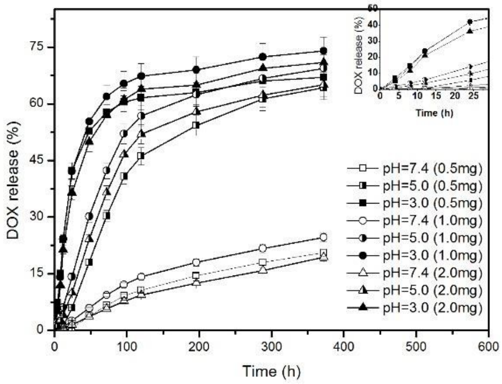


**Fig. 6** The release behavior of the hydrogel (sample 2) with different buffers. Insert: the DOX release at first 24h.

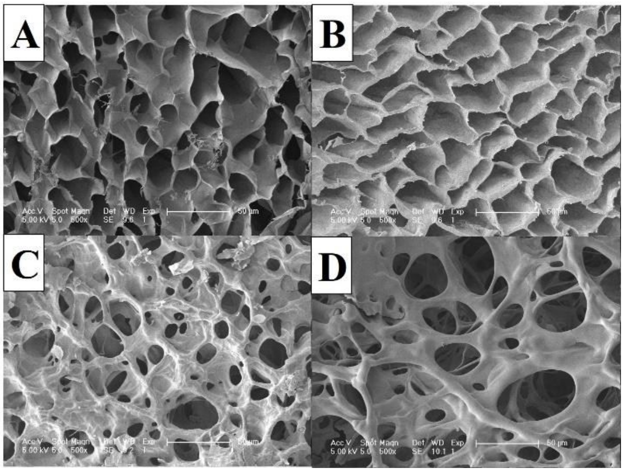
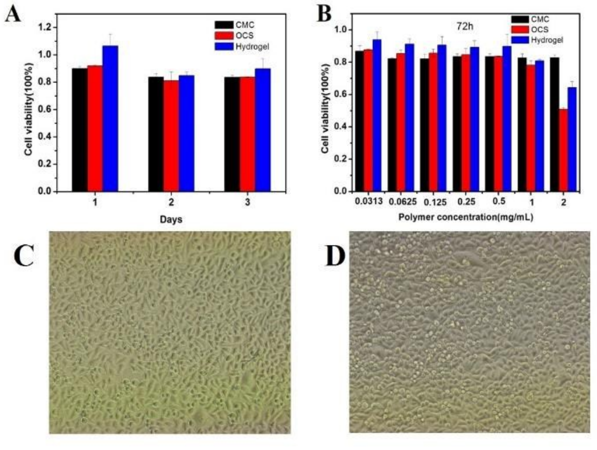


**Fig. 7** the morphology of the hydrogel after immerged into various solutions for 24h. A, distill water; B, PBS with pH=7.4; C, PBS with pH=5.0 and D, PBS with pH=3.0.

useful tool to transmit the target drug to the right place[51]. Here, DOX was used as model drug to investigate the control and release properties of the hydrogel. As was shown in Fig. 6 and the insert picture, there was no dramatic initial burst release for drug-loaded hydrogel at different pH conditions. And the hydrogel present a pH-sensitive released profiles. The lower the pH value, the faster the drug was released. The hydrogel showed sustained release of DOX at pH=7.4, not more than 30% of DOX was released in 15 days. In the same time period, about 70% of DOX was released at pH=5.0 and pH=3.0. We attributed it to the hydrolysis of the Schiff base reaction[52]. The imine group was fragile at acid condition and the stability of these bonds decrease as the pH decrease. In order to confirm the hypotheses, the SEM was be used as a power tool to investigate the morphology of the hydrogel during the hydrolysis process. As had been seem in Fig. 7, the hydrogel was regular multi-pore structure before exposed to various PBS solutions. When the hydrogels immersed into the PBS solution with pH=7.4 for 24h, the pore diameter and dispersion did not exhibited obvious change and the pores were bridged each other with wall. However, when the hydrogel was immerged into the PBS with pH=5.0, there was appearing vast different on the morphology compared with the morphology of the hydrogel immerged into the PBS (7.4) and distill water. The pores was changing irregular for each other and the bridged wall was corroded. Moreover, the erosion was more obvious for the hydrogel immerged into pH=3.0. It demonstrated further that the imine groups was more easily hydrolysis in acid condition. So the behaviours of the control and release could be explained as follow: DOX was restricted in the passageway of the gel and was diffused to the outside through one-dimensional direction in PBS=7.4. With the pH declining, the imine groups was hydrolysis and the networks were corroded. So the barrier of the pores were disappearing, resulting to the fast release of DOX in acid environment.

Moreover, the varieties doses of the DOX were also studied for the control and release. Although the doses of the DOX was different, the behaviour of release for the DOX was similar in various PBS solution. It demonstrates the control and release behavior of the DOX in hydrogel was independence of the concentration of the DOX. The uniform dispersion of the pore and the hydrolysis of imine group in solution might play key role during those process. Therefore, the drug-loaded hydrogel had potential for biomedical applications, since tumor tissues were known to be acidic.

### 3.5. Cell Cytotoxicity



control 1mg/mL

**Fig. 8** A: The cell viability of the L929 cell inside of the hydrogel after 3 days for the concentration of CMC and OCS were 0.5mg/mL respectively. B: The cell viability of L929 cell in varies concentration of the polymer after 3 days. C: the cells morphologies in saline after 72h for control. D: the cells morphologies in hydrogel, where the concentration of CMC and OCS were 1mg/mL after 72h.

The cytotoxicity potential, as reflected by the viability of cells encapsulated in the hydrogels, was evaluated by MTT assays. Cells were grown in the presence of 200μL cylindrical CMCT/OCS gels, for up to 3 days with media changes every 24 hours. Cell viability was examined at 1, 2 and 3 days and some of the results were depicted in Fig. 8A. No significant difference was found between the cells incubated with hydrogel and the controls, suggesting that the hydrogel did not have any adverse effect on cell growth; this could be inferred as material noncytotoxicity.

In order to further confirm the noncytotoxicity of the hydrogels, we take the concentration as a factor to evaluate the cell viability. Fig. 8B shows that all CMCT and OCS solutions and hydrogels induced only minimum decreases in relative cell viability over the concentration range from 0.0313 to 1 mg/mL after 3days of cell exposure. The relative viability of L929 cells was 0.85±0.05 for 1 mg/mL CMCT solutions, 0.86±0.04 for 1mg/mL OCS solutions and 0.92±0.05 for the represent hydrogel, indicating that none of the hydrogel exhibited significant cytotoxicity. Moreover, we also observed cells morphologies at optical microscope with the concentration of the OCS and CMCT were 1mg/mL respectively in Fig. 8C and D. If we increased the OCS concentration from 1mg/mL to 2mg/mL, the relative viability of L929 cells was decreasing due the toxicity of the aldehyde groups.

All in all, the hydrogel may have potential for biomedical applications, although more thorough testing with human cell lines, as well as in vivo testing, would be required to confirm this potential.

## 4. Conclusions

We demonstrated a method to synthesize in situ hydrogel based on natural polysaccharides derivates. The starch-based nanoparticles and the modified chitosan in situ cross-linked to form hydrogels via Schiff base reaction. The dried hydrogel have well dispersion pore structure and the swelling ratio in PBS solutions was increasing with pH increasing. Moreover, we used DOX as a model drug to simulate the controlled-release properties of the hydrogel. The result showed that the DOX-loaded hydrogels prefer to release in an acid environment. And the curve of the release wasn't relevant to the loaded-amount of the drug. In further, the cytotoxicity potential of the hydrogels was determined by an in vitro viability assay using L929 cell as a model cell, and the results reveal that the hydrogels are noncytotoxic. The hydrogels would be a promising drug carrier for biomedical applications.

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## Notes and references

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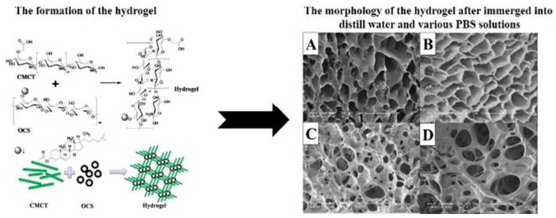

The degradable behavior of the hydrogel in various pH condition was observed by SEM.