Bioorganic & Medicinal Chemistry Letters 20 (2010) 4147–4151

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis, characterization and preliminary analysis of in vivo biological activity of chitosan/celecoxib microcapsules

Shuk-Yan Cheng [a], Marcus Chun-Wah Yuen [a], Pik-Ling Lam [a], Roberto Gambari [b], Raymond Siu-Ming Wong [c], Gregory Yin-Ming Cheng [c], Paul Bo-San Lai [d], See-Wai Tong [c], Kit-Wah Chan [c], Fung-Yi Lau [c], Stanton Hon-Lung Kok [e,*], Kim-Hung Lam [a,*], Chung-Hin Chui [a,c,*]

[a] Institute of Textiles and Clothing, The Hong Kong Polytechnic University, Hong Kong, China
[b] BioPharmaNet, Department of Biochemistry and Molecular Biology, The University of Ferrara, Ferrara, Italy
[c] Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong, China
[d] Department of Surgery, The Chinese University of Hong Kong, Hong Kong, China
[e] Department of Chemistry, The Chinese University of Hong Kong, Hong Kong, China

ARTICLE INFO

Article history:
Received 12 April 2010
Revised 13 May 2010
Accepted 14 May 2010
Available online 9 June 2010

Keywords:
Celecoxib
Chitosan
Cyclooxygenase 2
Oral administration

ABSTRACT

The use of chitosan as the wall of microcapsule designed for delivery of encapsulated celecoxib is reported. Microcapsules were characterised with respect to size and encapsulation efficiency of celecoxib. In vivo animals demonstrated that both free celecoxib administration and chitosan/celecoxib microcapsules administration lead to a significant inhibition of cyclooxygenase-2 protein expression in the hepatocytes when compared with vehicle control mice. Interestingly, microcapsule containing celecoxib showed a better inhibition of cyclooxygenase-2 protein expression when compared with a simple oral administration of free celecoxib. Gas-chromatography–mass-spectrometry analysis showed that in mice treated with free celecoxib or chitosan/celecoxib microcapsules, their plasma concentration of celecoxib was similar. Microcapsules-based biomaterials as oral drug delivery vehicles may help to improve the absorption efficiency of therapeutic drugs.

Microencapsulation is a micropackaging technique involving the production of microcapsules which act as barrier walls of solids or liquids. The microcapsules are produced by depositing a thin polymer coating on small solid particles or liquid droplets, or on dispersions of solid in liquids. The release mechanisms of the core content vary depending on the selection of wall materials and, more importantly, its specific end uses. The core content is released from the microcapsules under a variety of controlled conditions, such as friction, pressure, change of temperature, diffusion through the polymer wall, dissolution of the polymer wall coating or biodegradation.[1–3] Particles in micro-size prepared by the microencapsulation technique are called microcapsules, microspheres or microparticles.[1,4,5]

The structure of microcapsules generally consists of two major components: (1) wall shell and (2) core material, respectively. The wall shell is usually a polymer coating that surrounds and protects the core materials. It can be natural polymer, semi-synthetic polymer or synthetic polymer.[6] Chitosan, a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit), is a natural polymer which is produced commercially by deacetylation of chitin, which is the structural element present in the exoskeleton of crustaceans, for example, crabs and shrimp etc. Chitosan is non-toxic, bio-degradable and bio-compatible, and has long been used as a bio-polymer or crude material in pharmaceutical and medical fields, papermaking and food processing. Numerous scientific reports, patents and commercial developments concerning the preparation of chitosan based microcapsules have been published.[6–12] Most of the recent developments of chitosan microcapsules are focused on the encapsulation of drugs for biological applications. The core material, which may be in the form of liquid or solid, refer to the substance to be encapsulated by the wall material and is the main component to be delivered to human body. In this present study, a simple and low-cost coacervation technique has been used to produce chitosan microcapsules. Celecoxib, the new generation of a non-steroid anti-inflammation drug, was used as a tool to be encapsulated into the chitosan based microcapsule. In order to verify possible applications of the produced chitosan/celecoxib microcapsules, analytical studies as well as in vivo biological assays were performed.

We first report data showing that chitosan based microcapsules containing celecoxib were successfully produced.[13] Morphological

* Corresponding authors. Tel.: +852 2766 6456; fax: +852 2766 1432 (S.H.-L.K.); tel.: +852 3400 8705; fax: +852 2364 9932 (K.-H.L.); tel.: +852 766 6456; fax: +852 2766 1432 (C.-H.C.).
E-mail addresses: stantonkok@hotmail.com (S.H.-L. Kok), bcjoelam@inet.polyu.edu.hk (K.-H. Lam), chchui@graduate.hku.hk (C.-H. Chui).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.05.054

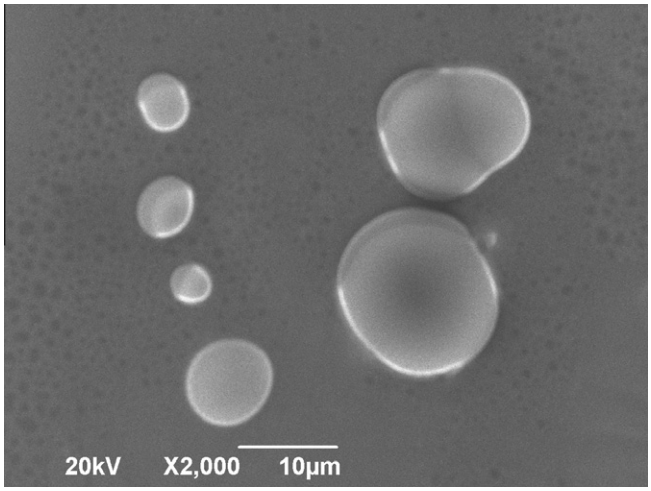

Figure 1. Scanning electron microscopic analysis of the chitosan/celecoxib microcapsules.

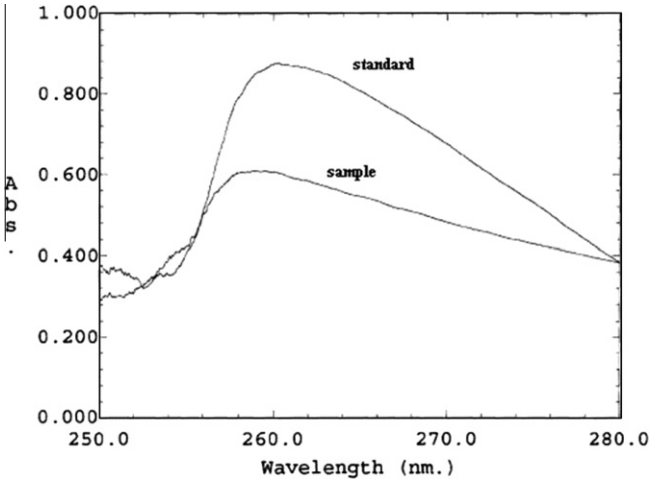

Figure 2. Absorption spectra of free reference standard celecoxib and celecoxib isolated from microcapsules.

Table 1
Analysis of the plasma liver enzyme levels

	Control	Celecoxib	Chitosan/celecoxib	Reference (U/L)
ALT	40.33 ± 4.73	46.33 ± 4.93	53.34 ± 15.95	(28–132)
AST	52.00 ± 5.12	49.67 ± 7.23	47.33 ± 6.43	(59–247)

Results are shown as mean ± standard deviations from each group. No statistical significant difference is found between the free celecoxib or the chitosan/celecoxib group in respect to the control group (p >0.05). Student t-test was used and data was considered to be statistically significant when p value was smaller than 0.05.

analysis using scanning electron microscopy was performed (Fig. 1). The particle size of microcapsules was ranging from 2.0 μm to 15 μm and the mean particle size was 5.6 μm. The dry weight of chitosan/celecoxib microcapsules was determined to be a mean of 6.2 g obtained from three independent measurements. Analytical chemistry assays showed that the concentration of celecoxib in the microcapsules was 0.09 μg ± 0.03 μg per ml (mean value from three independent experiments) (Fig. 2). Based on these results, drug loading of the chitosan microcapsules was estimated to be 0.0341 g per dry weight of microcapsules and the encapsulation efficiency was determined to be 68.2%.[14]

In order to determine the biological activity, we have treated mice orally with chitosan/celecoxib microcapsules at a concentration of 4 μg/g for a continuous of 4 days. After the treatment protocol, all the mice were sacrificed and liver autopsy was performed for section preparation and immunohistochemistry analysis.[15] As demonstrated by the representative experiment shown in Figure 3, samples from vehicle control group displayed consistent expression of cyclooxygenase-2 protein from their liver sections (Fig. 3 A and B). Both of free celecoxib administration (Fig. 3C and D) and chitosan/celecoxib microcapsules administration (Fig. 3E and F) showed a significant inhibition of cyclooxygenase-2 protein expression. Interestingly, microcapsule containing celecoxib showed a stronger inhibition of cyclooxygenase-2 protein expression from mice hepatocytes when compared with a simple oral administration of celecoxib.[16]

Plasma liver functional enzyme markers demonstrated that our chitosan based microcapsule system did not exert toxicological adverse effects on the mice livers (Table 1).[17] Further semi-quantitative GC–MS analysis from isolated plasma samples of mice

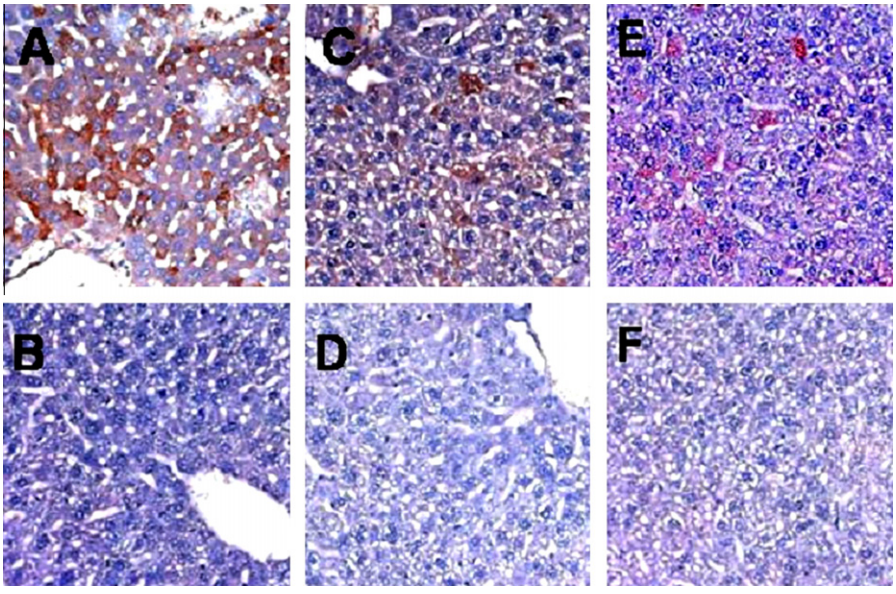

Figure 3. Representative immunohistochemistry study for the expression of cyclooxygenase-2 protein on the liver section of mice after oral administration of free celecoxib (C and D), celecoxib containing microcapsules (E and F). Treatment protocol was started from day 1 to day 4 days at 4 μg/g body weight/day and mice were sacrificed on day 5. Livers were collected for analysis. (B), (D) and (F) are their correspondence negative controls showing the high integrity of the mice hepatocytes.

showed that celecoxib was detected in mice after oral administration of celecoxib containing microcapsules and free celecoxib with similar plasma concentrations (Fig. 4).[18]

Chitosan based microcapsule is found to be a good delivery model for oral drug delivery. Under acidic condition, chitosan based microcapsules would decompose and release its content. This is the physiological situation in the stomach, owing to the continuous secretion of gastric juice, including hydrochloric acid. The released content will be delivered to the small intestine, being absorbed in the jejunum. This is really a good pathway for the release of oil from the microcapsule in the stomach and oil is being absorbed in the jejunum as small oil droplets after the emulsification of bile salt and being transported to the peripheral lymphatic system. However, most the research work by others seldom

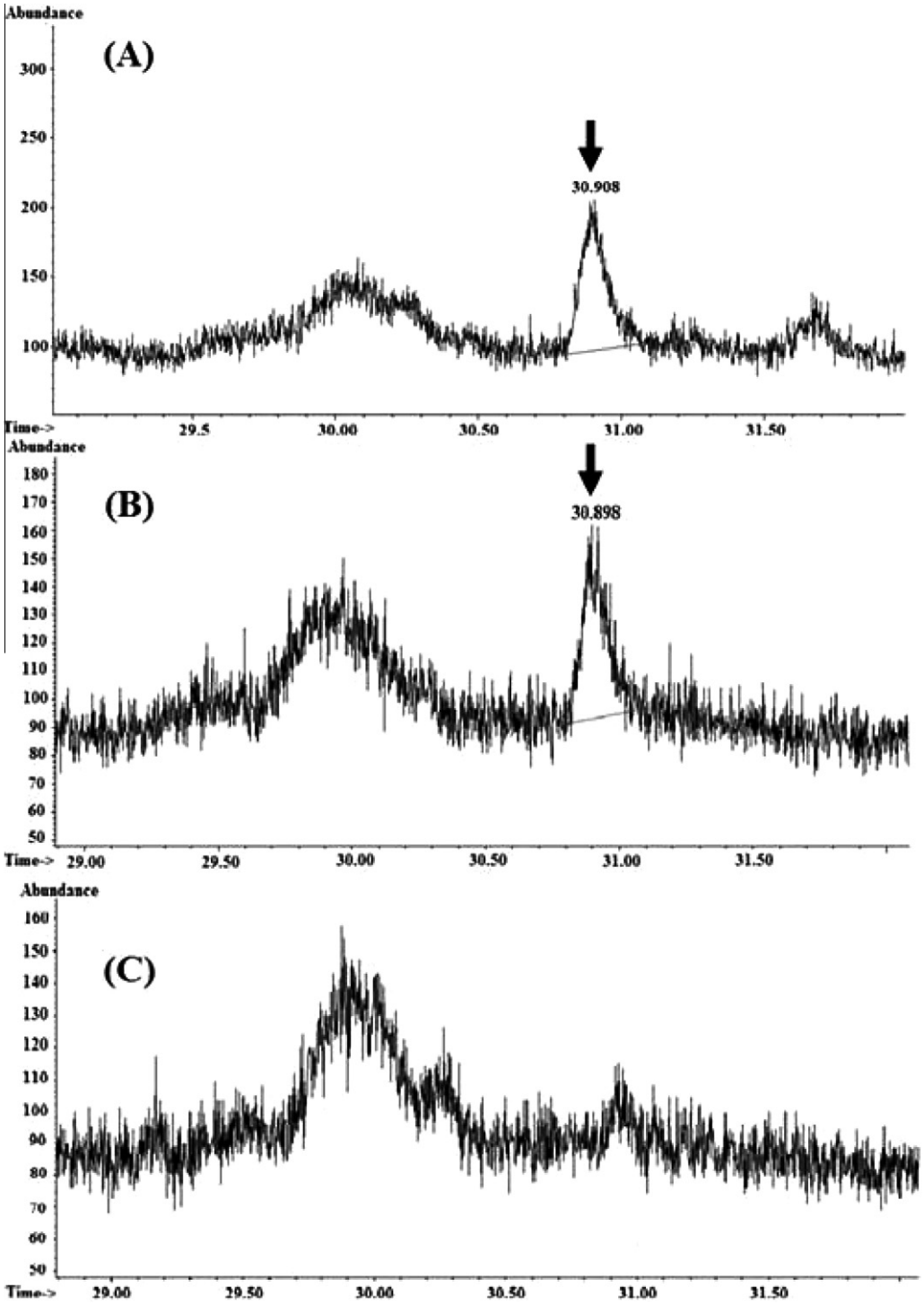

Figure 4. GC–MS analysis to detect the presence of celecoxib from plasma sample of mice after oral administration of celecoxib containing microcapsules (A), free celecoxib (B) and control (C). The calculated concentration of celecoxib from celecoxib containing microcapsules and free celecoxib were 44 ± 50 and 42 ± 60 ppb, respectively. Treatment protocol was started from day 1 to day 4 days at 4 μg/g body weight/day and mice were sacrificed on day 5. Plasma was collected for analysis. Arrow indicates the peak corresponding to celecoxib in the samples analyzed.

reports the subsequent pharmaceutical evaluation for the bioactivity of those microcapsule containing ingredients.

Celecoxib is among the second generation of non-steroid anti-inflammatory drugs being widely used worldwide. However, its low water solubility affects its gastrointestinal absorption.[19] Here, we have successfully produced chitosan based microcapsules and encapsulated celecoxib in jojoba oil as a demonstration model for our oral delivery model. Pathologically, cyclooxygenase-1 protein is continuously expressed, while it is suggested that cyclooxygenase-2 will be expressed only under inflammation condition. However, it was recently reported that the mRNA of cyclooxygenase-2 could be detected in liver tissues of normal mice.[20] Our results show that chitosan/celecoxib microcapsules display better inhibitory activity on cyclooxygenase-2 protein from liver sections of treated mice when comparison is done with oral administration of free celecoxib. The plasma concentration of celecoxib was comparable after a four-day treatment protocol. More importantly, this drug delivery model did not exert additional load on the liver function. We used a treatment protocol of 4 μg/g/day for four days, which really resembled the actual clinical condition in which patient with inflammation is prescript with celecoxib at 200 mg/day for four days, assuming an adult body around 50 kg. However, what we still need to discuss includes the further improvement of drug loading efficiency. There are several factors presumably involved, including the choice of oil, which should mediate good drug solubility, but at the same time should be beneficial to the cardio-vascular system. In this respect, the safety oral administration of jojoba oil was reported.[21] Despite the fact that the solubility of celecoxib in jojoba oil was considerably high, different therapeutic drugs may have variable solubility in different types of oils so the balance of drug solubility and health beneficial factors would be one of the important criteria to be considered. Other factors to be considered are the chitosan concentration used, the ratio of dissolved chitosan and drug dissolved oil as well as the procedure including coacervation, centrifugal extrusion, emulsion hardening, which still need to be optimized.

Currently, many patients suffering from various diseases need to receive oral administration and/or intravenous injection of therapeutic regimens. Unfortunately, some of the orally administrated drugs are found to have poor water solubility while some patients are found to have allergic response towards intravenous injection. Here we describe the use of the microencapsulation technology to encapsulate celecoxib as a preliminary example of obtaining an improved pharmacological activity with the free counterpart when animal models are employed. The development of the microcapsules related biomaterials as oral drug delivery vehicles may be an alternative administrative pathway to improve the efficiency of the therapeutic actions from the time-course-release efficiency and absorption point of view.

Collectively, we have successfully produced chitosan based microcapsule and encapsulated celecoxib. The chitosan/celecoxib microcapsules were characterised with respect to their biological activity in comparison to that displayed by free celecoxib. The produced chitosan/celecoxib microcapsules showed a significant inhibition of cyclooxygenase-2 protein expression when compared with vehicle control mice. Interestingly, microcapsule containing celecoxib showed a better inhibition of cyclooxygenase-2 protein expression in mouse hepatocytes when compared with a simple oral administration of celecoxib. Our chitosan based microcapsule system did not exert toxicological adverse effect on mice livers. Furthermore, celecoxib was detected at similar plasma concentration levels in mice after oral administration of celecoxib containing microcapsules and free celecoxib. Further developments of the microcapsules related biomaterials as oral drug delivery vehicles may help to improve the efficiency of the therapeutic actions from the convenience and absorption point of view.

Acknowledgements

The authors acknowledge the financial support (1ZV-5L) to C.-H.C. R.G. is sponsored by AIRC (Italian Association for Cancer Research). This work forms part of the Ph.D. thesis of S.-Y.C.

References and notes

1. Nelson, G. *Rev. Prog. Color.* **2001**, *31*, 57.
2. Yoshizawa, H. *Kona.* **2004**, *22*, 23.
3. Nelson, G. *Int. J. Pharm.* **2002**, *242*, 55.
4. Mei, W. P. *J. China Text. Inst.* **1995**, *5*, 188.
5. Simon, B. *Microencapsulation: Methods and Industrial Applications*; New York: CRC Press, 2006.
6. Wang, L. Y.; Ma, G. H.; Su, Z. G. *J. Controlled Release* **2005**, *106*, 62.
7. Yang, Z. Q.; Song, B. Z.; Li, Q. X.; Fan, H. F.; Fan, O. Y. *China Particuology* **2004**, *2*, 70.
8. Wang, L. Y.; Gu, Y. H.; Su, Z. G.; Ma, G. H. *Int. J. Pharm.* **2006**, *311*, 187.
9. Hsieh, W. C.; Chang, C. P.; Gao, Y. L. *Colloid Surf., B Biointerfaces* **2006**, *53*, 209.
10. Kosaraju, S. L.; D'ath, L.; Lawrence, A. *Carbohydr. Polym.* **2006**, *64*, 163.
11. Peng, X. H.; Zhang, L. *J. Biobased Mater. Bioenerg.* **2008**, *2*, 64.
12. Hafner, A.; Filipovic-Grcic, J.; Voinovich, D.; Jalsenjak, I. *Drug Dev. Ind. Pharm.* **2007**, *33*, 427.
13. *Chemicals and reagents*. Unless specified, all the chemicals and reagents were purchased from Sigma–Aldrich. 1% Chitosan (CTS, practical grade, >85% deacetylation) was dissolved in 1% acetic acid followed by filtration to remove insoluble impurities. Celecoxib, as used in the treatment of osteoarthritis, rheumatoid arthritis, acute pain, painful menstruation and menstrual symptoms, and to reduce numbers of colon and rectum polyps in patients with familial adenomatous polyposis, was obtained from Pzifer pharmaceutical company. Jojoba oil (obtained from *Simmondsia chinensis*) was obtained from the Easy Creation Asia Limited.
14. *Synthesis of chitosan/celecoxib microcapsules*. Fifty micrograms of celecoxib was firstly dissolved in 5 ml of jojoba oil. The mixture of celecoxib/jojoba oil was poured into 50 ml of acidic chitosan solution followed by intermixing for 10 min using a magnetic stir plate, Heidolph MR3001 (Heidolph, Germany), at a speed of 1200 rpm to form o/w emulsion. The emulsion was then further mixed using an ultrasonic processor (Vibra Cell™ VCX 750, Sonics and Materials Inc., USA) with the ultrasonic amplitude being 85% for 1 min to break down the emulsion into smaller droplets. Fine emulsion was produced without coalescence. 1% sodium hydroxide solution was slowly dipped into the emulsion for precipitation; and the precipitates were formed when the pH value reached around 7.5. Afterwards, pH value was further adjusted to pH 10.5 by sodium hydroxide solution to ensure complete precipitation. The precipitates, that is, chitosan/celecoxib microcapsules were rinsed with hexane followed by deionised water and spun down using a centrifuge in order to remove the unwanted materials including excess chitosan and uncapsulated oil as well as celecoxib. Analytical work for chitosan/celecoxib microcapsules. The morphology of novel synthesized chitosan/celecoxib microcapsules was recorded by scanning electron microscopy. The range and mean of particle size of microcapsules was evaluated with a zetasizer (Malvern Instruments, Zetasizer 3000 HSA). The microcapsules were firstly diluted with deionised water at the ration of 1:20 and 4 ml of the solution was taken for evaluation. Encapsulated celecoxib was extracted with ethanol and purified from known amount of microcapsules. The concentration of isolated celecoxib was further determined by absorption spectrophotometry and compared with the celecoxib positive reference curve.
15. *Animal care and mice treatment*. Eight weeks old C57BLC mice, weighing approximately 20–25 g, were purchased from the animal unit of The Chinese University of Hong Kong and maintained in a conventional sanitary facility, in accordance with the institutional guidelines on animal care, with the required consistent temperature and relative humidity. All the procedures were approved by the Animal Research Ethics Committee. A total of nine mice were included in our preliminary study and they were divided into three groups, respectively. The free celecoxib was mixed directly with a physiological saline solution containing 0.5% methylcellulose and 0.025% Tween 20 while the chitosan/celecoxib microcapsules were also mixed with the physiological saline solution. From day one to day four, treatment groups received celecoxib or chitosan/celebrex microcapsules once daily at a concentration of 4 μg/g of body weight. The concentration of free celecoxib solution was adjusted to the same as the celecoxib in the chitosan/celecoxib. Control group received the same volume of physiological saline orally. The animals were monitored and recorded if there had any abnormal behaviors. On day 5, all the mice were sacrificed and autopsies was performed to collect peripheral blood and livers for analytical chemistry and pathological analysis.
16. *Immunohistochemistry analysis of cyclo-oxygenase 2 protein expression*. Sections of mouse liver from autopsy samples were dewaxed with xylene and gradient concentrations of ethanol. Possible endogenous peroxidase was blocked and slides were washed with PBS. Slides were then blocked again and treated with diluted primary antibody (rabbit anti-rat cyclo-oxygenase 2) in PBS. Slides were washed with PBS and then treated with the secondary antibody CSA II rabbit link. After washing, slides were further treated with amplification reagent and anti-fluorescein-HRP. Afterwards, slides were incubated with DAB

substrate. Nuclei were stained with haematoxylin and finally slides were inspected under a light microscope.
17. Whole blood was collected after mice were sacrificed and plasma was isolated after centrifugation. Afterwards, plasma liver enzymes including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by the Vet biochemistry assay kits for the IDEXX laboratories machine in order to determine whether there are any liver failure phenomena from chitosan/celecoxib microcapsules treated groups of mice by comparing with the free celecoxib group and vehicle treatment group as well as the normal control ranges.
18. *Plasma concentration of celecoxib*. Plasma concentration of celecoxib from mice after the four day treatment protocol was determined semi-quantitatively by gas-chromatography–mass-spectrometry (GC/MS) and compared with the celecoxib positive reference curve.
19. Babu, G. V. M. M.; Shankar, V. G.; Sankar, K. H.; Seshasayana, A.; Kumar, N. K.; Murthy, K. V. R. *Indian J. Pharm. Sci.* **2001**, *6*, 588.
20. Okamoto, T.; Yamamura, K.; Hino, O. *Jpn. J. Pharmacol.* **2000**, *83*, 359.
21. Verschuren, P. M.; Nugteren, D. H. *Food Chem. Toxicol.* **1989**, *27*, 48.