

# 1 **Porous Chitosan Microspheres as Microcarriers for 3D Cell Culture**

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21 **ABSTRACT:** Highly porous chitosan microspheres (CSM) were prepared through emulsion-  
22 based thermally induced phase separation (TIPS) without using toxic crosslinkers and chemical  
23 porogenic agents other than ice. The CSM had an average diameter of ~150  $\mu\text{m}$  with  
24 interconnected pores varying from 20~50  $\mu\text{m}$  in size. Due to their excellent biocompatibility and  
25 unique porous structure, high-performance hepatocyte culture in three-dimensional (3D) space  
26 was achieved using the CSM as microcarriers, as cell growth also took place within the internal  
27 pores of the CSM, besides their external surface, and multidirectional cell–cell interactions were  
28 observed. Enhanced cellular activity and functions were obtained with the CSM microcarriers as  
29 compared with 2D cell culture. It is believed that these CSM microcarriers provide a promising  
30 platform for 3D cell culture *in vitro*.

31 **KEYWORDS:** Chitosan, Porous microspheres, Microcarriers, 3D cell culture, Hepatocytes.

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45 **Graphical abstract:**

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49 **Highlights**

- 50 • CSM were prepared by a mild procedure, free of toxic crosslinking agents and porogens.
- 51 • The CSM have excellent biocompatibility and a unique porous structure.
- 52 • Convincing evidence for 3D cell culture was obtained using the CSM as microcarriers.
- 53 • The CSM microcarriers are favorable to maintaining cellular activity and functions.

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## 60 1. INTRODUCTION

61 Traditional cell plate culture has been questioned because of potential changes in  
62 morphology and gene expression, often resulting in distortions in cell behavior and biological  
63 functions of cultured cells as compared with cells in natural organisms and tissues (Asghar et al.,  
64 2015). To better maintain cell morphology, behavior and functions, the concept of three-  
65 dimensional (3D) cell culture was proposed, that is cell growth in a 3D environment with  
66 sufficient and multidirectional cell–cell interactions mimicking the *in vivo* architecture of natural  
67 organs and tissues (Achilli, Meyer, & Morgan, 2012; Pampaloni, Reynaud, & Stelzer, 2007).  
68 Natural polymer-based scaffolds have received broad attention for 3D cell culture due to their  
69 good biocompatibility and designed structure (Higuchi et al., 2014; Kehr, 2016; Mazza et al.,  
70 2015; Motealleh, Hermes, Jose & Kehr, 2018; Motealleh & Kehr, 2017; Song et al., 2017). For  
71 example, the group of Kehr developed alginate-based 3D hydrogel scaffolds containing periodic  
72 mesoporous organosilica or zeolite nanoparticles functionalized with the chiral biopolymers  
73 poly(L-lysine) and poly(D-lysine). Chirality-dependent cell adhesion and cell migration were  
74 investigated in these hydrogel scaffolds (Kehr, 2016; Motealleh et al., 2017; Motealleh et al.,  
75 2018).

76 The microcarrier culture technique, first introduced by van Wezel (1967), relies upon  
77 microspheres derived from various materials, with either a porous or a non-porous structure, as  
78 supports for anchoring cell lines. The main advantages of the microcarrier technology include a  
79 high surface area to mass ratio, the use of stirred microcarrier suspension culture, and easy scale-  
80 up making it possible to culture a wide range of animal cells in high yield (Chen, Reuveny, &  
81 Oh, 2013). A wide range of commercial and experimental microcarriers have emerged over the  
82 past few decades (Duan et al., 2015; Fang et al., 2014; Healthcare & Biosciences, 2005; Yu,

83 Kornmuller, Brown, Hoare, & Flynn, 2017). However, it should be noted that most of the  
84 existing microcarriers only enable cell attachment and growth on their outermost surface, or else  
85 on their external pore surface, with either no or only unidirectional cell–cell interactions. This is  
86 in essence very similar to monolayer cell culture in plates, and thus may not be considered 3D  
87 cell culture in the strict sense. Indeed, real 3D cell culture with microcarriers should enable cell  
88 growth within the microcarriers in addition to their surface, with sufficient and multidirectional  
89 cell–cell interactions, to mimic more closely the *in vivo* environment experienced by cells in  
90 natural organs and tissues (Achilli et al., 2012). This is considered crucial for the cells to present  
91 normal behavior and function. Chung et al. (2008) fabricated amine-functionalized microcarriers  
92 with a well-interconnected pore structure from poly(lactic-*co*-glycolic acid) (PLGA), through  
93 gas-promoted foaming in a water-in-oil-in-water double emulsion, for cell cultivation and  
94 injectable delivery. Unfortunately, the seeded cells barely infiltrated the inner pores of these  
95 supports but rather attached onto their outer surface for the most part. The authors hypothesized  
96 that the highly porous skeletal structure of the microcarriers likely did not provide sufficient  
97 anchoring surface area for cell attachment within the interior void spaces. Many other  
98 microcarriers were reported that only achieved cell growth on their outer surface (Duan et al.,  
99 2015; Yu et al., 2017; Zhang et al., 2018). More recently, Yan et al. (2018) reported porous  
100 microcarriers fabricated from strontium-substituted hydroxyapatite-*graft*-poly( $\gamma$ -benzyl L-  
101 glutamate) nanocomposites. The adhesion and infiltration of rabbit adipose-derived stem cells  
102 (ADSCs) within the internal cavities of these microcarriers were confirmed by confocal  
103 microscopy. It was shown that labeled ADSCs could be detected at 1/8, 2/8, 3/8 and 4/8 diameter  
104 depths of the microcarriers, which implied that cell attachment and growth were allowed in the  
105 innermost regions of these microcarriers.

106 The biopolymer chitosan, obtained by the deacetylation of chitin, has been widely applied in  
107 the biomedical field because of its non-toxicity and good biodegradability (Anitha et al., 2014;  
108 Pellá et al., 2018). Different chitosan-based porous macroscopic scaffolds were developed for 3D  
109 cell culture (Tripathi & Melo, 2015; Westin, Trinca, Zuliani, Coimbra & Moraes, 2017). As an  
110 example, Tripathi et al. (2015) synthesized sponge-like composite agarose–chitosan scaffolds in  
111 various forms including monolith and disc shapes. Primary hepatocyte growth and cell–cell  
112 interactions within the interconnected pore network of these scaffolds were observed. Chitosan-  
113 based microcarriers were also reported for cell culture (Custódio, Cerqueira, Marques, Reis &  
114 Mano, 2015; Tedesco et al., 2018; Wu et al., 2011; Zhang et al., 2018). For example, Zhang et al.  
115 (2018) developed chitosan-based microcarriers reinforced with graphene oxide through an acid-  
116 dissolution/alkali-precipitation approach. These hybrid microspheres were able to support stem  
117 cell expansion, growth and proliferation. Custódio et al. (2015) also developed chitosan  
118 microcarriers, bioconjugated on their surface with monoclonal antibodies favoring cell capture  
119 and subsequent cell expansion. Although multiple chitosan-based microcarriers have been  
120 investigated over the past decade, there is only one report, by Fang et al. (2014), for which 3D  
121 cell culture was supported in the true sense, enabling cell migration to the inside of the carrier  
122 and multidirectional cellular interactions. This may be because porous chitosan microspheres are  
123 usually prepared by crosslinking emulsions, i.e. chitosan emulsion droplets are first formed, and  
124 then stabilized with a crosslinking reagent such as glutaraldehyde. Unfortunately, the pores of  
125 chitosan microspheres obtained by that method are not large enough to accommodate cells (Wu  
126 et al., 2011). Fang et al. (2014) first prepared porous chitosan microspheres with large pores  
127 ( $47.5 \pm 5.4 \mu\text{m}$ ) by a phase separation technique. Microcarriers, named PEC, were then  
128 fabricated by coating the chitosan microspheres with poly(L-glutamic acid) *via* electrostatic

129 interaction and evaluated for cartilage regeneration. The authors confirmed that the poly(L-  
130 glutamic acid)-coated PEC microcarriers enabled the proliferation potential of chondrocytes,  
131 with cell infiltration and survival in the inner regions of the microcarriers. However they also  
132 stated that the poly(L-glutamic acid) modification played a vital role in 3D cell culture, as it  
133 enhanced the hydrophilicity of the microcarriers. We previously reported a simple and reliable  
134 emulsion-based thermally induced phase separation (TIPS) procedure to fabricate chitosan  
135 microspheres with large pores (J. Li et al., 2017). By adapting this preparation method for the  
136 current investigation, chitosan microspheres (CSM) with superior biocompatibility and a highly  
137 porous structure were obtained and fully characterized. In particular, the suitability of these as-  
138 prepared chitosan microspheres as microcarriers for 3D hepatocyte culture was confirmed,  
139 without requiring surface modification.

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## 141 **2. Materials and Methods**

### 142 **2.1. Materials and cell line.**

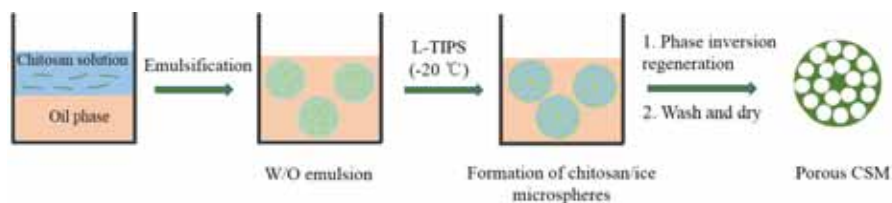
143 Chitosan (degree of deacetylation > 95%) was obtained from Sigma-Aldrich. Its  
144 viscosity-average molecular weight, determined with an Ubbelohde viscometer at 25°C and the  
145 Mark-Houwink-Sakurada equation  $[\eta] = KM^\alpha$ , where  $K = 1.8 \times 10^{-6}$  and  $\alpha = 0.93$  (Costa,  
146 Teixeira, Delpech, Souza, & Costa, 2015), was  $M_v = 1.02 \times 10^6$ . The surfactants Span 80 (S80)  
147 and Tween 60 (T60), acetic acid, petroleum ether and sodium hydroxide (NaOH) were purchased  
148 from Shanghai Sinopharm Chemical Reagent Co. Ltd. All the reagents were analytical grade and  
149 were used without further purification.

150 A human fetal hepatocyte line, L-02, obtained from Tongji Medical School, Huazhong  
151 University of Science and Technology (Wuhan, China) was cultured in RPMI-1640 medium

152 with 10% fetal bovine serum and 1% antibiotics (100 mg/mL streptomycin and 100 U/mL  
153 penicillin), by incubation at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

## 154 2.2. Preparation of porous chitosan microspheres (CSM).

155 To fabricate porous CSM appropriate as 3D culture supports for the L-02 cells, a  
156 combination of micro-emulsification and low temperature thermally induced phase separation  
157 (L-TIPS) techniques was selected and adapted from the procedure described in our previous  
158 work (J. Li et al., 2017). Specifically, a 1% (w/v) aqueous chitosan solution was prepared by  
159 dissolving chitosan powder into 1% (w/v) aqueous acetic acid solution (ca. 0.167 M), to serve as  
160 the dispersed phase. The continuous phase was petroleum ether containing S80 and T60  
161 emulsifiers in a mass ratio (S80/T60) of 4.8/0.2, and a total mass content of 5% (w/v). The  
162 chitosan solution (10 mL) was added drop-wise into 50 mL of the continuous phase at 40°C  
163 under magnetic stirring at 1000 rpm to obtain a w/o emulsion. After stirring for 3 h, the emulsion  
164 was quenched and stored at -20°C for 3.5 h. A phase inversion liquid, in the form of 100 mL 1%  
165 (w/v) NaOH solution in ethanol and water (14:1, v/v), was then poured into the quenched  
166 emulsion under mild stirring. The chitosan microspheres that formed were collected by  
167 centrifugation and were successively washed with water and ethanol until the supernatant  
168 became neutral, before drying under vacuum at 30°C. The as-prepared CS microspheres are  
169 identified as CSM (Scheme 1).



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171 Scheme 1. Fabrication procedure for the porous chitosan microspheres (CSM).



### 172 **2.3. Characterization of the CSM.**

173 The morphology of the CSM was observed on a scanning electron microscope (SEM)  
174 (Quanta 200, FEI) at an accelerating voltage of 20.0 kV after sputter-coating with gold. To  
175 characterize the distribution of pores within the particles, the cross-sectional morphology of the  
176 CSM was visualized as follows. The CSM were first stained with 1% (w/v) Rhodamine B  
177 solution in ethanol. After vacuum drying at 30°C for 10 h, they were embedded in paraffin and  
178 cut into 5 µm thick slices on a microtome (Leica RM2235). The cross-section structure was then  
179 observed on an Olympus BX51 optical microscope. The specific surface area was characterized  
180 by nitrogen adsorption-desorption isotherm analysis at 77 K (Micromeritics ASAP 2020  
181 apparatus). Other physical properties that were characterized include the porosity, pore size  
182 distribution, density, water absorption capacity and the elastic modulus. The protocols for these  
183 studies are provided in the Supplementary Information.

### 184 **2.4. Biocompatibility assay.**

185 The contact cytotoxicity and non-contact cytotoxicity of the CSM were evaluated with L-  
186 02 cells. Blood compatibility assays were also performed for the CSM through determination of  
187 the hemolysis rate. The specific protocols used for these studies are provided in the  
188 Supplementary Information.

### 189 **2.5. Cell attachment on/in the CSM.**

190 A CSM suspension in water (4 mg/mL), sterilized by autoclaving, was added to a L-02  
191 cell suspension in culture medium ( $1 \times 10^5$  cells/mL) in a volume ratio of 1:1. After gentle mixing  
192 on a shaker for 1 h, the cell/CSM suspension was transferred to a 24-well plate at 1 mL/well.  
193 After incubation for 10 h the cells were washed with PBS three times, followed by progressive

194 dehydration in 30%, 50%, 70%, 90%, 95% and 100% ethanol for 10 min each. After freeze-  
195 drying, the samples were carefully removed with forceps and placed on conductive adhesive tape  
196 on a platform for SEM (SU8010, Hitachi) observation after sputter-coating with gold. The  
197 fractured surface of the CSM/cell complex was also obtained and imaged by SEM after the same  
198 pretreatment. To determine the cell distribution within the CSM, the cells were stained with  
199 Calcein-AM (2  $\mu$ M) and observed by laser scanning confocal microscopy (Olympus FV1000,  
200 Japan) at different depths of the CSM after incubation for 48 h.

## 201 **2.6. Cell activity evaluation.**

202 The viability of cells cultured on the CSM microcarriers was assessed using a live/dead  
203 assay kit (Dojindo, Japan). The L-02 cells were cultured with 2 mg/mL of CSM in a 35 mm  
204 confocal dish (In Vitro Scientific, USA) for 12, 24, 48 or 72 h, respectively. Working solutions  
205 of Calcein-AM (2  $\mu$ M) and PI (4  $\mu$ M) were prepared from stock solutions. After washing the  
206 cells with PBS solution three times, an equimolar mixture of dyes was added at 500  $\mu$ L/well.  
207 After incubation at 37°C for 30 min, the samples were observed by laser scanning confocal  
208 microscopy (Nikon A1, Japan) using an excitation wavelength of 490 nm and the green emission  
209 at 515 nm to observe the live cells, while an excitation wavelength of 535 nm and the red  
210 emission at 615 nm were used to observe the dead cells. To further evaluate cell activity, the cell  
211 shape was determined by laser scanning confocal microscopy (Olympus FV1000, Japan) after  
212 incubation with the CSM for 48 h. The cell nuclei and cell membranes were stained with  
213 Hoechst 33258 (Beyotime, China) (10  $\mu$ g/mL) and DiI (Beyotime, China) (10  $\mu$ M), respectively.  
214 An excitation wavelength of 405 nm and the blue emission were used to observe the cell nuclei,  
215 while an excitation wavelength of 559 nm and the red emission served to observe the cell  
216 membranes.

217 **2.7. Albumin and urea secretion.**

218 The concentration of albumin (ALB) and urea in the culture medium was determined by  
219 the competitive enzyme linked immunosorbent assay (ELISA). The cell culture supernatants  
220 were collected in a sterile tube after centrifugation for 20 min at 2000 rpm. Aliquots of standards  
221 and diluted supernatants (100  $\mu$ L each) were added to the antibody-coated wells. After  
222 incubation for 1 h, the wells were washed three times and incubated with ALB antibody labeled  
223 with horseradish peroxidase (HRP) for 1 h. After washing three times, 3,3',5,5'-  
224 tetramethylbenzidine (TMB) substrate solution was added before incubation in the dark for 30  
225 min. The OD values were measured spectrophotometrically at 450 nm. The concentration of  
226 ALB in the samples was determined by comparing the OD values for the samples to a standard  
227 curve.

228 **2.8. Gene expression.**

229 Total RNA extraction from the L-02 cells was achieved with a RNApure Tissue & Cell  
230 Kit (CW BIO, China), after the cells were cultured with 2 mg/mL of CSM for 48 h in a 6-well  
231 plate at a seeding density of  $30 \times 10^4$  cells/well. The total RNA was adjusted to 1.0  $\mu$ g/sample  
232 before it was transcribed into cDNA with a First Strand cDNA Synthesis Kit (TOYOBO, Japan)  
233 according to the instructions. Fluorescence-based quantitative PCR was conducted using a 7500  
234 Fast Real-time PCR system (Applied Biosystems, USA) and SYBR Select Master Mix (Applied  
235 Biosystems, USA) according to the manufacturers' guidelines. The specific quantitative primers  
236 were synthesized by BGI (Shenzhen, China) with sequences as follows:

237 GAPDH: 5'- ACAACTTTGGTATCGTGGAAGG-3'(sense) and 5'-  
238 GCCATCACGCCACAGTTTC-3'(antisense);

239 CYP2E1: 5'-GGGAAACAGGGCAATGAGAG-3'(sense) and 5'-  
240 GGAAGGTGGGGTCGAAAGG-3'(antisense);  
241 GST: 5'-AACCTCAACTGAACAGCATCC-3'(sense) and 5'-  
242 GGTTGGTCTTGGTCCTCCTAT-3'(antisense);  
243 MRP: 5'-AAGGAGGTACTAGGTGGGCTT-3'(sense) and 5'-  
244 CCAGTAGGACCCTTCGAGC-3'(antisense);  
245 ASS1: 5'-TCCGTGGTTCTGGCCTACA-3'(sense) and 5'-GGCTTCCTCGAAGTCTTCCTT-  
246 3'(antisense).

## 247 **2.9. Proteomics analysis.**

248 The cells were lysed and total protein extraction was achieved by the same treatment  
249 described above. The protein concentration was determined by the Bradford protein assay. Then  
250 the samples were thermally denatured, reduced and alkylated prior to trypsin (Progmega)  
251 digestion for 15 h. The digested proteins were labelled with an iTRAQ reagent kit (8 plex,  
252 Applied Biosystems) according to the protocol supplied. The fractionated peptide mixtures were  
253 analyzed on a Q-Exactive mass spectrometer equipped with an EASY-nLC 1000 System  
254 (Thermo Fisher Scientific). The parameters for the spray voltage, capillary temperature and  
255 declustering potential of the source ionization were set at 2.1 kV, 250°C and 100 V,  
256 respectively.

## 257 **2.10. Western blot analysis.**

258 For western blot analysis, equal amounts of proteins (20 µg/sample) were separated on  
259 SDS-PAGE and transferred onto a PVDF membrane. After blocking overnight, the membrane  
260 was successively incubated with primary antibody and HRP-conjugate secondary antibody at

261 37°C for 1 h with shaking. The bands were visualized using Pierce ECL Western Blotting  
262 (Thermo Scientific, USA).

### 263 **2.11. Statistical analysis.**

264 Quantitative data are expressed as the arithmetic mean value  $\pm$  standard deviation (SD).  
265 All the quantitative results were obtained from at least triplicate samples. A t-test was used to  
266 detect differences between groups, with  $p < 0.05$  considered statistically significant and  $p <$   
267  $0.001$  considered highly statistically significant.

## 268 **3. RESULTS AND DISCUSSION**

### 269 **3.1. Morphological and physical properties of the CSM.**

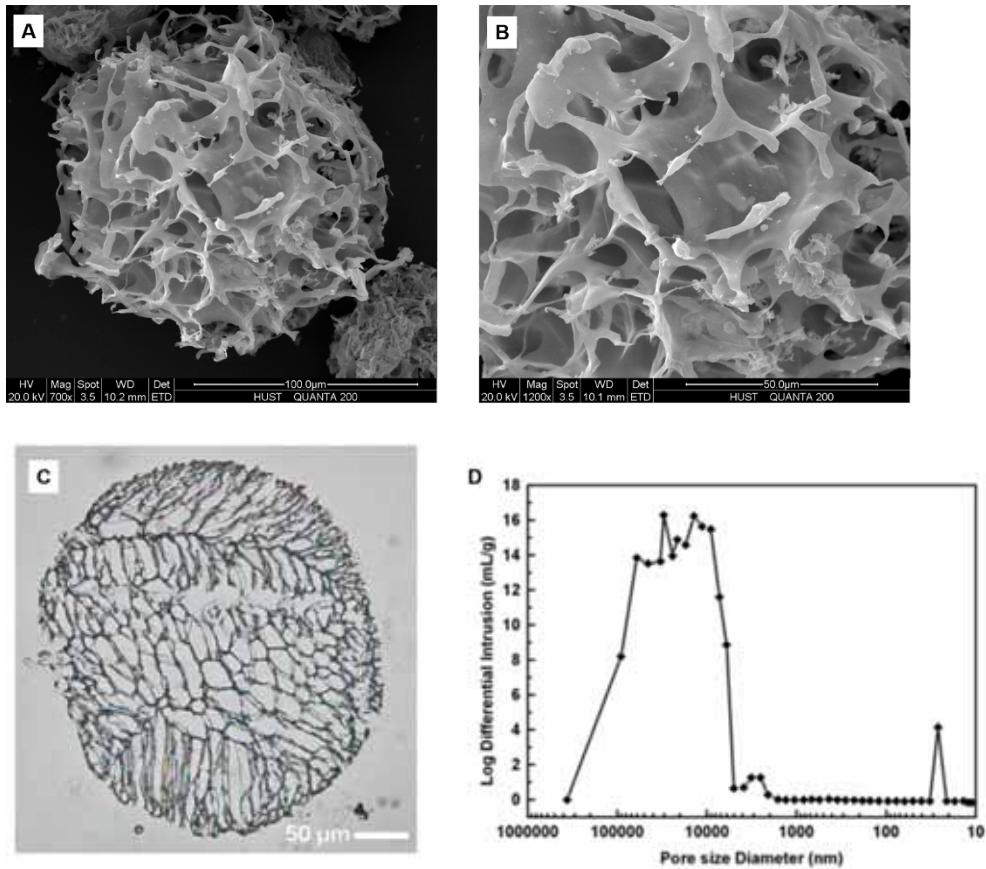
270 The structure of the CSM obtained by the method described is visible in Figure 1. The  
271 microspheres have a spherical topology, a diameter of ca. 150  $\mu\text{m}$  and a large number of  
272 irregular pores varying from 20 to 50  $\mu\text{m}$  in diameter (Figure 1A). The pores are interconnected,  
273 as can be seen more clearly from the enlarged image section provided in Figure 1B. This was  
274 further confirmed by optical microscopy of the microtomed material providing cross-section  
275 views of the CSM as exemplified in Figure 1C, likewise revealing the presence of multiple  
276 interconnected pores in the 20~50  $\mu\text{m}$  range throughout the whole particles. It was demonstrated  
277 in our previous study (J. Li et al., 2017) that such a structure is due to the mechanism of TIPS.  
278 Upon quenching the emulsion droplets to  $-20^\circ\text{C}$ , solid-liquid phase separation occurs within  
279 them, and the water/acetic acid solvent mixture undergoes slow crystallization allowing the ice  
280 crystals to gradually grow larger, leaving behind irregular pores on and within the CSM after the  
281 ice crystals are removed. It was further verified that the pore size in the microspheres could be  
282 controlled by adjusting the quenching temperature (Fang et al., 2014; J. Li et al., 2017): The pore

283 size decreases as the quenching temperature is lowered, because a relatively fast crystallization  
284 process produces smaller crystals. TIPS is a well-established method for the preparation of  
285 porous materials. Crystalline polymers such as polylactide (PLA) and PLGA are often employed  
286 to prepare porous materials by the TIPS technique (Ahmadi, Mordan, Forbes, & Day, 2011;  
287 Nina, Raheleh, & M., 2015; Zhang & Ma, 2015), but chitosan-based porous materials have been  
288 also obtained by the same approach (J. Li et al., 2017; Tripathi et al., 2015).

289 The porosity of the CSM, determined by a liquid displacement method, was as high as  
290  $98.0\pm 0.5\%$ , which leads to a very low density of  $0.040\pm 0.006\text{ g/cm}^3$  for the dry CSM. The  
291 distribution of pore sizes was also estimated by mercury injection. The results in Figure 1D  
292 demonstrate that most pores are within a range of 10~60  $\mu\text{m}$ , with an average diameter of 27.8  
293  $\mu\text{m}$ , which is in good agreement with the SEM observations. The specific surface area was  
294 measured to be  $30.0\pm 0.7\text{ m}^2/\text{g}$ , much higher than for the commercial Cytopore microcarriers  
295 (reported to be 1.0~3.0  $\text{m}^2/\text{g}$ ) (Healthcare & Biosciences, 2005; Ikonomou, Drugmand, Bastin,  
296 Schneider, & Agathos, 2002; Xiao et al., 1999). It is believed that microcarriers with a high  
297 specific surface area can provide a large number of anchoring sites for the cells, which is crucial  
298 to achieve high density cell culture (Yanagi, Miyoshi, Fukuda, & Ohshima, 1992). It was indeed  
299 suggested that large pores and a high pore interconnectivity within engineered scaffolds promote  
300 oxygen/nutrients diffusion, facilitating the in-growth and distribution of cells throughout the  
301 constructs (Chiu et al., 2011; Huang et al., 2012). The CSM possess a high water absorption  
302 capacity of as much as 2300%, enabling the microcarriers to form stable suspensions in the cell  
303 culture medium.

304 Elasticity is one of the most important characteristics of cell microcarriers. On the one hand, it  
305 can influence cell behaviors such as differentiation and messenger expression; on the other hand,

306 sufficient elasticity is required for the microcarriers to withstand shear forces in stirred  
307 bioreactors (Cha, Liechty, Khademhosseini, & Peppas, 2012; K. Li et al., 2004; Radaei,  
308 Mashayekhan, & Vakilian, 2017). The elastic modulus of the CSM measured by atomic force  
309 microscopy (AFM) in the wet state was 153 kPa (see Supplementary Information), which is  
310 slightly higher than for Cytopore microcarriers. Elastic modulus analysis for soft microspheres  
311 was also conducted by other researchers using AFM. For instance, Radaei et al. (2017) measured  
312 elastic modulus values of 34~133 kPa for gelatin/chitosan blend microspheres, depending on  
313 their composition.



314  
315 Figure 1. Structural and physical properties of the CSM: (A) SEM image; (B) Enlarged section  
316 of SEM image; (C) Optical microscopy image for a cross-section view; (D) Pore size  
317 distribution.

318 When the CSM are applied as microcarriers for cell culture, the highly porous structures with  
319 interconnected pores, mainly within a size range of 20~50  $\mu\text{m}$ , are expected to provide a large  
320 surface area for cell attachment and a good 3D environment for cell in-growth. Their elasticity  
321 should allow them to remain intact under shear, in addition to supporting the cells. Moreover, the  
322 CSM preparation is free of toxic crosslinking agents (such as glutaraldehyde) and porogens  
323 (other than ice), making the CSM microcarriers totally toxin-free in composition, which is  
324 favorable for growth in cell cultures. Consequently, these as-prepared microcarriers were  
325 expected to have a good performance in 3D cell culture.

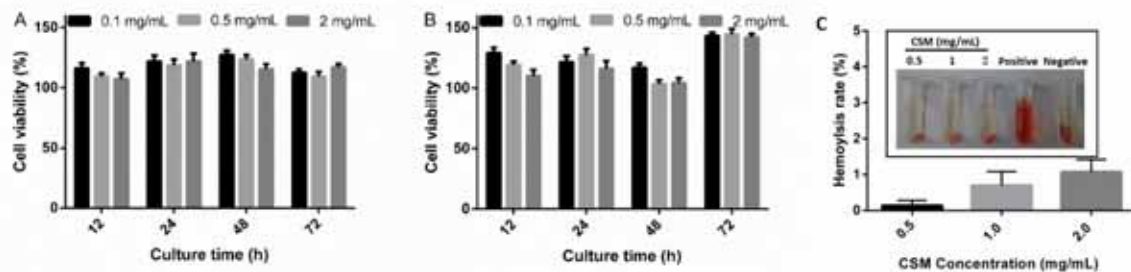
326

### 327 **3.2. Cytotoxicity and blood compatibility.**

328 The *in vitro* cytotoxicity of the CSM to L-02 cells was studied using the CCK-8 assay,  
329 and the results obtained are provided in Figure 2. The viability of L-02 cells incubated with CSM  
330 extract (non-contact; Figure 2A) and CSM suspensions (contact; Figure 2B) displayed no  
331 significant change at all the concentrations tested, with cell viability over 100% in all cases.  
332 Moreover, it is noted that the viability of L-02 cells incubated with the CSM was higher than for  
333 cells incubated with the CSM extract, which suggests that the CSM provided good anchorage  
334 facilitating cell adhesion, growth and proliferation. The blood compatibility of the CSM was  
335 evaluated by the hemolysis test (Figure 2C). It can be seen that the hemolysis rate with CSM at  
336 different concentrations (0.5, 1 and 2 mg/mL) was ca. 0.14%, 0.70% and 1.07%, respectively.  
337 According to standard ASTM-F/756-08 (2000), a hemolysis rate < 2% is defined as non-  
338 hemolytic (Seyfert, Biehl, & Schenk, 2002). The CSM are therefore considered non-hemolytic  
339 over the concentration range investigated. The trend of increasing hemolysis rate with CSM  
340 concentration is ascribed to the positive surface charges on the CSM, which could interact



341 electrostatically with the red blood cells. These superior cell and blood compatibility results  
342 indicate that the CSM are highly biocompatible and suitable for biomedical applications  
343 involving cell and blood contact.



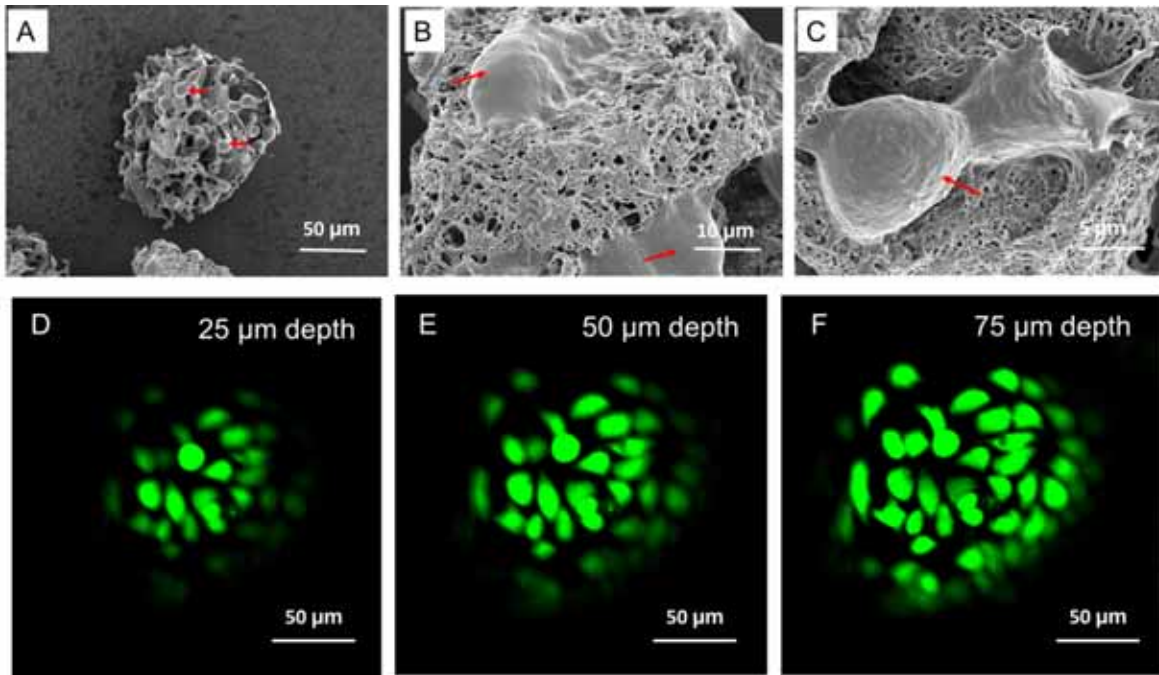
344  
345 Figure 2. Biocompatibility evaluation: (A) Non-contact and (B) contact cytotoxicity of the CSM  
346 by CCK-8 assay with L-02 cells; (C) Hemolysis test for the CSM at different concentrations.

347

### 348 3.3. Hepatocyte culture with the CSM microcarriers and evaluation.

349 SEM images for L-02 cells incubated with the CSM microcarriers for 10 h are shown in  
350 Figures 3A~C. It is clear that the L-02 cells (indicated by the red arrows) were attached and grew  
351 within the internal pores of the microcarriers, in addition to the outermost surface and the  
352 external pore surface (Figure 3A). Multidirectional cell–cell interactions were established and  
353 extracellular matrix seems to be visible surrounding the cells (Figures 3B, C), suggesting that the  
354 cells adapted rapidly to the new environment in the CSM microcarriers and remained in good  
355 condition. The fractured surface of a CSM seeded with L-02 cells and incubated for 10 h was  
356 also imaged by SEM; it is presented in the Supplementary Information (Figure S2). It is clearly  
357 seen that cells are distributed across the whole fractured surface of the CSM, in addition to the  
358 edge. This indicates that cells were able to grow within the internal pores of the CSM, in addition  
359 to their external surface. To further confirm cell in-growth within the microcarriers, cell cultures

360 with the microcarriers were investigated by confocal microscopy after incubation for 48 h. As  
361 shown in Figures 3D~F, Calcein-AM labeled cells (green) were detected at different depths  
362 within the microcarrier from 25  $\mu\text{m}$  to 75  $\mu\text{m}$ . This indicates without any doubt that the cells  
363 were able to infiltrate and grow within the inner pores of the CSM.

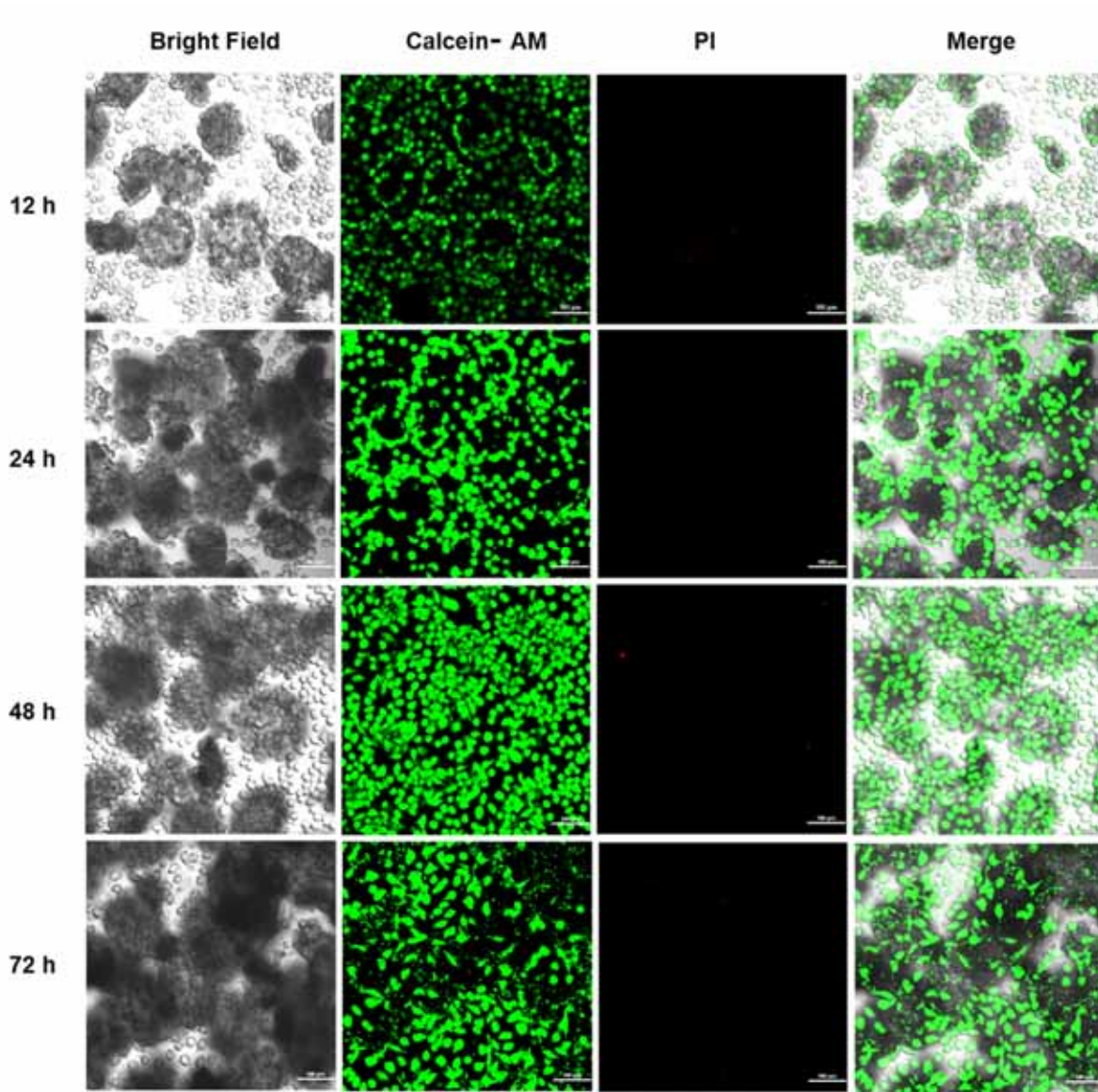


364  
365 Figure 3. Hepatocyte culture with the CSM microcarriers: A~C. SEM images confirming the  
366 attachment and growth of L-02 cells on/in the CSM microcarriers after incubation for 10 h. The  
367 red arrows indicate L-02 cells; D~F. Confocal microscopic images showing cell infiltration and  
368 growth at depths of 25, 50, and 75  $\mu\text{m}$  within the microcarriers after incubation for 48 h.

369 To evaluate cell activity, the Live/Dead assay was used to monitor the growth and proliferation  
370 of L-02 cells on/in the CSM microcarriers. Confocal laser scanning microscopy images for L-02  
371 cells with specific live-dead staining after 12, 24, 48 or 72 h of incubation with the microcarriers  
372 are provided in Figure 4. The live cells were stained with Calcein-AM (green), while the dead  
373 cells were stained with Propidium Iodide (PI) (red). It can be seen that the cells incubated with

374 the microcarriers were highly active, with dead cells hardly observed over the time interval  
375 studied. The cell number in the microcarriers increased with time from 12 to 72 h, as reflected in  
376 the increased green fluorescence. These results clearly indicate that the CSM microcarriers can  
377 support cell survival and proliferation. To further examine cell activity, the cell shape was  
378 determined after incubation with the CSM for 48 h, by staining the cell nuclei and membranes  
379 with Hoechst 33258 and DiI, respectively. As shown in Figure 5, the L-02 cells exhibited an  
380 epithelial-like morphology, which implies the cells had good growth and proliferation status (Hu  
381 et al., 2013).

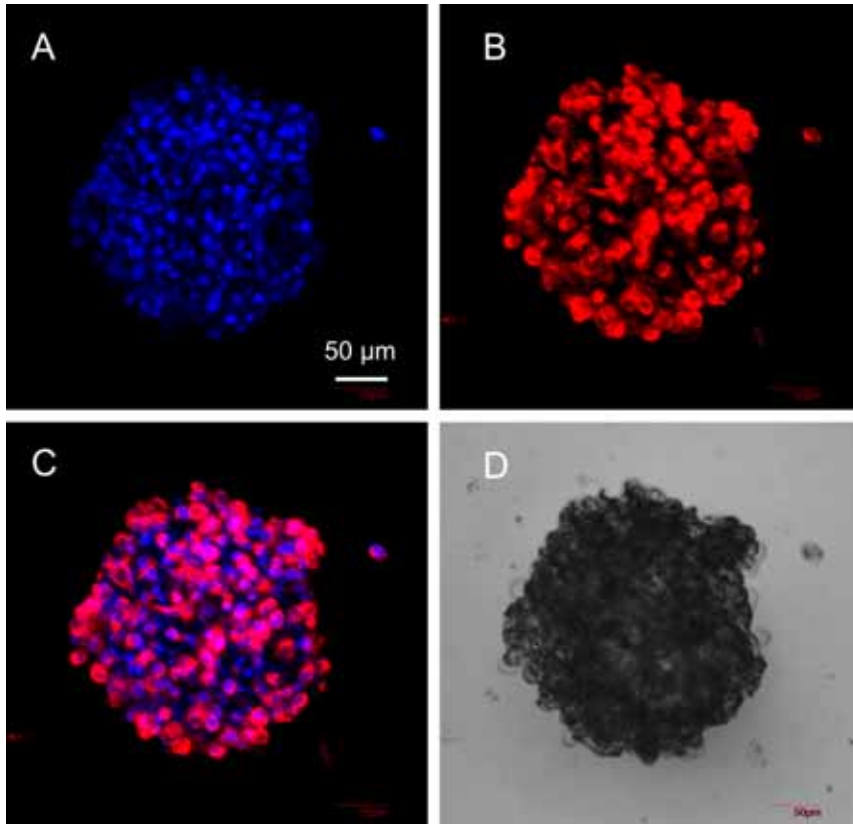
382 The success of the 3D cell culture achieved with the CSM microcarriers is attributed to their  
383 unique porous structure, with interconnected pores of 20~50  $\mu\text{m}$ , in addition to the good  
384 biocompatibility of chitosan, both of which promote good cell adhesion, growth and  
385 multidirectional cell–cell interactions. It was indeed suggested that a porous structure plays a  
386 vital role in cell culture, because it promotes not only the adhesion, migration and distribution of  
387 cells, but also for the exchange of nutrients and metabolic waste (Choi, Xie, & Xia, 2009). To  
388 achieve high-performance cell culture with porous microcarriers or scaffolds, the pore size  
389 should fall within an appropriate range (Murphy, Haugh, & O'Brien, 2010; O'Brien, Harley,  
390 Yannas, & Gibson, 2005; Yannas, Lee, Orgill, Skrabut, & Murphy, 1989). If the pore size is too  
391 small, cell migration is limited, resulting in the formation of a cellular capsule around the  
392 microcarriers. Conversely, if the pore size is too large, there is a decrease in surface area limiting  
393 cell adhesion (Murphy et al., 2010). The optimal pore size can vary from tens to hundreds of  
394 microns for different cell lines, however (O'Brien et al., 2005; Yannas et al., 1989). The pores  
395 should also be interconnected to allow sufficient multidirectional cell–cell interactions within the  
396 microcarriers.



398

399 Figure 4. Confocal laser scanning microscopy images for L-02 cells after 12, 24, 48 and 72 h of  
400 incubation with the CSM microcarriers. Scale bar: 100  $\mu\text{m}$ .

401



402

403 Figure 5. Cellular morphology observation by confocal microscopy after incubation with the  
404 CSM microcarriers for 48 h: A. Cell nuclei stained with Hoechst 33258; B. Cell membranes  
405 stained with DiI; C. Merged image of A and B; D. Bright field image. Scale bar: 50 μm.

406

### 407 **3.4 Liver function investigation.**

408 The mRNA expression of liver function-related genes including CYP2E1  
409 (cytochromeP450, family2, subfamilyE, polypeptide1), GST (glutathione-S-transferase), MRP  
410 (multidrug resistance-related proteins) and ASS1 (argininosuccinate synthase 1) was investigated  
411 in L-02 cells cultured with the CSM microcarriers for 48 h. As can be seen in Figure 6A, the  
412 mRNA expression levels for the above genes in the CSM-cultured L-02 cells were not  
413 significantly different from L-02 cells not exposed to the microcarriers. This indicates that the

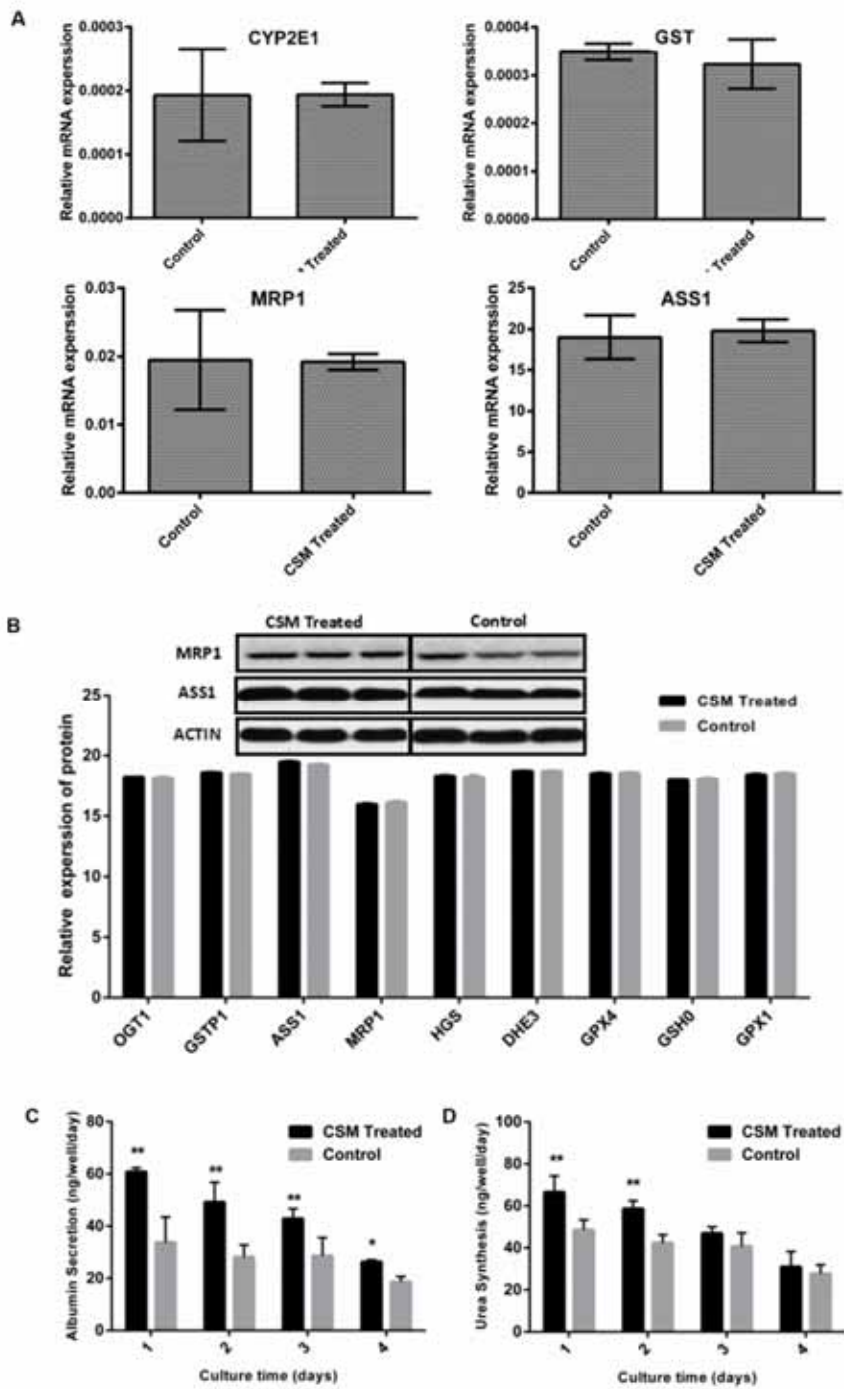
414 CSM microcarriers had no negative effects on the expression of liver function-related genes in  
415 the L-02 cells.

416 The expression of proteins related to liver functions in L-02 cells cultured on the CSM  
417 microcarriers was studied by proteomics analysis. The relative quantitative expression of the  
418 liver function-related proteins OGT1 (UDP glucuronosyl transferase), GST, HGS (hepatocyte  
419 growth factor-regulated tyrosine kinase substrate), DHE3 (glutamate dehydrogenase 1,  
420 mitochondrial), GPX 1,4 (glutathione peroxidase 1,4) and GSH0 (glutamate-cysteine ligase  
421 regulatory subunit 0) is depicted in Figure 6B. The results of western blot analysis for MRP and  
422 ASS1 are shown in the upper part of Figure 6B. Similarly to gene expression, protein expression  
423 related to liver functions in L-02 cells cultured on the CSM microcarriers was almost the same as  
424 for L-02 cells not exposed to the microcarriers. This indicates that the CSM microcarriers had no  
425 negative effects on the expression of liver function-related proteins in the L-02 cells.

426 To further examine the hepatocellular functions, the albumin secretion and urea synthesis  
427 ability were investigated in L-02 cells cultured on the CSM microcarriers. The results, shown in  
428 Figure 6C,D, demonstrate that as compared with cells cultured in plates, cells seeded on the  
429 CSM produced significantly more albumin each day over the 4-day period investigated, while  
430 urea synthesis was also significantly enhanced over the first two days. These results suggest that  
431 L-02 cells cultured on the CSM microcarriers had a higher activity than cells cultured in plates,  
432 which is probably due to the formation of cell-cell interactions in 3D space in the former case.  
433 With regards to the expression of the different hepatocyte functions, it is known that the  
434 establishment of adequate intracellular communication between cells is important (Nakazawa,  
435 Izumi, & Mori, 2009); because the L-02 cells cultured on the CSM microcarriers presumably had  
436 better intercellular communication than in the cell monolayers, they may be able to express

437 higher hepatocyte functions. It is noted that the secretion of albumin and the synthesis of urea  
438 decreased over time for cells cultured on both the microcarriers and in plates (Figure 6C,D).  
439 Similar results for hepatocyte functions *in vitro* were reported in the literature (Chua et al., 2005;  
440 Seo et al., 2005). For example, Chua et al. (2005) reported hepatocyte culture on galactosylated  
441 poly( $\epsilon$ -caprolactone-*co*-ethyl ethylene phosphate) nanofiber and film scaffolds. They also found  
442 that the albumin and urea synthesis functions of the hepatocytes decreased over a 5-day period.  
443 However, other studies demonstrated that the hepatocyte functions were maintained or even  
444 increased over time (Isoda et al., 2004). This shows the complexity of hepatocyte function  
445 maintenance, in that the expression of hepatocyte functions could be influenced by different  
446 biochemical and topological cues, among others.





447

448 Figure 6. Liver function evaluation for L-02 cells cultured with the CSM microcarriers: (A)  
 449 mRNA expression of liver function-related genes; (B) Liver function-related protein expression;  
 450 (C) Albumin secretion; (D) Urea synthesis.



451 **4. CONCLUSIONS**

452 Microcarriers based on porous chitosan microspheres (CSM) appropriate for 3D cell  
453 culture were developed, by adapting a simple emulsion-based thermally induced phase  
454 separation (TIPS) process to produce interconnected pores with a size appropriate to  
455 accommodate the cells. The mild preparation conditions used for the CSM, free of toxic reagents,  
456 ensured superior biocompatibility of the carriers. 3D cell culture was achieved using these  
457 microcarriers with hepatocytes, as cell growth was allowed within the internal pores, in addition  
458 to the outermost surface and the external pore surface, and multidirectional cell–cell interactions  
459 were observed. These CSM microcarriers are favorable to maintain the activity and functions of  
460 the cells in comparison with 2D cell culture in plates. On the basis of the results obtained, it is  
461 believed that these CSM microcarriers have great potential as efficient platforms for 3D cell  
462 culture.

463

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470

471

472 **SUPPLEMENTARY INFORMATION**

473 Supplementary information associated with this article can be found in the online version. The  
474 following files are available free of charge: Protocols for the determination of the porosity,  
475 density, pore size distribution, water absorption, elastic modulus, and the biocompatibility  
476 studies of the CSM, SEM image of a fracture surface of the CSM seeded with L-02 cells  
477 (DOCX).

478

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625 1741.

# Supplementary Information

## **Porous Chitosan Microspheres as Microcarriers for 3D Cell Culture**

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## Porosity and density

The porosity of the CSM was determined *via* a liquid displacement method with ethanol as the displacement liquid, as reported in previous studies (Guan, Fujimoto, Sacks, & Wagner, 2005; Li et al., 2017). Briefly, a certain amount of CSM ( $M_S$ ) was added to a pycnometer containing absolute ethanol ( $M_1$ ). Then the bottle was subjected to low vacuum until the air in the scaffold was completely removed and replaced with ethanol. The bottle was then completely filled with ethanol and was weighed as  $M_2$ . Then the CSM saturated with ethanol were collected by carefully draining the ethanol, the remaining ethanol on the CSM surface was removed with filter paper. The pycnometer containing wet CSM was weighed as  $M_3$ . The volume of the CSM was calculated as  $V_S = (M_1 - M_2 + M_3 - M) / \rho$  (ethanol). The density ( $\rho$ ) and porosity ( $P$ ) were calculated using Equations (S1) and (S2):

$$\rho = M_S \rho(\text{ethanol}) / (M_1 + M_3 - M - M_2) \quad (\text{S1})$$

$$P = V_P/V_S = [(M_3 - M - M_S) / (M_1 - M_2 + M_3 - M)] \times 100\% \quad (\text{S2})$$

## Pore size distribution

The pore size distribution was estimated with a mercury injection apparatus (AutoPore IV 9500, Micromeritics Instrument Corporation). The equipment operated at pressures of 10 kPa to 300 MPa, to probe pores with diameters ranging from 150  $\mu\text{m}$  to 0.005  $\mu\text{m}$ . The CSM samples were dried with silica gel prior to the measurements.

## **Water absorption capacity**

The water absorption capacity of the CSM was investigated as described previously [S1]. A certain amount of dry CSM ( $M_d$ ) was immersed into phosphate-buffered saline (PBS, pH 7.4) solution. The glass beaker containing the mixture was evacuated for 3 min, to allow filling of the pores in the CSM by the solution, and allowed to stand for 24 h. Subsequently, the PBS solution was discarded and excess liquid was removed with filter paper. The mass of the wet CSM ( $M_w$ ) was measured. Triplicate measurements were made for each sample. The water absorption ratio (A) was calculated using Equation (S3):

$$A = [(M_w - M_d) / M_d] \times 100\% \quad (S3)$$

## **Elastic modulus**

The dry CSM were put into PBS solution and stirred at 400 rpm for 30 min. The elastic modulus (E) of the wet CSM was determined by analysis of the force-displacement curve on an atomic force microscopy (AFM) apparatus. The plot of force versus distance between the probe and the CSM surface was recorded as shown below (Figure S1). The set point was selected as 14 nN for a quadratic pyramid tip and the cantilever was lowered at a velocity of 1  $\mu\text{m/s}$ . The constant spring of the cantilever ( $K_c$ ) was 0.68 N/m. The local elastic modulus of the sample was calculated using the equations below (Kuznetsova, Starodubtseva, Yegorenkov, Chizhik, & Zhdanov, 2007; Radaei, Mashayekhan, & Vakilian, 2017; Hertz, 1881).



$$E = \frac{F}{\pi a^2} \quad (S4)$$

$$F = K_c \times K_s \times \Delta V, \text{ in which } K_s = \Delta Z_1 / \Delta V_1 \quad (S5)$$

$$a = \sqrt[3]{\frac{3FR}{4E}} \quad (S6)$$

In these equations,  $E$  is elastic modulus of the CSM;  $F$  is the force applied;  $a$  is the Hertzian contact radius;  $K_c$  is the constant spring of cantilever (in this study, it was 0.68N/m.);  $K_s$  is the displacement sensitivity, which is the displacement of the scanner when the operating point changes by one volt.  $R$  is the radius of the CSM tested. Since the values of  $\Delta V$ ,  $\Delta V_1$ ,  $\Delta Z_1$  are known for the force curve (Figure S1), the elastic modulus ( $E$ ) of the CSM can be calculated as 153 kPa.

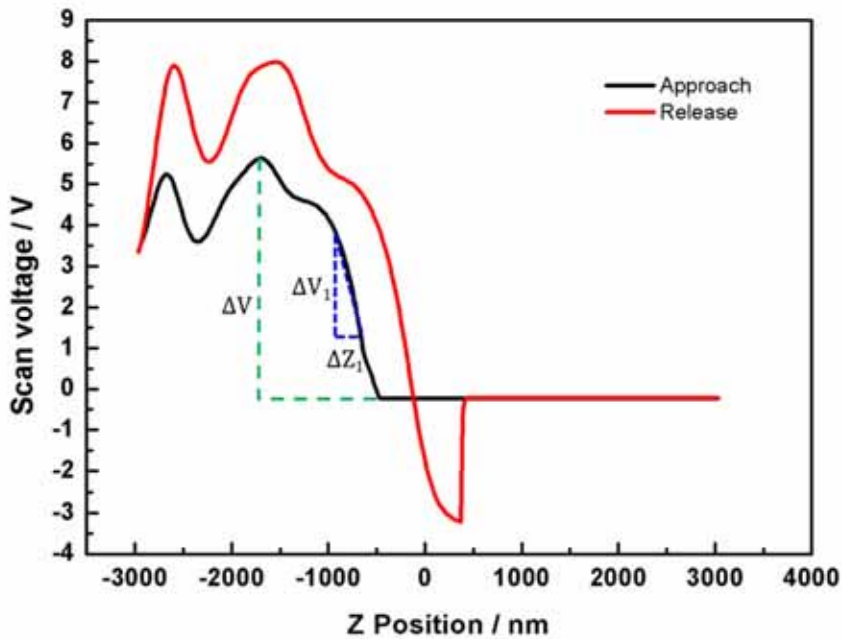


Figure S1. Analysis of the AFM force curve.

## Biocompatibility assay

**Cytotoxicity assay.** For the non-contact cytotoxicity assays, a CSM extract was prepared according to ISO 10993-5. Briefly, sterilized CSM were immersed in cell culture medium at different concentrations of 0.5, 1.0 and 2.0 mg/mL at 37 °C for 72 h. Solid components were then removed by centrifugation at 10000 rpm for 5 min. The collected supernatant was used as CSM extract after filtration through a 0.22 µm filter membrane. The L-02 cells were seeded at  $1 \times 10^4$  cells/well in a 96-well plate and incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere overnight. The medium was then replaced with the CSM extract, using the cell culture medium as control. After incubation for 12, 24, 48 or 72 h, the cells were treated with 10 µL/well of CCK-8 (Dojindo, Japan) solution with shaking for 5 min and incubated for another 30 min. Absorbance values were measured in triplicate at 450 nm using a microplate reader (Multiskan GO, Thermo Fisher, USA). For contact cytotoxicity, the cells were seeded with the CSM at concentrations of 0.5, 1.0 and 2.0 mg/mL, and the above procedures for the assessment of non-contact cytotoxicity were followed. Cell viability was calculated using Equation (S7), where OD<sub>S</sub>, OD<sub>B</sub> and OD<sub>N</sub> are the OD values for the sample, the blank control and the negative control, respectively.

$$\text{Cell viability} = \frac{OD_S - OD_B}{OD_N - OD_B} \times 100\% \quad (S7)$$

**Hemolysis rate test.** To evaluate the blood compatibility of the CSM, the hemolysis rate was determined by the method described below. Fresh blood was collected from a healthy rabbit and mixed with heparin immediately to prevent clotting, before dilution with normal saline at a volume ratio of 1:1.25. The CSM samples at different concentrations (i.e. 0.5, 1.0 and 2.0 mg/mL) were

pre-swelled in normal saline overnight. Distilled water and normal saline were used as positive and negative controls, respectively. All the samples (1.5 mL) were stored at 37°C in the presence of 50 µL of diluted blood for 30 min. The samples were then centrifuged at 1000 rpm for 5 min and the supernatant was subjected to absorbance measurements at 545 nm on a microplate reader (Multiskan GO, Thermo Scientific). The hemolysis rate was calculated using Equation (S8), where  $OD_S$ ,  $OD_N$ , and  $OD_P$  are the absorbance values for the sample, the negative control and the positive control, respectively.

$$\text{Hemolysis Rate} = [(OD_S - OD_N) / (OD_P - OD_N)] \times 100\% \quad (\text{S8})$$

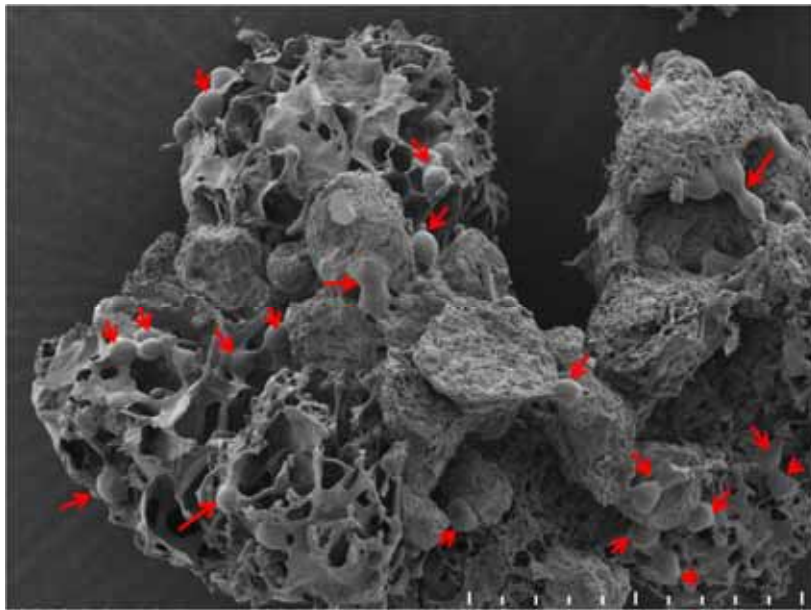


Figure S2 SEM image for a fractured surface of the CSM seeded with L-02 cells (indicated with red arrows) after 10 h of incubation. Scale bar: 100 µm.

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