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– Supplementary Data –

# 3D printing-mediated microporous starch hydrogels for wound hemostasis

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## Materials and methods

### Main experimental materials

Food-grade native maize starch (NMS) was purchased from Qinghuangdao Pengfei Starch Co., Ltd. (China). Sodium Phosphate, citric acid and hydrochloric acid, all analytically pure, were supplied by Guangzhou Chemical Reagent Factory. Sodium hydroxide, absolute ethanol, trihydrate sodium acetate, and acetic acid, all analytically pure, were supplied by Tianjin Baishi Chemical Co., Ltd., (China). 2,2,6,6-Tetramethyl-1-piperinedinyloxy (TEMPO), laccase and 8-amino-1,3,6-pyrenetrisulfonic acid were obtained from Sigma Company (USA). Cell proliferation kit (methyl thiazolyl diphenyl tetrazolium bromide, MTT), fibronectin (FN) ELISA Kit, tumor necrosis factor (TNF-α) ELISA Kit, epidermal growth factor (EGF) ELISA Kit and vascular endothelial growth factor (VEGF) ELISA Kit were purchased from Sigma Company (USA). EDTA antigen retrieval solution, VEGF antibody, EGF antibody were provided by Annoron Medical Device Co. (China). All other chemicals and reagents were of analytical grade.

### Preparation of OMS

The protocol for the preparation of OMS was adapted from previously reported methods [1]. Firstly, 5g NMS powder was dispersed in 100 ml of 0.1 M citrate phosphate buffer of pH 5. Then TEMPO (15% w of the NMS) and laccase (6% w of the NMS) were added to the solution for 8 h at room temperature under stirring at 200 rpm and oxygen bubbling. After reaction, the product was washed with water and ethanol, then

dried at 45 °C and ground in a mortar to obtain OMS. The analysis of OMS was showed in supporting information.

**Cytocompatibility**

"NIH 3T3 cells were cultured with DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FCS (fetal calf serum) at 37 °C at 96-well plate ($5 \times 10^4$ cells per well) in a humidified incubator containing 5% $CO_2$ atmosphere for 24 h. After removing the medium, 300 uL of DMEM with different concentrations of 3D-OMS and CaOMS with 0-800 μg/ml was added to 96 well plate and cultured for 48 h. After incubation, the medium in each well was replaced with 180 μL of DMEM and 20 μL of MTT (5 mg/mL) solution and cultured for 4 h for MTT testing. Finally, the culture medium was removed, dimethyl sulfoxide (150 μL) was added to each well. Cell viability (%) was estimated by the optical density values at 490 nm using the resulting formula of (Eq. (8)).

$$\text{Cell viability (\%)} = \frac{A_{sample490}}{A_{blank490}} \times 100\%\#\ (1)$$

Where $A_{sample490}$ is the absorbance of 3D-OMS and $A_{blank490}$ is the absorbance of the untreated cells as blank sample at 490 nm.

***In vitro* coagulation**

Briefly, 10-40 mg of the sample (dry basis) powder was weighed into a centrifuge tube and then mixed with 1 mL of mouse blood containing anticoagulant. The concentration of each sample was 10, 15, 20, 25, 30, 35, 40 mg/mL. The centrifuge tubes were inverted every 1 minute and the time required for the blood to clot was recorded. The coagulation effect was further evaluated by optical densitometry. 3 mL

of deionized water at 5, 10, 15, and 20 min slowly added after adding the anticoagulant, and shacked gently 3 times. Optical density values (OD) were determined at 540 nm after standing for 5 minutes.

**ELISA test**

Briefly, add 100 μL of ELISA protein standard (RABRTNFAS, RAB0150C, RABVEGFFS and RAB1011C for TNF-α, EGF, VEGF, FN, respectively) and test samples to a 96-well plate containing rat ELISA antibody (RABRTNFAAEA, RAB0150A-EA, RABMVEGFAEA and RAB1011A-1EA for TNF-α, EGF, VEGF, FN, respectively) for 2 h at 25°C, the plates were washed 4 times with 300 ul washing buffer (RABWASH4), and 100 μl RABRTNFAF was added to each well. The plates were then incubated at 25°C for 1 h. After washing 4 times with RABWASH4 again, 100 μl of 1 μg/ml biotinylated (RABHRP5, RAB0150D, RABMVEGFF and RAB1011F for TNF-α, EGF, VEGF, FN, respectively) in blocking buffer was added to each well and incubated at 25°C for another 45 min. Following another 4 washes with RABWASH4, the RABTMB3 (100 μl/well; Sigma-Aldrich) was added to each well and incubated for 30 min at 25°C for color development. Finally, 50 μL of reaction stop solution (RABSTOP3) was added to each well. Optical density (OD) values of different targeted molecules were recorded with a microplate reader at a wavelength of 450 nm. The concentration of each protein was estimated from the standard curve determined by serial dilution.

**Result and disscusion**

The expression level of several cytokines may provide wound healing details *in vivo*[2]. The assessment of Fibronectin (FN) content in mice serving as wound models may also indicate the degree of wound healing [3]. The results showed that the FN level in the blood of mice in each group gradually increased during the healing period (**Figure S7(a)**). Meanwhile, the content of FN in the CaOMS groups was greater than that in other two groups at 1st and 3rd day, indicating that CaOMS could stimulate the production of fibronectin during the early phase (1-3 days) of wound healing. Tumor necrosis factor α (TNF-α), a multifunctional inflammatory cytokine involved in the regulation of tissue homeostasis and local immune responses [4], is often used to evaluate changes in wound viability overtime after injury [5]. The contents of TNF-α in three groups were the highest on the 3rd day after surgery **(Figure S7(b))**, indicating that the inflammatory reaction was the most conspicuous. Consistent with FN content, there was no significant difference in the content of TNF-α in different groups of wound models.

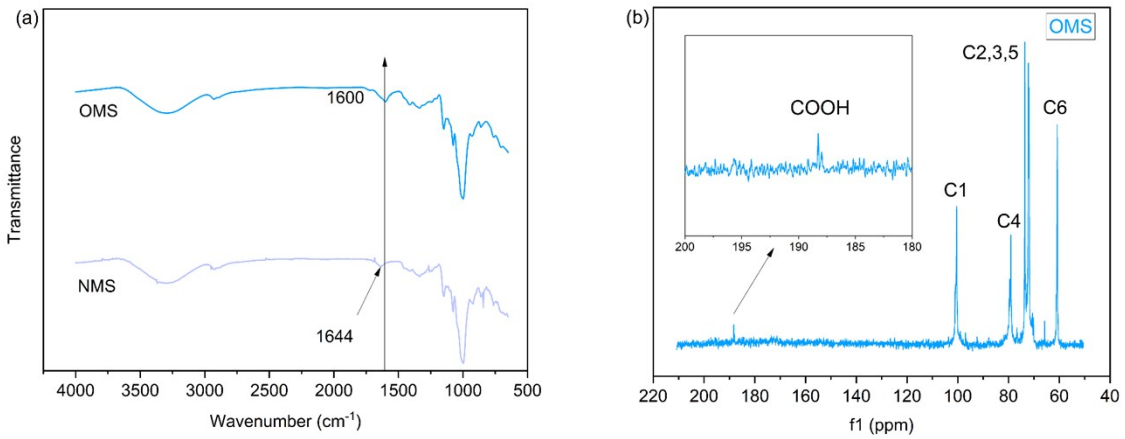


**Figure S1**. ATR-FTIR spectra (a) of NMS and OMS; $^{13}C$ NMR spectra (b) of OMS;

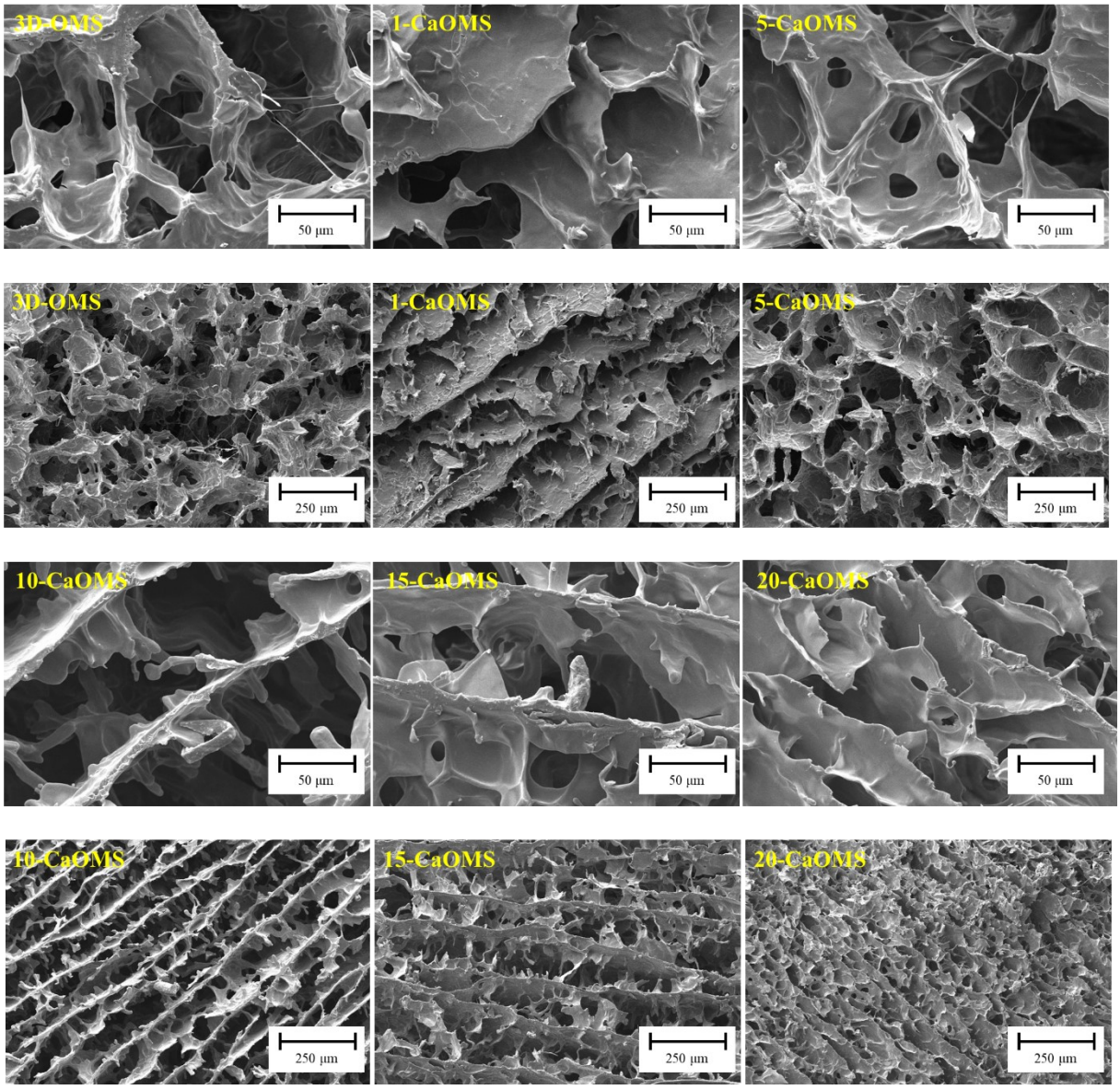


**Figure S2.** The morphology structure of 3D-OMS and CaOMS with different calcium ion concentration

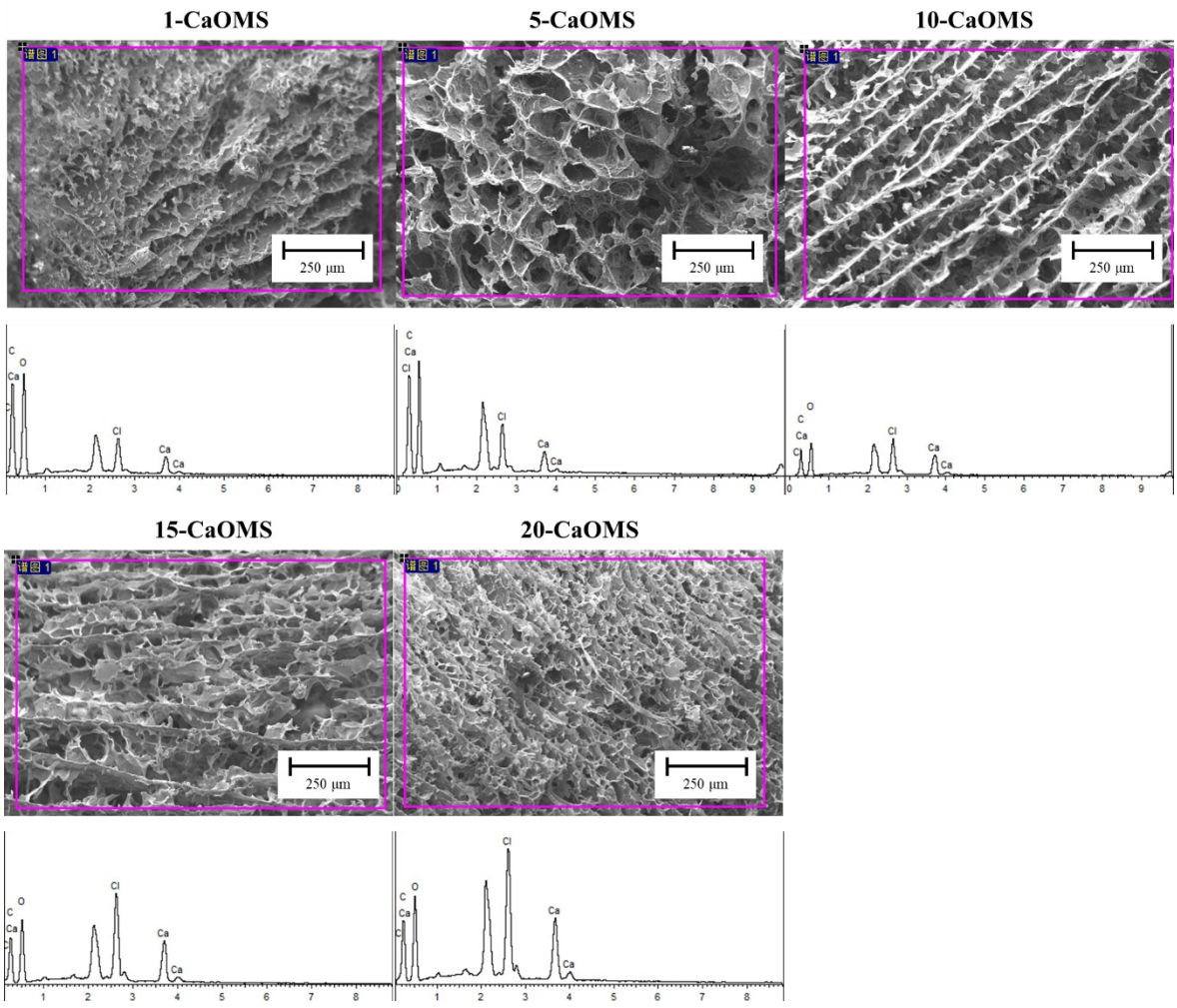


**Figure. S3.** The energy spectrum diagram of CaOMS with different calcium ion concentration

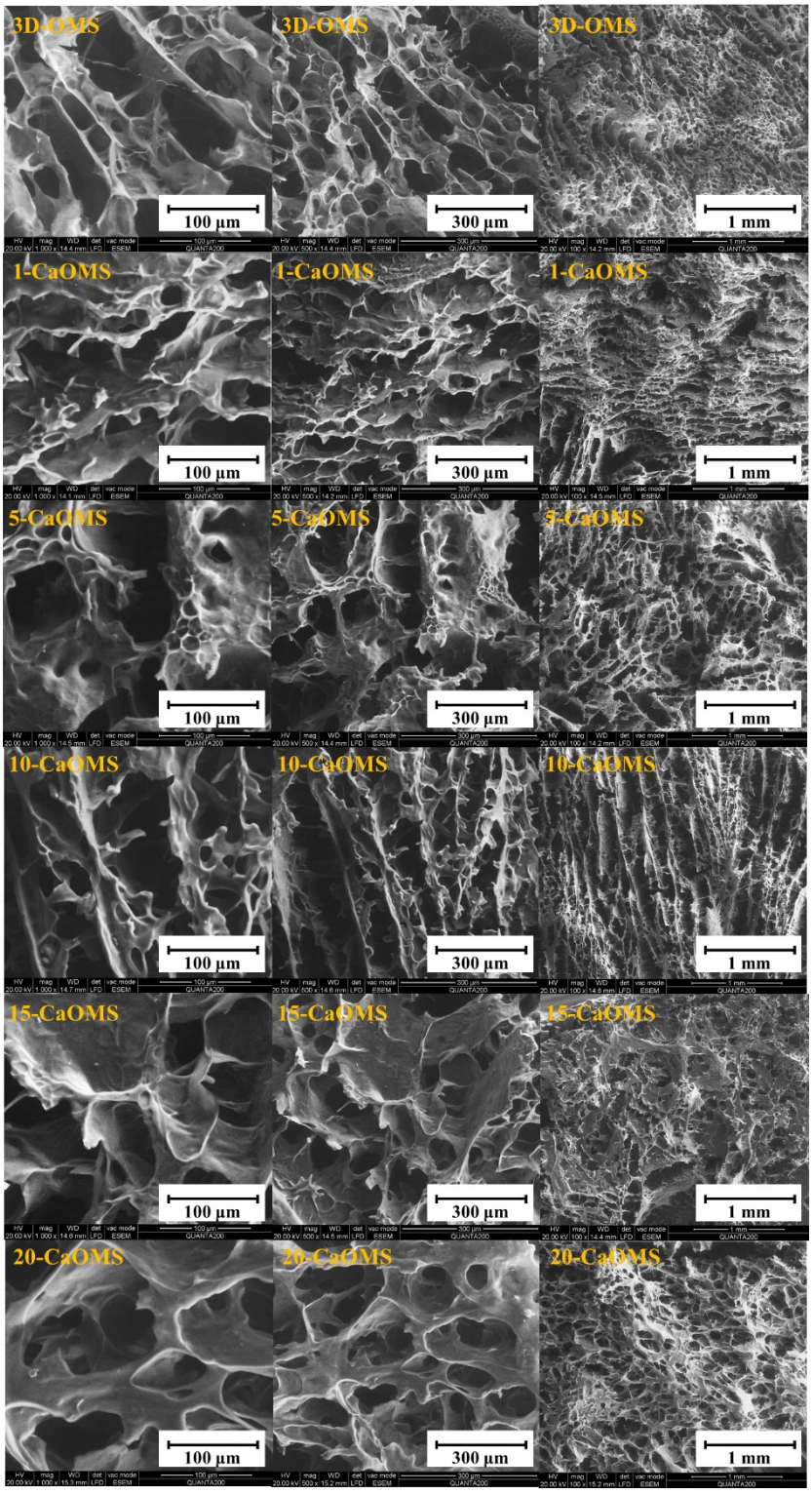


**Figure S4.** The morphology structure of 3D-OMS and CaOMS with different calcium ion concentration (wet basis) with different calcium ion content

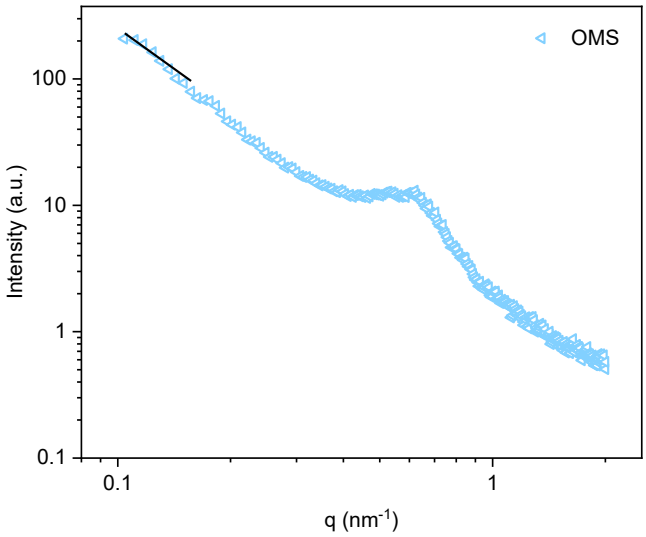


**Figure S5.** Kratky curves of OMS.

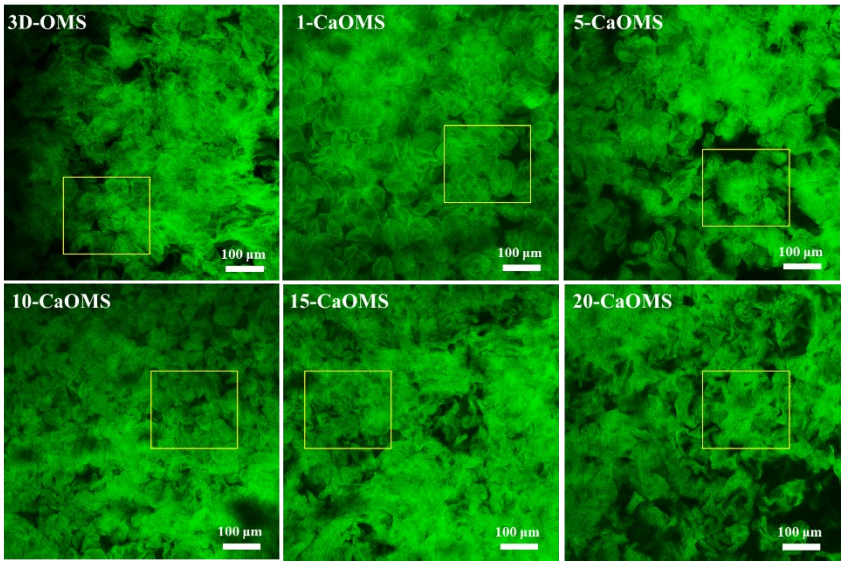


**Figure S6.** The microstructure of 3D-OMS and CaOMS with different calcium ion concentration after absorption.

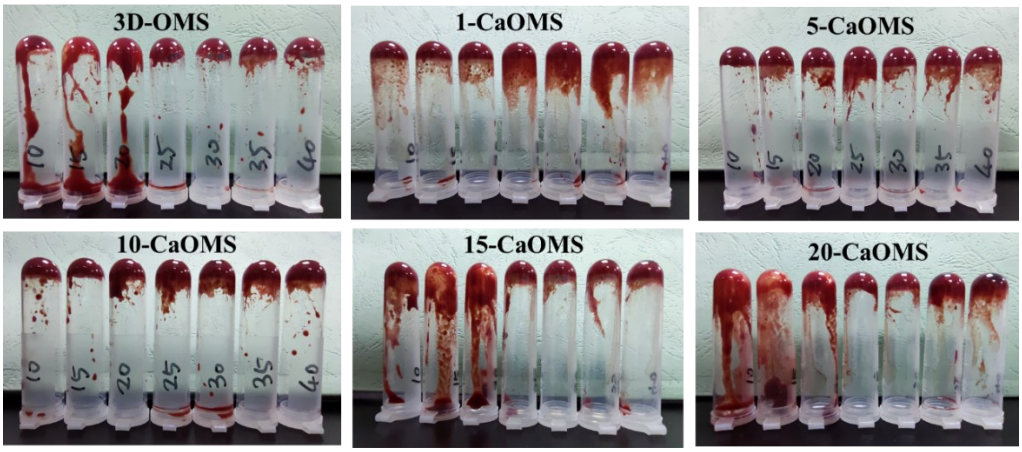


**Figure S7.** *In vitro* coagulation of 3D-OMS and CaOMS, 3D-OMS photo was taken at 60 min;1-CaOMS and 5-CaOMS photos were taken at 7 min;10-CaOMS photo was taken at 12 min;15-

CaOMS and 20-CaOMS photos were taken at 30 min

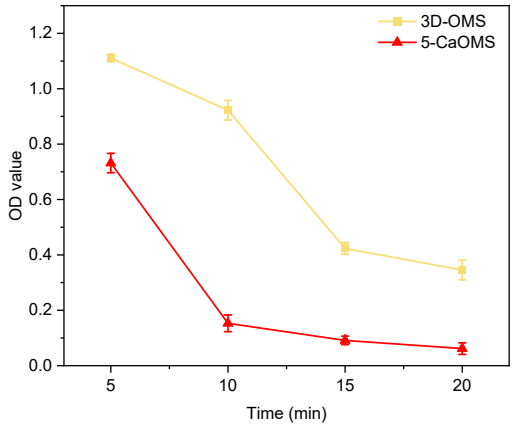


**Figure S8.** dynamic clotting time of 3D-OMS and 5-CaOMS

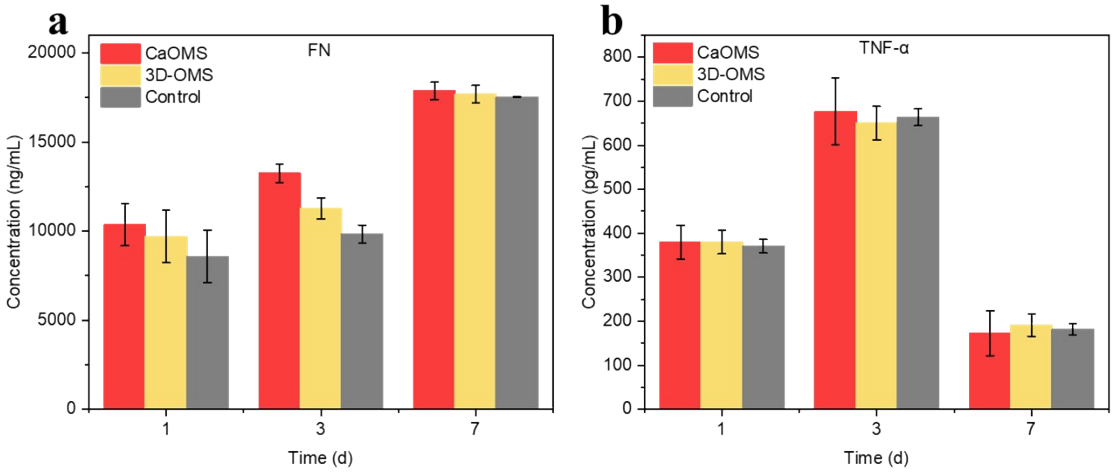


**Figure S9.** Quantitative analysis results of FN (a) and TNF-α (b) mean IOD as assessed by Image-Pro Plus software for each group

**Table S1.** The calcium ion distribution of CaOMS with different calcium ion concentration

| Samples | Mass (%) | Atom (%) |
|---|---|---|
| 1-CaOMS | 2.30 | 0.82 |
| 5-CaOMS | 2.37 | 0.85 |
| 10-CaOMS | 6.33 | 2.40 |
| 15-CaOMS | 6.38 | 2.44 |
| 20-CaOMS | 6.62 | 2.54 |

**Table S2.** The SAXS fitting parameters of 3D-OMS and CaOMS with different calcium ion concentration

| Samples | $\Xi$ (nm) | $\xi$ (nm) | $R^2$ |
|---|---|---|---|
| 3D-OMS | 1.866±0.013$^{d}$ | 0.428±0.013$^{b}$ | 0.999 |
| 1-CaOMS | 1.972±0.027$^{c}$ | 0.543±0.016$^{a}$ | 0.998 |
| 5-CaOMS | 1.989±0.028$^{c}$ | 0.559±0.017$^{a}$ | 0.998 |
| 10-CaOMS | 2.041±0.028$^{bc}$ | 0.558±0.017$^{a}$ | 0.998 |
| 15-CaOMS | 2.102±0.035$^{ab}$ | 0.570±0.018$^{a}$ | 0.997 |
| 20-CaOMS | 2.141±0.035$^{a}$ | 0.548±0.017$^{a}$ | 0.997 |

The data are presented as average value ± SD. Different superscript letters in the same column indicate different groups with significant difference ($p < 0.05$).

**Table S3.** The gel strength parameters of 3D-OMS and CaOMS with different calcium ion concentration

| Samples | Breaking Force(g) | Distance to Rupture(cm) | Gel Strength(g/cm) |
|---|---|---|---|
| 3D-OMS | 3849.42±406.11$^{b}$ | 0.21±0.05$^{a}$ | 788.7±120.72$^{b}$ |
| 1-CaOMS | 5181.6±152.96$^{a}$ | 0.22±0.01$^{a}$ | 923.75±12.11$^{a}$ |
| 5-CaOMS | 5146.22±103.32$^{a}$ | 0.22±0.01$^{a}$ | 923.88±16.9$^{a}$ |
| 10-CaOMS | 5152.43±187.95$^{a}$ | 0.2±0.01$^{a}$ | 928.41±8.37$^{a}$ |
| 15-CaOMS | 5278.8±161.78$^{a}$ | 0.19±0$^{a}$ | 919.49±10.63$^{a}$ |
| 20-CaOMS | 5262.82±301.31$^{a}$ | 0.18±0.01$^{a}$ | 921.27±5.90$^{a}$ |

The data are presented as average value ± SD. Different superscript letters in the same column indicate different groups with significant difference ($p < 0.05$).

**Table S4.** The gel strength parameters of hydrated 3D-OMS and CaOMS with different calcium ion concentration

| Samples | Breaking Force(g) | Distance to Rupture(cm) | Gel Strength(g/cm) |
|---|---|---|---|
| 3D-OMS | 59.26±4.86$^{b}$ | 0.20±0.02$^{a}$ | 11.44±1.19$^{b}$ |
| 1-CaOMS | 67.91±5.1a | 0.19±0$^{a}$ | 19.19±1.13$^{a}$ |
| 5-CaOMS | 70.09±6.02$^{a}$ | 0.2±0.01$^{a}$ | 18.87±0.54$^{a}$ |
| 10-CaOMS | 69.44±2.98$^{a}$ | 0.18±0.01$^{a}$ | 18.27±0.61$^{a}$ |
| 15-CaOMS | 72.48±9.09$^{a}$ | 0.21±0.01$^{a}$ | 18.18±1.28$^{a}$ |
| 20-CaOMS | 70.98±2.03$^{a}$ | 0.2±0.01$^{a}$ | 18.31±1.27$^{a}$ |

The data are presented as average value ± SD. Different superscript letters in the same column indicate different groups with significant difference ($p < 0.05$).

**Table S5.** *In vitro* coagulation time of 3D-OMS and CaOMS with varying addition

| Samples | Sample addition (mg) | | | | | |
|---|---|---|---|---|---|---|
| | 10 | 15 | 20 | 25 | 30 | 35 |
| 3D-OMS | - | - | - | 60±5$^{a}$ min | 30±3$^{a}$ min | 10±1$^{b}$ min |
| 1-CaOMS | 7±1$^{b}$ min | 7±0$^{b}$ min | 7±0$^{b}$ min | 6±0$^{d}$ min | 5±0$^{d}$ min | 5±0$^{d}$ min |
| 5-CaOMS | 7±1$^{b}$ min | 7±1$^{b}$ min | 6±0$^{b}$ min | 5±1$^{d}$ min | 2±0$^{e}$ min | 0±0$^{e}$ min |
| 10-CaOMS | 12±2$^{a}$ min | 12±2$^{a}$ min | 12±1$^{a}$ min | 10±2$^{c}$ min | 8±1$^{c}$ min | 7±1$^{bc}$ min |
| 15-CaOMS | * | * | * | 10±2$^{c}$ min | 9±2$^{c}$ min | 8±1$^{bc}$ min |
| 20-CaOMS | * | * | * | 17±2$^{b}$ min | 16±2$^{b}$ min | 15±2$^{a}$ min |

*: No coagulation for 30 min; -: No coagulation for 60 min. The data are presented as average value ± SD. Different superscript letters in the same column indicate different groups with significant difference ($p < 0.05$).

**Table S6.** The *in vitro* biodegradation of 3D-OMS and CaOMS with different calcium ion concentration

| Samples | Biodegradation (%) | | | | |
|---|---|---|---|---|---|
| | 10 min | 30 min | 60 min | 90 min | 120 min |
| 3D-OMS | 7.25±0.28$^{a}$ | 7.24±0.17$^{a}$ | 49.81±0.24$^{a}$ | 100±0$^{a}$ | N |
| 1-CaOMS | 6.55±0.10$^{b}$ | 6.60±0.06$^{b}$ | 30.32±0.27$^{d}$ | 68.49±0.61$^{c}$ | 100±0$^{a}$ |
| 5-CaOMS | 6.41±0.05$^{b}$ | 6.79±0.12$^{b}$ | 32.88±0.04$^{b}$ | 68.54±0.20$^{c}$ | 100±0$^{a}$ |
| 10-CaOMS | 6.48±0.02$^{b}$ | 6.78±0.31$^{b}$ | 32.59±0.34$^{bc}$ | 70.02±0.24$^{b}$ | 100±0$^{a}$ |
| 15-CaOMS | 6.47±0.06$^{b}$ | 6.57±0.15$^{b}$ | 31.67±0.56$^{c}$ | 68.57±0.19$^{c}$ | 100±0$^{a}$ |
| 20-CaOMS | 6.51±0.06$^{b}$ | 6.56±0.06$^{b}$ | 31.53±0.47$^{c}$ | 69.10±0.04$^{c}$ | 100±0$^{a}$ |

The data are presented as average value ± SD. Different superscript letters in the same column indicate different groups with significant difference ($p < 0.05$).

**Table S7.** Cell viability of 3D-OMS and CaOMS with different calcium ion concentration

| Samples | Cell viability (%) | | | | |
|---|---|---|---|---|---|
| | 0 μg/mL | 50 μg/mL | 200 μg/mL | 600 μg/mL | 800 μg/mL |
| 3D-OMS | 96.76±1.73[a] | 93.35±9.03[a] | 96.77±7.98[a] | 99.62±5.79[a] | 97.94±5.25[a] |
| 1-CaOMS | 95.81±3.27[a] | 97.2±8.08[a] | 99.36±4.10[a] | 98.24±7.50[a] | 98.30±8.51[a] |
| 5-CaOMS | 97.09±2.02[a] | 97.53±8.55[a] | 98.55±0.88[a] | 96.79±8.18[a] | 97.95±0.04[a] |
| 10-CaOMS | 96.88±0.98[a] | 98.25±7.87[a] | 99.85±0.73[a] | 98.58±5.46[a] | 99.60±4.66[a] |
| 15-CaOMS | 98.51±2.36[a] | 98.63±4.48[a] | 98.89±1.08[a] | 99.83±3.99[a] | 98.29±7.63[a] |
| 20-CaOMS | 95.08±1.80[a] | 99.67±2.01[a] | 98.79±5.47[a] | 97.49±9.62[a] | 96.06±4.05[a] |

The data are presented as average value ± SD. Different superscript letters in the same column indicate different groups with significant difference ($p < 0.05$).

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