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計畫編號：NHRI-GT-EX89E815P

國家衛生研究院八十九年度整合性醫藥衛生科技研究計畫

靈芝子實體作為皮膚取代物之探討研究

年度成果報告

執 行 機 構：台北醫學大學

計 畫 主 持 人：蘇慶華

執行期間：88 年 7 月 1 日 至 89 年 12 月 31 日

計畫名稱：靈芝子實體作爲皮膚取代物之探討研究

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研究人員：附表於後

關鍵字：Ganoderma , N-acetyl-D-glucosamine , Chitin , artificial skin

壹、八十九年度計畫研究成果摘要

總摘要：

靈芝長久以來爲藥用真菌之重要成員。本研究利用靈芝子實體做爲材料以酒精、熱水、熱鹼及漂白過程逐步萃取。由各層萃取物中取得可供利用之成份，改變過去於熱水萃取後無法利用之靈芝廢料，並轉成爲高價值之人工皮膚創傷覆被材。其成分以 poly-β-N-Acetylglucosamine 以及 β-1-3-glucan 爲主之纖維狀材質，經冷凍乾燥後編織成膜做爲皮膚創傷覆被材之研究材料。

本計畫以第一子計畫，「靈芝子實體菌絲成分組成及備製」爲核心以 18 種不同種靈芝製成之覆被材薄膜，進行成分分析及促進癒傷效用之比較，結果發現各種靈芝均可製成薄膜其成份之組成並無顯著差異，因此本研究以栽培最廣，材料取得最容易之松杉靈芝(Ganoderma tsugae)製備成薄膜供其他子題實驗材料。成分分析分別利用酸水解法，酵素水解法，再以 HPLC 及 TLC 分析單糖組成，並以 NMR，IR 圖譜分析發現靈芝薄膜爲 60%之 Chitin 及 40%之β-1-3-glucan 共構組成，故命名爲 SACCHACHITIN。

SACCHACHITIN 提供第二子計畫「靈芝薄膜之機械性及物理性質分

析」顯示 SACCHACHITIN 具有當良好之吸水性，並在拉力及張試驗顯示在使用上達到滿意之作用，爲增加 SACCHACHITIN 於溼潤狀態下之拉力，以 LiCl/Dimethylamine(DMA)雙極溶劑系統溶解後，再以水置換成爲均質之透明膜，在拉力，厚度及成品外觀上更加改善，其成分因溶解之關係且 Chitin 含量較高並另名爲 GANOCHITIN，亦爲本研究之另一進展。

SACCHACHITIN 在癒傷的作用於本計畫第三子題研究計畫「靈芝薄膜癒傷效果之評估」中進行，以大白鼠及天竺鼠爲動物模式在動物之背部以脊椎爲中心切除 $2\times2cm^2$ 之對稱傷口後敷被以 SACCHACHITIN，日製 BESCHITIN(以蟹殼純化之 Chitin 商業化產品)，及紗布進行貼敷，比較傷口面積減少之過程，發現 SACCHACHITIN 之傷口癒合最爲迅速，此紗布之動物平均完成癒合的時間縮短 6-10 天。SACCHACHITIN 亦提供某獸醫院做爲動物皮膚受傷之治療，目前共有 10 例對直徑 20 公分以上之受傷家犬，於貼覆後均可在 35 天癒合重新長毛。而人體試驗部分亦正於台北醫學大學萬芳醫院外科部進行對於褥創之治療果評估，預計於本期計畫到結束即可完成試驗。

SACCHACHITIN 之癒會機轉於本研究計畫之第四子計畫中，以酵素，細胞及動物模式進行，顯示 SACCHACHITIN 具有 1)對於基質金屬型蛋白質水解酶(Matrix metalloproteinase， MMP)第 1，8，9 型均有結合及抑制作用，避免由 Neutrophils 或微生物分泌之此類酵素過度分解傷口中之膠原蛋白，促進傷口癒合。2)傷口中以免疫酵素染色法中，顯示 SACCHACHITIN 促進第I型膠原蛋白之累積，也印證第一項之結果。3)天竺鼠纖維母細胞(Fibroblasts)及人類角質細胞(Keratinocytes)在 SACCHACHITIN0.1%共同培養下，不但不具毒性，且有促進增殖之作用，毒性試驗亦證實

SACCHACHITIN 不具有細胞毒性。4)SACCHACHITIN 在大白鼠試驗中，證實不具免疫性反應，也就是不具過敏反應或排斥。5)比較 PCNA(Proliferating cell nuclear antigen)易顯示 SACCHACHITIN 促進傷口癒合之作用。

本研究之結果顯示 SACCHACHITIN 爲一來源豐富，成品低廉，製作簡單，製造回收率高，並具有功效之皮膚創傷覆被材。同時與其他類似產品比較(如螃蟹殼來源)，由於製作流程更爲簡單，成本更爲低廉，SACCHACHITIN 及 GANOCHITIN 爲本研究之兩項產品。同時亦可衍生成爲 SACCHACHITOSAN 做爲 Chitosan 之來源，更可提高靈芝廢物之利用價值及產品之多樣性。

貳、八十九年度計畫著作一覽表

群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

若爲群體計畫，請勾選本表屬於：☐子計畫　　■總計畫(請自行整合)

列出貴計畫於本年度中之所有計畫產出於下表，包含已發表或已被接受發表之文獻、已取得或被接受之專利、擬投稿之手稿（manuscript）以及專著等。「計畫產出名稱」欄位請依「臺灣醫誌」參考文獻方式撰寫；「產出型式」欄位則填寫該產出爲期刊、專利、手稿或專著等，舉例如下：

序號	計畫產出名稱	產出型式	SCI*	致謝與否
例	Chang SF, Cheng CL. The suppression effect of DNA sequences within the C4A region on the transcription activity of human cyp21. Endocrine research. 1998, 24(3&4):625-630	期刊	V	V
1.	Su CH, Sun CS, Juan SW, Ho HO, Hu CH, Sheu MT. Development of fungal mycelia as skin substitutes:effects on wound healing and fibroblast.Biomaterials 1999.20:61-68	期刊	V	
2.	Su CH, Sun CS, Juan SW, Ho HO, Hu CH, Sheu MT. Development of fungal mycelia as skin substitutesII:effects on wound healing.1999.1(1):40-46	期刊		
3.	Tseng H, Lu JL, Su CH. Evaluation of Chemical Properties of Membrane from Ganoderma. Proceding of The 4th Biomedical Materials & Technology Symposium 1997, p13	研討會		
4.	Lin SC, Su CH, Tseng H. Study on Preparation of Membrane from Fungal Ganoderma Residue as Skin Substitute and its Solubility and Mechanical Properties. 臺北醫學院 87 年度師生聯合學術研究發表會 1998, A30	研討會		
5.	Analysis of cytotoxicity of Sacchachitin and its effects on local immuno-modulation and inflammation during skin wourd healing	研討會		
6.	Utilization of Fruiting Bodies from Genus *Ganoderma*.FUNGAL SCIENCE.	手稿		

*SCI：Science Citation Index，若發表之期刊爲 SCI 所包含者，請打勾。

*「致謝與否」欄位：若該成果產出有註明衛生署或國家衛生研究院委託資助字樣者，請打勾。

參、八十九年度計畫重要研究成果

群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料
若爲群體計畫，請勾選本表屬於：□子計畫　　■總計畫(請自行整合)

1. 計畫之新發現或新發明

本研究之成果推出三種由靈芝子實體料中發展出三種產品分別爲：

a. SACCHACHITIN：爲靈芝纖維編織成多孔性皮膚傷口癒合促進覆蓋膜。

b. GANOCHITIN：爲 SACCHACHITIN 之衍生產品，具同樣之皮膚傷口癒合促進作用，其強度更強，於施用時更爲穩固不易崩解。

c. SACCHACHITOSAN：由 SACCHACHITIN 衍生之 Chitosan 產品，爲具免疫調節功能之可溶性多糖。

以上三種均已申請專利或申請專利中。

本研究亦發現靈芝子實體廢料製成之薄膜對於大白鼠，天竺鼠及家犬均有傷口癒合促進之功能其機轉可解釋爲 1)促進組織纖維母細胞及角質細胞之增殖及轉移。2)促進傷口中膠原蛋之形成。3)抑制基質金屬型蛋白水解酶第I型，第VIII型，第IX之酵素活性。

2. 計畫對學術界或產業界具衝擊性（impact）之研究成果

本研究之成果可提供產業界：

a. 靈芝在萃取三萜類及水溶性多糖後之廢料可發展成更高價值之皮膚覆被材，並具顯著的傷口癒合作用。亦即本研究提供產業界一個「完全利用靈芝子實體」有效而產率高之技術及方法。

b. 由於靈芝子實體可完全利用，產業界對於靈芝產品之總單位成本下降也就是提升各項產品之價值及競爭力。

c. 靈芝過去只做爲健康食品，本研究延伸並提升靈芝的用途至 Chitin 及 Chitosan，並提供 Chitin 及 Chitosan 廉價的來源。

對學術而言，本研究爲創先以靈芝解釋其癒傷的機轉。

3. 計畫對民眾教育之研究成果：

靈芝可做爲健康食品以外的醫藥用途，在提出苦味的三萜類及多糖體後剩下的殘渣做一些簡單的純化過程，即可變成人工皮膚功能的癒傷促進薄膜。對皮膚受到燒燙傷，挫創，或者久傷不癒的褥創，糖尿病病人不易癒合的傷口，提供一種治療的途徑。

肆、八十八年度計畫所培訓之研究人員

群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料若為群體計畫，請勾選本表屬於：□子計畫　　■總計畫(請自行整合)

種類			人數	備註
專任人員	1. 博士後研究人員	訓練中		
		已結訓		
	2. 碩士級研究人員	訓練中	4	
		已結訓		
	3. 學士級研究人員	訓練中	4	
		已結訓		
	4. 其他	訓練中	2	臨時工
		已結訓		
兼任人員	1. 博士班研究生	訓練中	1	
		已結訓		
	2. 碩士班研究生	訓練中	2	
		已結訓		
醫師		訓練中	1	
		已結訓		
特殊訓練課程				

伍、八十八年度重要研究成果產出統計表

群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

若爲群體計畫，請勾選本表屬於: □子計畫　　■總計畫(請自行整合)

(係指執行八十八年度計畫之所有研究產出成果)

<table>
<tr><td colspan="3">科技論文篇數</td><td colspan="3">技術移轉</td><td rowspan="2">技術報告
篇</td></tr>
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篇</td><td>篇</td><td>技　術
輸　入</td><td>千元</td><td>項</td><td>技術創新
項</td></tr>
<tr><td>研討會
論　文</td><td>1
篇</td><td>篇</td><td>技　術
輸　出</td><td>千元</td><td>項</td><td>著作權
(核准)
項</td></tr>
<tr><td>專　著</td><td>篇</td><td>篇</td><td>技　術
擴　散</td><td>千元</td><td>項</td><td>專利權
(核准)
項</td></tr>
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期刊論文：指在學術性期刊上刊登之文章，其本文部份一般包含引言、方法、結果、及討論，並且一定有參考文獻部分，未在學術性期刊上刊登之文章（研究報告等）與博士或碩士論文，則不包括在內。

研討會論文：指參加學術性會議所發表之論文，且尚未在學術性期刊上發表者。

專　　著：爲對某項學術進行專門性探討之純學術性作品。

技術報告：指從事某項技術之創新、設計及製程等研究發展活動所獲致的技術性報告且未公開發表者。

技術移轉：指技術由某個單位被另一個單位所擁有的過程。我國目前之技術轉移包括下列三項：一、技術輸入。二、技術輸出。三、技術擴散。

技術輸入：藉僑外投資、與外國技術合作、投資國外高科技事業等方式取得先進之技術引進國內者。

技術輸出：指直接供應國外買主具生產能力之應用技術、設計、顧問服務及專利等。我國技術輸出方包括整廠輸出、對外投資、對外技術合作及顧問服務等四種。

技術擴散：指政府引導式的技術移轉方式，即由財團法人、國營事業或政府研究機構將其開發之技術擴散至民間企業之一種單向移轉（政府移轉民間）。

陸、參與八十八年度計畫所有人力之職級分析

群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

若爲群體計畫，請勾選本表屬於：□子計畫　　■總計畫(請自行整合)

職級	所含職級類別	參與人次
第一級	研究員、教授、主治醫師	4人
第二級	副研究員、副教授、總醫師	3人
第三級	助理研究員、講師、住院醫師	5人
第四級	研究助理、助教、實習醫師	人
第五級	技術人員	人
第六級	支援人員	人
合計		12人

〔註〕

第一級：研究員、教授、主治醫師、簡任技正，若非以上職稱則相當於博士滿三年、碩士滿六年、或學士滿九年之研究經驗者。

第二級：副研究員、副教授、助研究員、助教授、總醫師、薦任技正，若非以上職稱則相當於博士、碩士滿三年、學士滿六年以上之研究經驗者。

第三級：助理研究員、講師、住院醫師、技士，若非以上職稱則相當於碩士、或學士滿三年以上之研究經驗者。

第四級：研究助理、助教、實習醫師，若非以上職稱則相當於學士、或專科滿三年以上之研究經驗者。

第五級：指目前在研究人員之監督下從事與研究發展有關之技術性工作，且具備下列資格之一者屬之：具初（國）中、高中（職）、大專以上畢業者，或專科畢業目前從事研究發展，經驗未滿三年者。

第六級：指在研究發展執行部門參與研究發展有關之事務性及雜項工作者，如人事，會計、秘書、事務人員及維修、機電人員等。

柒、參與八十八年度計畫所有人力之學歷分析

群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

若爲群體計畫，請勾選本表屬於：□子計畫　　■總計畫(請自行整合)

類別	學歷別	參與人次
1	博士	6人
2	碩士	2人
3	學士	3人
4	專科	人
5	博士班研究生	人
6	碩士班研究生	1人
7	其他	2人
合計		14人

捌、參與八十八年度計畫之所有協同合作之研究室

群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

若爲群體計畫，請勾選本表屬於：□子計畫　　■總計畫(請自行整合)

機構	研究室名稱	研究室負責人
台北醫學大學	生物醫學材料研究所	蘇慶華
台北醫學大學	細胞及分子生物學研究所	蔡郁惠
台北醫學大學	醫學研究所材料研究室	曾厚
台北醫學大學	附設醫院皮膚科	胡俊弘
台北醫學大學萬芳醫院	外科	簡正義

玖、八十八年度之著作抽印本或手稿

依「貳、八十八年度計畫著作一覽表」所列順序附上文獻抽印本或手稿。

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Development of Fungal Mycelia as Skin Substitutes II: Effects on the Wound Healing Process

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Key Words
Ganoderma tsugae
Fungal mycelia
Wound healing
Macrophage
Inflammation
Implantation

ABSTRACT

The effect of SACCHACHITIN membrane, a skin substitute prepared from the residue of the fruiting body of *Ganoderma tsugae*, on wound healing was evaluated in this study. Two mirror image wound areas were excised on the back of female Wistar rats by dissecting a 2.0 × 2.0 cm^2 skin area of full thickness. SACCHACHITIN membrane was placed randomly on one of the wounds and gauze on the other. Changes in the wound area were examined after a predetermined amount of time postoperatively. Histological examination of the wound and surrounding tissue was also performed but only on the 4th, 7th, and 16th days post-operation. The interaction of tissue with the dressing was evaluated by the implantation of these 2 materials. The results show that the wound area covered with SACCHACHITIN membrane was statistically significantly smaller than that covered with gauze for all time points measured. Histological examination revealed that SACCHACHITIN membrane induced mild inflammation and stimulated aggregation of polymorphonuclear leukocytes around the margin of the wound. The large number of macrophages and giant cells which infiltrated into the wound area covered with gauze indicates a response to a foreign body.

INTRODUCTION

One of the most urgent goals in the treatment of skin trauma is to provide an effective way of protecting any surface exposure of the skin. By covering with a suitable wound dressing, loss of body fluids is minimized, body temperature is maintained, infection of the wound area is controlled, and pain is relieved. Optimally, when the wound-healing process is accelerated, the original appearance and functions of the skin can be restored. Nowadays, the major method of treatment is skin grafting.However, the source of the

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skin and the cost of the medical treatment are problems that still need to be resolved. Substitution of skin with different materials as dressings appears to be an alternative choice.[1] Chitin (polymeric N-acetyl-D-glucosamine) is well known for its wound healing characteristics and has become one of the most important wound dressings in recent years.[2-5] Dating back to 1970, Prudden *et al.* demonstrated by their standard technical assay that chitin possesses an excellent acceleratory capacity for topical use, which was definitely superior to that of cartilage.[6] As a result, a whole new era was opened up for studying the wound-healing ability of chitin isolated from crab shell.[7-10] A product made of chitin from crab shell under the trade name BESCHITIN® W has been marketed by the Morihita Resere Co. of Japan.

Ganoderma tsuage, whose Chinese name is *Lingzhi*, has long been an important member of the medicinal fungi used in the Asian area, including Taiwan and Japan.[11-13] However, after hot water extraction of the water-soluble fraction of *Ganoderma*, the resulting water-insoluble part (more than 90%) remains unused and is treated as waste. Recently, the mycelia components of Ganoderma were analyzed and revealed to be 40% chitin with 60% β-1,3-D-glucan.[14] Therefore, the possible functions of β-1,3-D-glucan and its synergetic effects with chitin could possibly make it an ideal biomaterial for use in wound dressings. In addition, since the extracted waste from *Ganoderma* contains the fibril structure of mycelia, it could be directly knitted into a membrane without requiring dissolving and fibril separation processes. The potential usefulness of this biomaterial as a wound dressing and its inherent advantages encouraged us to investigate its possible effects on the wound-healing process.

MATERIALS AND METHODS

Materials

The residue of the fruiting body of *Ganoderma tsugae*, a generous gift from a factory in Natuao, Taiwan, was collected after hot water extraction twice. Ketamine HCl was supplied by Sigma Co. (St. Louis, MO). Pentobarbital sodium was purchased from Siegfried Zofingen (Switzerland). Female Wistar rats, weighing from 300 to 410 g, were obtained from the Animal Center, National Taiwan Univ. Analytical-grade reagents were obtained from Merck Co. (Germany).

Preparation of the SACCHACHITIN Membrane

The purification of fibers to form the SACCHACHITIN membrane followed a similar procedure to that reported in a previous paper.[14] The fibers, with lengths ranging from 10 to 50 μm, were collected and dispersed in deionized water to form a suspension, which was subsequently filtered. The membrane formed on the filter paper was then freeze-dried (EYELA, model FD-5N) to obtain a porous membrane with a diameter of 7 cm and thickness of 0.1 to 0.2 mm for the following studies. The chemical constituents of the final product were determined to be 40% N-acetyl-D-glucosamine and 60% β-1,3-D-glucan. The membranes were autoclaved and kept under aseptic conditions until use.

Wound-Healing Studies

Prior to the study, rats were anesthetized separately with ketamine (35mg/kg) and pentobarbital (12 mg/kg) dissolved in water for injection via the abdominal route. The dorsal and abdominal hair of the rats was removed with an electric razor. The method proposed by Kaufman was followed to prepare skin trauma.[15] Two equal mirror-image areas were marked in between the 12th rib and iliosacral joint on the dorsal area of the rats and 1 cm from the spinal cord. Two pieces of full-thickness skin, each with a surface area of about 2.0 × 2.0 cm^2, were excised. The method of excision was similar to that reported by Smahel *et al.*[16] The depth of the excised area was as deep as the panniculus carnosus. After cleaning off the blood residues with gauze and 0.9% saline solution, one of the lesions was randomly chosen and covered with cotton gauze for comparison. The other side was covered with a SACCHACHITIN membrane as prepared above, being equal in size to the cotton gauze. Both dressings were hydrated with 0.9% saline solution to promote adhesion of the dressings to the wound surface. Treated rats were placed in individual cages with an air-filtering device in a temperature range between 22 and 28 °C with no humidity control.

After surgery, changes in the area of the wounds were measured on the 4th, 8th, 12th, 16th and 20th days,

after which fresh dressings were applied. A modified method of Nangia[17] to calculate the wound area was employed. Generally, the outline of the wound area was marked on a transparent piece of paper and then transcribed to another piece of ordinary paper. A hand-held scanner (Proscan Gray) was used to capture the image, and data were stored as a monochromic BMP file. With proper adjustment, a computer program written with Visual Basic was employed to calculate the wound area so obtained. A total of 15 rats was included in this study, and results were reported as means with standard deviation. The statistical significance of any differences was analyzed by paired Student's *t*-test. Another 8 rats were treated by the same procedure as that above except that the wound area was not measured until the 12th day. The results were also examined by paired Student's *t*-test. This served as a basis for examining the effects of mechanical injury on changes in the wound areas due to dressing replacement.

Histological Analysis

On days 4, 7, and 16, one of the 15 rats from the above study was sacrificed with pentobarbital (100 mg/kg). Lesions with the surrounding tissue were excised in a deep-V manner. Specimens were then fixed in 10% formalin for 24 h and embedded in paraffin. Sections of appropriate thickness were sliced and examined using a Hitachi model S-2400 SEM.

Implantation

Implantation proceeded according to a modified method of Peluso.[18] All animals were anesthetized in the same way as described above for the relief of any suffering due to pain during operation. Autoclave-sterilized SACCHACHITIN membranes and gauze were separately implanted into the dorsal area of rats on 2 opposite sides of the spinal cord (in between the 12th rib and iliosacral joint, with a depth reaching the panniculus carnosus). The lesions were then sealed with 3-O nylon sutures. Rats were kept in individual cages and sutures were removed on day 7. On day 14, the implants were excised together with the surrounding tissue. Specimens were fixed in 10% formaldehyde for 24 h and embedded in paraffin. Sections of appropriate thickness were then sliced and examined by both light microscopy and scanning electronic microscopy (Hitachi model S-2400 SEM).

Scanning Electronic Microscopy (SEM) Examinations

Specimens were dried by immersion in a series of aqueous solutions of increasing alcohol content, followed by critical point drying using liquid CO_2 as the transfer medium. Dried samples were then loaded onto aluminum studs and coated with gold for 3 min at 8 mA under a pressure of 0.1 torr. The samples were scanned and examined using a Hitachi model S-2400 SEM.

RESULTS

Changes in the wound area covered with SACCHACHITIN or gauze were estimated on days 4, 8, 12, 16, and 20. The results were plotted as shown in Fig. 1 and demonstrate that the areas of wounds covered with SACCHACHITIN membrane measured at the above time points were significantly ($p < 0.05$) smaller than those of wounds covered with gauze. Effects of mechanical injury on the change in wound area due to the replacement of dressings were examined and the results are shown in Fig. 2. Comparison of the wound area measured on day 12 with the corre-

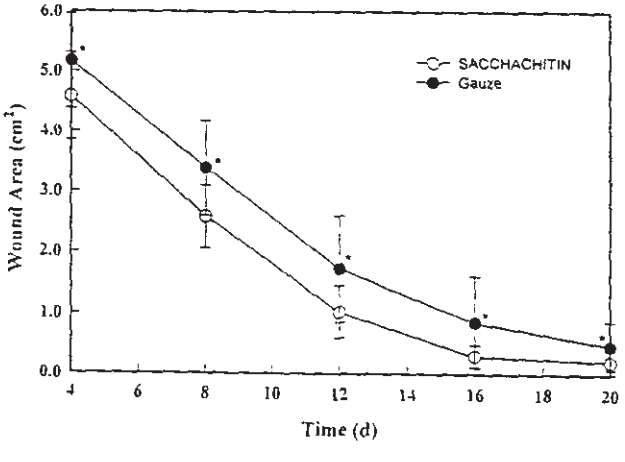


Fig. 1. Comparison of wound area changes between that covered with SACCHACHITIN membrane and with gauze at different time intervals. (*one-side paired *t*-test for significant difference with $p < 0.05$).

sponding data demonstrated no statistically significant effect of mechanical injury on the change in wound area ($p > 0.05$). This clearly indicates that any change in the wound area was not affected by mechanical stress imposed during the study, and that the data solely represents the difference between covering with SACCHACHITIN membrane and with gauze. Wound healing was thus accelerated when SACCHACHITIN membrane was used to cover the wound compared to when gauze was used.

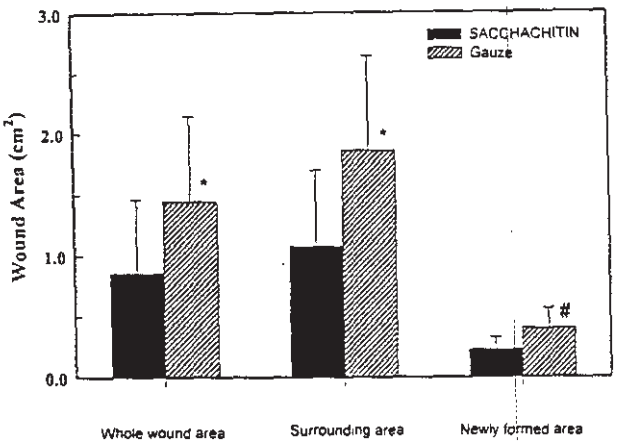
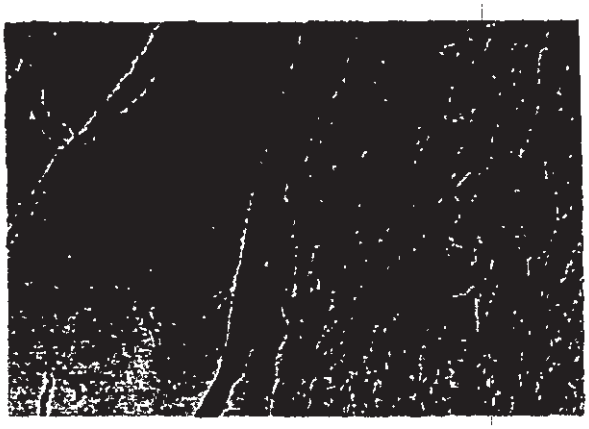


Fig. 2. Comparisons of the change of whole wound area, the area of newly-formed epithelia, and the surrounding area between that covered with SACCHACHITIN membrane and that with gauze on the 12th day. (*one-side paired t-test for significant difference with $p < 0.05$; # two-side paired t-test for insignificant difference with $p = 0.05$)

Histological examination of the wound tissue was conducted on days 4, 7, and 16. On the 4th day (Fig. 3a), a layer of exudate composed of polymorphonuclear cells and fibrous protein was found to have accumulated beneath the SACCHACHITIN membrane covering. Many new blood vessels had formed in the area close to the surface of the lesion, as well as underneath the newly growing epithelia. Inside the larger blood vessel, red blood cell stasis and margination of white blood cells were observed. A significant number of macrophages were present around the wound area. These phenomena indicate an acute inflammatory reaction and formation of granuloma granulation tissue. Fig. 3b shows similar phenomena for the wound area covered with gauze, except that a smaller amount of macrophages was observed.

By day 7, the growth of granuloma granulation tissue had become more apparent, and the presence of polymorphonuclear cells was observed in wounds covered with SACCHACHITIN membrane. Cell densities were higher and the matrix materials in the extracellular region had decreased. A photomicrograph is shown in Fig. 4a. In Fig. 4b, the existence of granuloma granulation tissue can be observed but not very clearly. In addition, the cell density was correspondingly lower in the wound covered with gauze compared to that covered with SACCHACHITIN membrane.

(a)



(b)

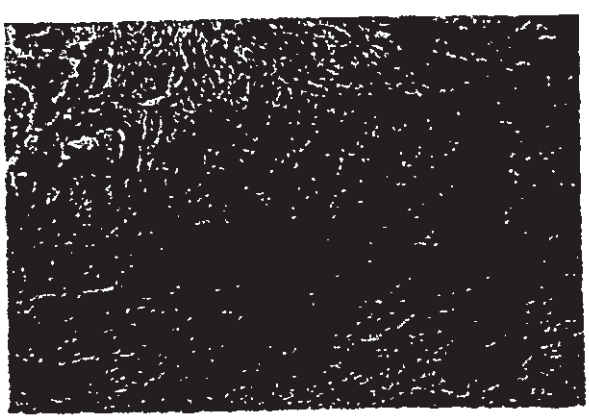


Fig. 3. Photomicrographs (× 100) of the wound area and its surrounding tissue covered with either SACCHACHITIN membrane (a) or gauze (b) for 4 days.

(a) (b)

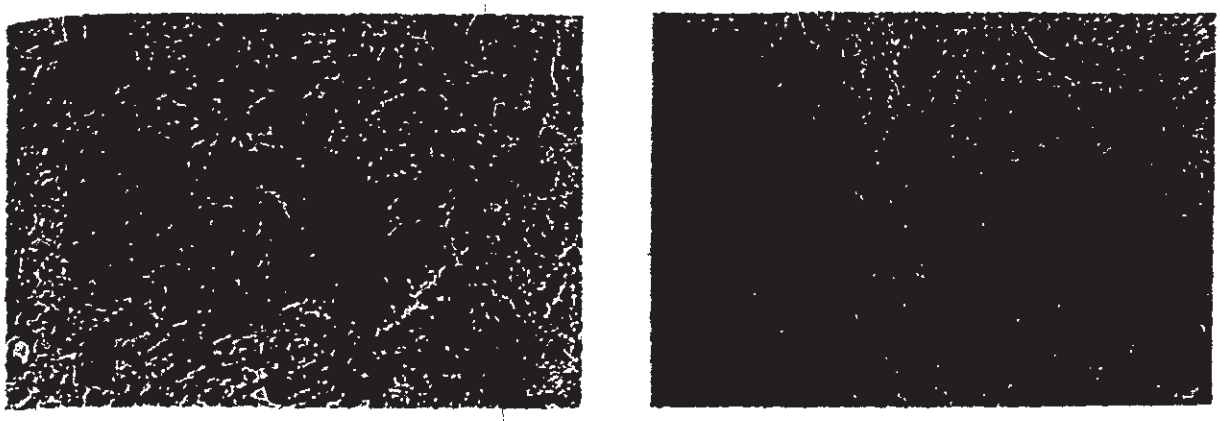


Fig. 4. Photomicrographs (× 100) of the wound area and its surrounding tissue covered with either SACCHACHITIN membrane (a) or gauze (b) for 7 days.

Figs. 5a and 5b show the results of the wound-healing process on day 16 when covered with SACCHACHITIN membrane for regions closer to and away from the center of the wound, respectively. The differentiation of the epithelia appeared to be quite good. A gradual change in the dermal region of the wound area was also observable: the closer the region to the center of the wound, the less the extent of differentiation of the epithelia observed, and granuloma granulation tissue appeared to be more obvious. Even then, the presence of mast cells fused with polymorphonuclear cells was observed; and in regions more distant from the center of the wound, the epithelia were well differentiated and a fibrous structure had formed with an abundance of matrix materials present extracellularly. On the contrary, granuloma granulation tissue was hardly observable on day 16 when the wound was covered with gauze (Fig. 6a). Only a small amount was found in the epithelia of regions closer to the wound. The dermal region had no mast cells and its major components were fibrous cells and extracellular matrix (Fig. 6b). However, the amount of extracellular matrix and level of cell density appeared to be lower compared to those observed with

(a) (b)

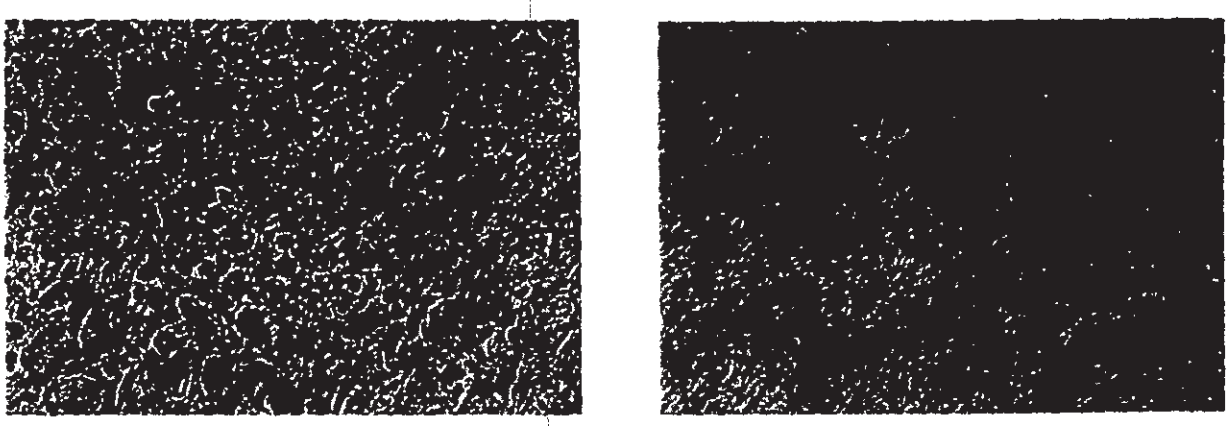


Fig. 5. Photomicrographs (× 100) of the region close to (a) or away from (b) the center of the wound area covered with SACCHACHITIN membrane for 16 days.

SACCHACHITIN membrane.

DISCUSSION

In 1977, Raju *et al.* suggested that an intense inflammatory response as a result of wound infection may greatly promote the differentiation of granuloma granulation tissue leading to better wound healing.[19] Franz *et al.* also discovered that a fetus wound infected by bacteria caused the same healing response as that observed in adults with the formation of a scar.[20] Although excessive inflammation leads to the death of tissue and a delay in wound healing, it is still recognized as beneficial to the body itself. It has been demonstrated that an appropriate inflammatory response can expel tissue debris, clean away foreign substances, minimize the chance of secondary infection, and promote the repair of tissue.[21-24] In the implantation study, an acute inflammatory reaction in the tissue was observed when the lesion was implanted with SACCHACHITIN membrane (Fig. 7a). It is believed that SACCHACHITIN membrane induces a similar phenomenon as a minor infection in the wound. This complies with what has been observed with the effect of SACCHACHITIN membrane on wound healing: on day 4, histological examinations revealed many more macrophages in the wound tissue. This was even more apparent on days 7 and 16. Furthermore, polymorphonuclear cells fused with macrophages were also apparent. This phenomenon of an accelerated wound-healing process is similar to that observed with the use of 20% benzoyl peroxide suspension[25] and also to that reported for chitin in the literature.[3]

In 1960, Prudden *et al.* discovered that cartilage was able to accelerate wound healing.[6] Furthermore, N-acetyl-D-glucosamine was shown by the same group to be responsible for acceleration of wound healing by chitin. Since SACCHACHITIN membrane is composed of 40% chitin and 60% β-1,3-D-glucan and a similar effect was observed in the wound healing process as with by chitin, its main mechanism of accelerating wound healing most likely can be attributed to its chitin component. Nevertheless, β-1,3-D-glucan itself is a strong activator of macrophages and is capable of attracting polymorphonuclear white cells.Since activated macrophages are known to accelerate wound healing,[26] the important role played by β-1,3-D-glucan can not be excluded.

Compared to SACCHACHITIN membrane, the macrophage reaction to foreign substances was the main response in the implantation study with gauze. Nevertheless, a smaller amount of macrophages was observed in a histological examination of the wound tissue covered with gauze. The main reason for this is still unclear. A possible reason is that a layer of thick crust, which was formed from the exudate secreted by the wound during an earlier stage, effectively prevented contact of the gauze with the wound.During the healing of the wound, the crust together with a major part of the gauze detached resulting in them not being recognized as a foreign substance (Fig. 7b).

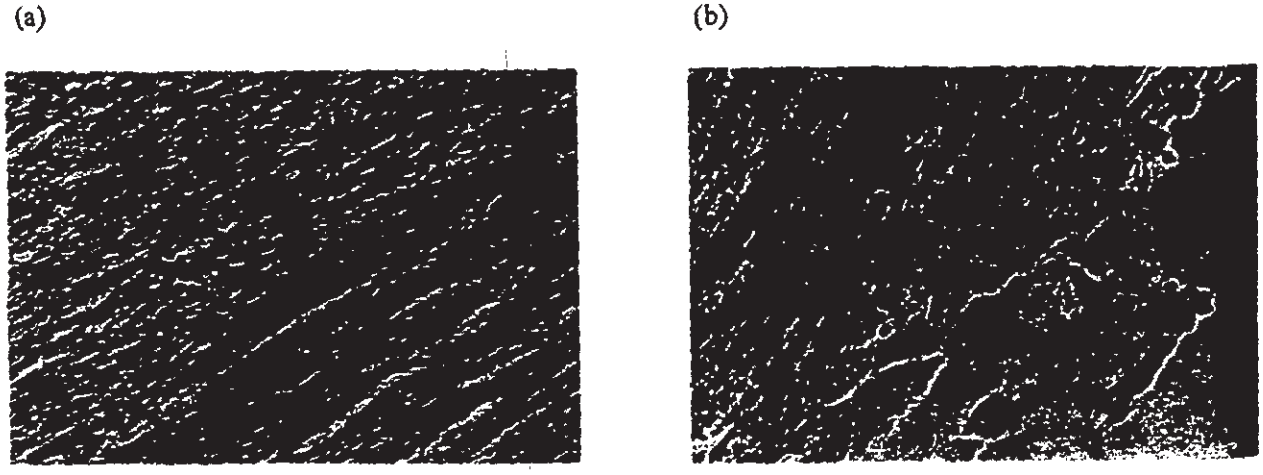


Fig. 6. Photomicrographs (× 100) of the region close to (a) or away from (b) the center of the wound area covered with gauze for 16 days.

A larger amount of exudate was found accompanying the wound when it was covered with SACCHACHITIN membrane. Furthermore, since chitin is able to attract an accumulation of polymorphonuclear white cells, a blue layer composed of cells involved in acute inflammation was found underneath the SACCHACHITIN dressing on day 4 during the implantation study. It appears likely that chitinase released by dead, active, or disintegrated granular white cells might be responsible for the disintegration of the SACCHACHITIN membrane. The debris from this disintegration was possibly eliminated mainly by macrophages. Along with drying of the wound, a solidified crust gradually formed and blocked the SACCHACHITIN membrane at the edges. Therefore, the main influence of the SACCHACHITIN membrane on the wound might be during the first few days. Furthermore, the disintegration of the SACCHACHITIN membrane by granulocytes might be a potential reason for the acceleration of the wound-healing process.[27]

Except for controlling infection and eliminating dead tissue, granulocytes have no obvious influence on the wound-healing process.[28] In a study on comparing the effect of GM-CSF and G-CSF on wound healing, GM-CSF simultaneously increased the number of both granulocytes and macrophages resulting in an acceleration of the wound-healing rate. Although G-CSF increases the number of granulocytes, it has no effect on the number of macrophages or the wound-healing rate.[29] Furthermore, for those patients with a low white cell count, the tolerance of wounds to tension during the healing process was found to be maintained normally. On the contrary, it has been recognized that macrophages play a more important role in the wound-healing process.[21,27,30,31] Not only are they involved in phagocytosis of foreign substances and the expression of antibodies, but secretion of cytokines by macrophages can also influence several different kinds of cell function. In the implantation study, an abundance of macrophages was found in the wound area covered with the SACCHACHITIN membrane. It would thus be reasonable to assume that the healing effect of SACCHACHITIN membrane on the wound was due to the activation of macrophages. The literature provides further evidence that chitin is able to activate as well as to enhance the function of ma-crophages.[18,32] In addition, it has been shown that chitin influences the epithelial cells of blood vessels, lymphocytes, fibrous cells, and the secretion of cytokines. Since the SACCHACHITIN membrane is similar in terms of chemical structure to glycosaminolycans, it may serve in guiding the reconstruction of the connective tissue.[33] All in all, the accelerating effects of SACCHACHITIN membrane on the wound-healing process can be attributed to an integrated response of all these functions.

CONCLUSIONS

In conclusion, a minor inflammatory reaction to-

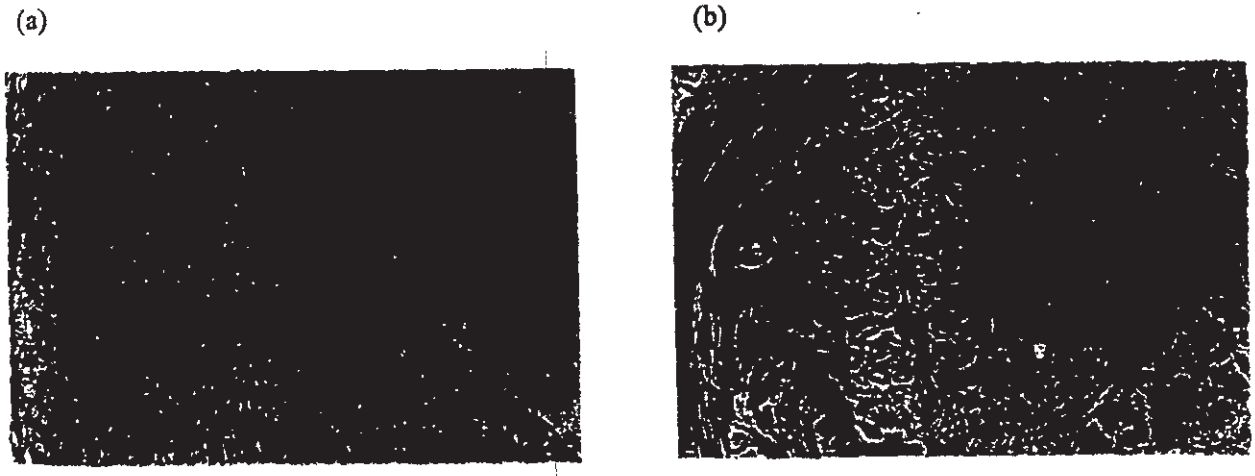


Fig. 7. Photomicrographs (× 100) of an acute inflammation response of the wound area to SACCHACHITIN membrane (a) and a foreign substance reaction of the wound area to gauze (b).

gether with the activation of macrophages to enhance the differentiation of granuloma granulation tissue are the possible mechanisms responsible for the acceleration of wound healing in the initial stage and for the increased tolerance in the wound area covered with SACCHACHITIN membrane. In several situations, such as chronic ulceration due to DM or bedsores, it would be preferable to have such a minor inflammatory reaction. On the contrary, excessive inflammation may cause the formation of scar tissue leading to a bad influence on the esthetic appearance of the skin. Fortunately, SACCHACHITIN membrane is just composed principally of fungal mycelia with no such differentiation ability as with bacteria.The self-limitating nature of SACCHACHITIN is another characteristic preventing excessive inflammation since it does not disintegrate markedly during healing of the wound.

ACKNOWLEDGEMENTS

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Development of fungal mycelia as skin substitutes: Effects on wound healing and fibroblast

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Abstract

In this study, Sacchachitin membrane, prepared from the residue of the fruiting body of *Ganoderma tsugae*, was estimated for its effects on wound healing and the proliferation and migration of fibroblast cells. Two mirror-image wounds were made on the back of female guinea pigs by dissecting a 1.5×1.5 cm^2 skin surface of full thickness. Sacchachitin membrane was placed randomly on one of the wounds and gauze or Beschitin® on the other. Changes in the wound area were measured and photographed after a predetermined amount of time postoperatively. Histological examination of the wound and surrounding tissue was also performed to reveal any interaction of tissue with the dressing. The results showed that the wound area covered with Sacchachitin membrane was statistically smaller than that covering with gauze on day 10, whereas there was no significant difference in the wound size compared to that with Beschitin®. Fibroblast cells from the dermis layer of guinea pigs were used. The number of fibroblast cells were counted on the predetermined days in the culture suspended with or without 0.01% w/v dressing materials. By layering on DMEM plates, the number of fibroblast cells migrating across the center line or outside of the central hole were counted after five days. All the results indicated that both 0.01% w/v of Sacchachitin and chitin significantly enhanced the proliferation and migration of fibroblast cells.
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Keywords: *Ganoderma tsugae*; Fungal mycelia; Wound healing; Fibroblast; Proliferation; Migration

1. Introduction

Wound healing is defined as the restoration of the continuity of living tissue and is an integrated response of several cell types to injury. It involves platelet aggregation and blood clotting, the formation of fibrin, an inflammatory response, alteration in the ground substance, endothelial and capillary proliferation and surface covering, regeneration of certain cell types, variable contracture and remodeling [1]. Healing is not complete until the disrupted surfaces are firmly knit by collagen. Generally, the use of a skin substitute to provide an environment conducive to healing is necessary [2]. In an endeavor to develop an ideal skin substitute, the performance of Sacchachitin membrane, prepared from the residue of the fruiting body of the medicinal fungus, *Ganoderma tsugae*, as an effective skin prosthesis has been examined [3]. This study evaluated the effectiveness of Sacchachitin membrane in the management of excised wounds in guinea pigs and compared its performance with gauze and Beschitin®.

A variety of wound models have been employed to study the wound healing process. The techniques that have been employed involve morphological examination of the wound size, histological examination of biopsied tissue samples, the detection of collagen content, the number of cells in the new connective tissue and epithelial layers [2], and the measurement of some biochemical parameters [1]. In conjunction with the area measuring technique and histological examination, evaluation of the effect of Sacchachitin on the proliferation and migration of fibroblasts in culture was included.

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2. Materials and methods

2.1. Materials

The residue of the fruiting body of *Ganoderma tsugae* was collected after hot water extraction twice and was a generous gift from a factory in Natuao, Taiwan. Beschitin-W®, made from crab chitin, is a commercial product of Morihita Resere Co. (Japan). Ketamine HCl and xylazine were supplied by Sigma Co. (St. Louis, MO, USA). Female guinea pigs, weighing from 380 to 480 g and aged from 8–10 weeks, were purchased from the Animal Center, National Taiwan University. Analytical-grade reagents were obtained from Merck Co. (Germany). Deacetylated Sacchachitin was obtained by dissolving Sacchachitin in 45% NaOH with heating. The resulting solution was dialyzed with tap water for two days and then with distilled water for one day resulting in a solution with a neutral pH. This solution was then freeze-dried to obtain deacetylated Sacchachitin. β-Glucan was the alkaline-soluble fraction of the polysaccharides obtained by treating the residue of the fruiting body of *Ganoderma tsugae* with 1 N NaOH at 90°C for 4 h.

2.2. Preparation of Sacchachitin membrane

The purification of fibers to form Sacchachitin membrane was followed a similar procedure that reported in the previous paper [3], except that the treatment with 1 N NaOH was conducted at 90°C for 4 h. The fibers with lengths in the range of 10–50 μm were then collected and dispersed in deionized water to form a suspension. The suspension was then filtered. The membrane formed on the filter paper was then freeze-dried (EYELA, model FD-5N) to obtain a porous membrane with a diameter of 7 cm and thickness of 0.1–0.2 mm for the following studies. The chemical constituents of the final product was determined to be 40% *N*-acetyl-D-glucosamine and 60% β-1,3-D-glucan. The membranes were autoclaved and kept under aseptic conditions until use.

2.3. Wound healing studies

Prior to the study, guinea pigs were anesthetized separately with Ketamine (35 mg kg^{-1}) and xylazine (5 mg kg^{-1}) by abdominal injection. The dorsal and abdominal hairs of the guinea pigs were removed with an electric razor. The method proposed by Kaufman was followed to prepare wounds [4]. Two equal mirror-image areas were marked on the dorsal area of the guinea pigs 1 cm apart from the spinal cord and in between the 12th rib and iliosaacral joint. Two pieces of full thickness skin, each with a surface area of about 1.5 × 1.5 cm^2, were excised. The method of excision was similar to that reported by Smahel [5]. The depth of the excised area was as deep as the panniculus carnosus. After cleansing off the blood residues with gauze and 0.9% saline solution, one of the lesions was randomly chosen and covered with an equal size of cotton gauze or Beschitin for comparison. The other side was covered with Sacchachitin membrane as prepared above. Both dressings were hydrated with 0.9% saline solution to promote the adhesion of the dressings to the wound surface. Treated guinea pigs were placed in individual cages with an air-filtering device in a temperature range between 22–28°C with humidity control.

After surgery, the area of the wound was measured on the 5th, 10th, 15th, and 20th days. Fresh dressings were replaced at the same time. A modified method of Nangia to calculate the wound area was employed [2]. Generally, the outline of the wound area was marked on a transparent paper and then transcribed to another piece of ordinary paper. A hand-held scanner (Proscan Gray) was used to capture the image and data were stored as a monochromic BMP file. With proper adjustment, a computer program written with Visual Basic was employed to calculate the wound area so obtained. A total of 15 guinea pigs were included in this study. The statistical significance of any difference was analyzed by a paired Student's *t*-test.

2.4. Histological analysis

On days 5, 10, 15, and 20, one of the guinea pigs from the above study was killed. Lesions with the surrounding tissue were excised in a deep-V shape. Specimens were then fixed in 36% formalin for 2–3 h and then cut into two halves to promote the infiltration of formalin into the tissue. Specimens were dehydrated with a tissue auto-treatment device (Sakura, RH-12E) and embedded in paraffin with a Paraffin Dispenser (Shadon, Lipshow). Sections of appropriate thickness (about 5 μm) were sliced (Sakura, IVS-400) and stained with haematoxylin and eosin. Entellan (Merck) was used to seal the specimen before examination using a Hitachi model S-2400 SEM.

2.5. Implantation

The implantation was according to the modified method proposed by Peluso [6]. All animals were anaesthetized in the same way as described above. A 1 cm square of autoclave-sterilized Sacchachitin was implanted into the lesion between the subcutaneous tissue and muscular membrane on the dorsal area of the guinea pigs 1 cm away from the spinal cord and in between the 12th rib and iliosaacral joint. On the opposite side, either gauze or Beschitin was implanted as a control. Both lesions were then closed with 3-O nylon sutures. Guinea pigs were kept in individual cages and the sutures removed on day seven. On day 14, the implants were excised together with the surrounding tissue. The specimens

were then fixed in 36% formaldehyde for 2–3 h and embedded in paraffin. Sections of appropriate thickness were then sliced and examined using a Hitachi model S-2400 SEM.

2.6. Scanning electronic microscopy (SEM) examinations

The Sacchachitin membrane samples harvested on day 9 from lesions were washed three times for 15 min with 0.1% cacodylate buffer solution (pH 7.4) containing 7% sucrose. The samples were then fixed in cacodylate buffer solution containing 1% OsO_4 for 1.5 h. After that, the samples were washed again according to the same procedure as described above. The specimens were dehydrated by immersion in a series of aqueous solutions of increasing alcohol content, followed by critical point drying using liquid CO_2 as the transfer medium (Hitachi, HCP-2). Dried samples were then loaded on aluminum studs and coated with gold for 3 min at 8 mA under a pressure of 0.1 Torr (Hitachi, IB2). The samples were scanned and examined using a Hitachi model S-2400 SEM. One control sample was prepared by simply immersing another Sacchachitin membrane in phosphate buffer for nine days and a second control sample was untreated Sacchachitin.

Part of the Sacchachitin membrane obtained from the wound healing studies was treated with 10% NaOH to remove blood clots and was then cleaned with distilled water until the pH was neutral. Membranes were pressed between two glass slides and treated with 0.5% periodic acid for 5 min. After that, they were washed with water several times and then incubated in Schiff's solution for 15 min. After washing with water for 10 min, a purple color could be visualized on these membranes. A control sample of Sacchachitin stored in phosphate buffer solution was also prepared.

2.7. Preparation of fibroblast cells

Fibroblast cells were acquired by the method of primary culture. A piece of skin was isolated from the dorsal area of a guinea pig and sterilized in iodine–alcohol solution for 30 s and then in 70% alcohol for 15 s. It was then cut into several pieces each with a surface area of about 2 mm^2. After attaching to a tissue culture dish for 20 min, 10 ml of culture medium (DMEM with 15% v/v fetal bovine serum, 1% v/v streptomycin) was added followed by storage for a couple of days in an incubator (Sanyo, MC0175) controlled at 37°C and with 5% CO_2. Fibroblast cells were harvested simply by removal of skin specimens [7].

2.8. Proliferation studies of fibroblast cells

Fibroblast cells at a concentration of about 3×10^4 cells ml^{-1} was placed in the DMEM medium in a petridish 35 mm in diameter. After incubation for 24 h, the medium was changed with fresh medium containing various materials at a concentration of 0.01% w/v. The materials tested included Sacchachitin powder, deacetylated Sacchachitin, chitin powder, *N*-acetyl-D-glucosamine, and β-1,3-D-glucan. On a predetermined day, 0.25% Trypsin-EDTA was added to detach the cells. About 50 µl of cell suspension was sampled and mixed with an equal volume of 0.5% Trypan blue. The total number of live cells was then counted using a hemocytometer under light microscopy [8]. A growth curve was plotted for each material added to the medium. The results were an average of replicated samples. At the same time of sampling, 70% of the medium was replaced with fresh medium. On day 9, another cell suspension was sampled and fixed with 70% aqueous alcohol solution followed by the addition of 300 µl of propidium iodide solution (50 µg ml^{-1}). The number of cells in the solution was counted for 5 min by a flowcytometer (FACScan, Becton Dickson). Data was analyzed with LYSIS 2 software. The effects of two different concentrations (0.05 and 0.1% w/v) of *N*-acetyl-D-glucosamine, deacetylated Sacchachitin and β-1,3-D-glucan, were also examined.

2.9. Migration studies of fibroblast cells

Fibroblast cells (3×10^5 cells ml^{-1}) were incubated in the DMEM medium containing 0.2% FCS for 72 h in a petridish. Aphidicolin (0.5 mg ml^{-1}) was added and the incubation continued for another 24 h. About 1.2×10^5 of these cells were incubated in a petridish of 35 mm in diameter until the cells were grown fully over the whole dish area. A cell lifter was used to mark a cross line in the center of the dish and those cells on one side were scratched off. The remaining cells on the other side were cleaned twice with phosphate buffer solution. Then, a medium containing 0.01% w/v of various materials (chitin, deacetylated Sacchachitin, and β-glucan) was added. Incubation was done at 37°C with 5% CO_2. The extent of migration was estimated by counting the number of cells growing across the central line at six different points each within an area of 600×600 μm^2. Differences among different materials were analyzed based on the Student's *t*-test with $P < 0.05$ [8–10].

Another way of estimating the migration of fibroblast cells was as followed. The test materials suspended in 1% agarose medium were placed in a petridish and a layer consisting of a precipitate of the test material later formed at the bottom of the petridish. A hole was punched with a Pasteur pipette in the center of the solidified agarose medium. About 1000–1500 cells were placed into the hole, 15 min later, the cells precipitated to the bottom of the hole. The hole was then covered with DMEM medium and incubated at 37°C with 5% CO_2 for a certain period of time. From the center of the hole, the cells that migrated along the interface between the layers of

agarose and of the test material were counted in six different areas of equal size but randomly chosen [11–12]. The Student's *t*-test was used to analyze any significant difference among the materials tested.

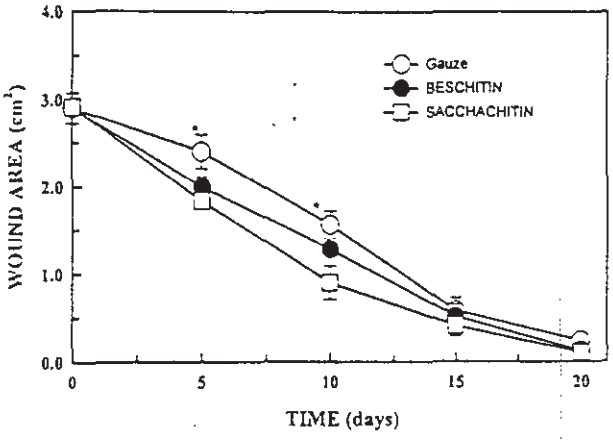

Fig. 1. Comparison of wound area changes at different time intervals when using Sacchachitin, Beschitin and gauze to cover the wound. (*: a paired *t*-test of significant difference with $P < 0.05$).

3. Results and discussion

Changes in the wound area covered either with Sacchachitin, Beschitin or gauze were estimated on days 5, 10, 15, and 20. The average change in the wound area is shown in Fig. 1 and demonstrates that the wound area covered with Sacchachitin measured at the above mentioned time was smaller than that covered with gauze. However, the difference between the area covered with Sacchachitin and that with Beschitin was not significant. Histological examination of wound tissue showed that new cells were apparently formed on day 10 in wounds covered with both Sacchachitin or Beschitin. On day 20, the differentiation of the hair follicles was also observed. However, the new cells only appeared on day 15 in wounds covered with gauze with shrinking of the wound area being observed also. Histological examination further revealed that the infiltration of numerous polymorphonuclear leukocytes into the wound area covered with Sacchachitin and Beschitin, whereas only a few monocytes were found to have infiltrated the wound area covered with gauze. These results are shown in Figs. 2–4.

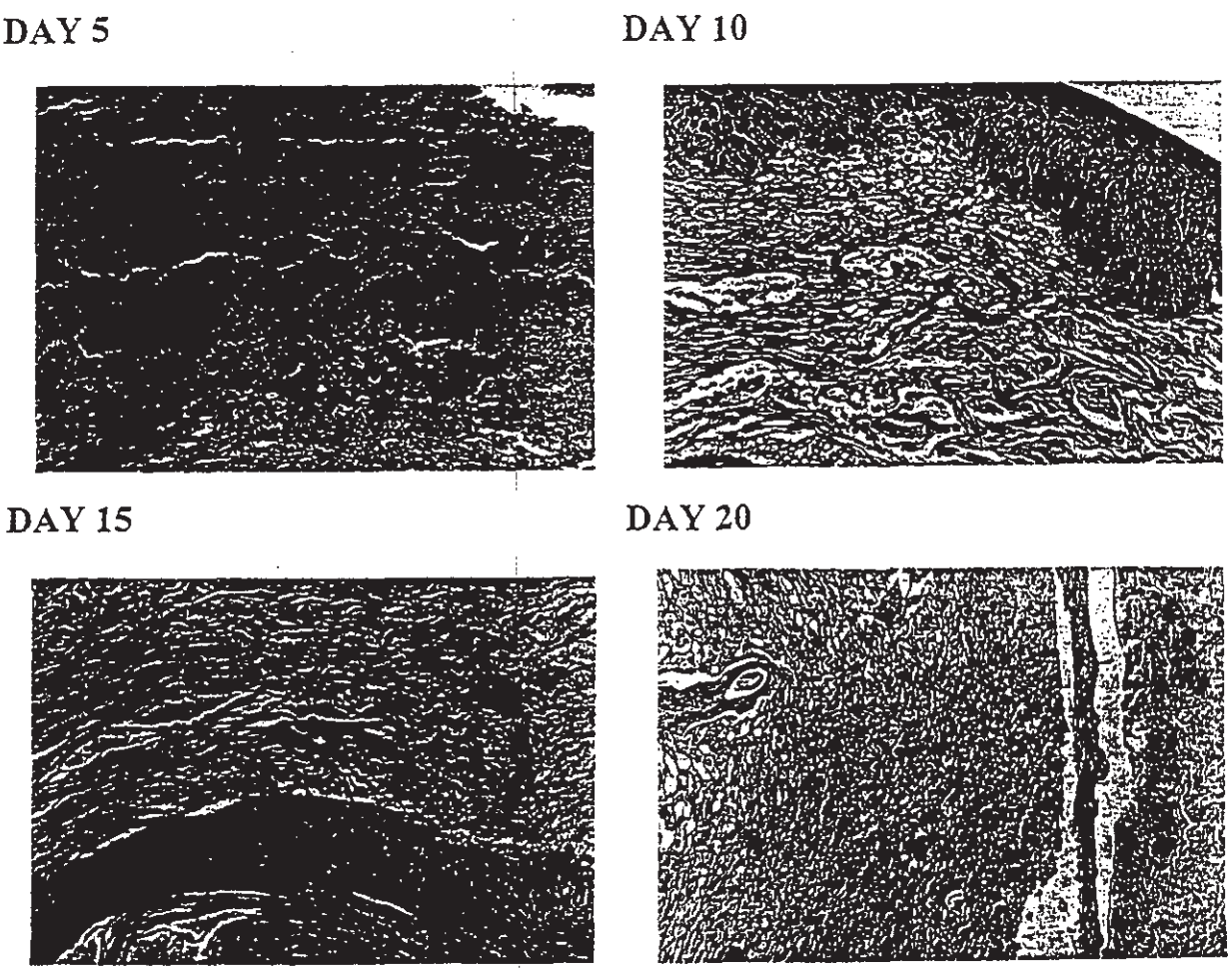

Fig. 2. Photomicrographs of the wound area and its surrounding tissue covered with Sacchachitin membrane at different time intervals.

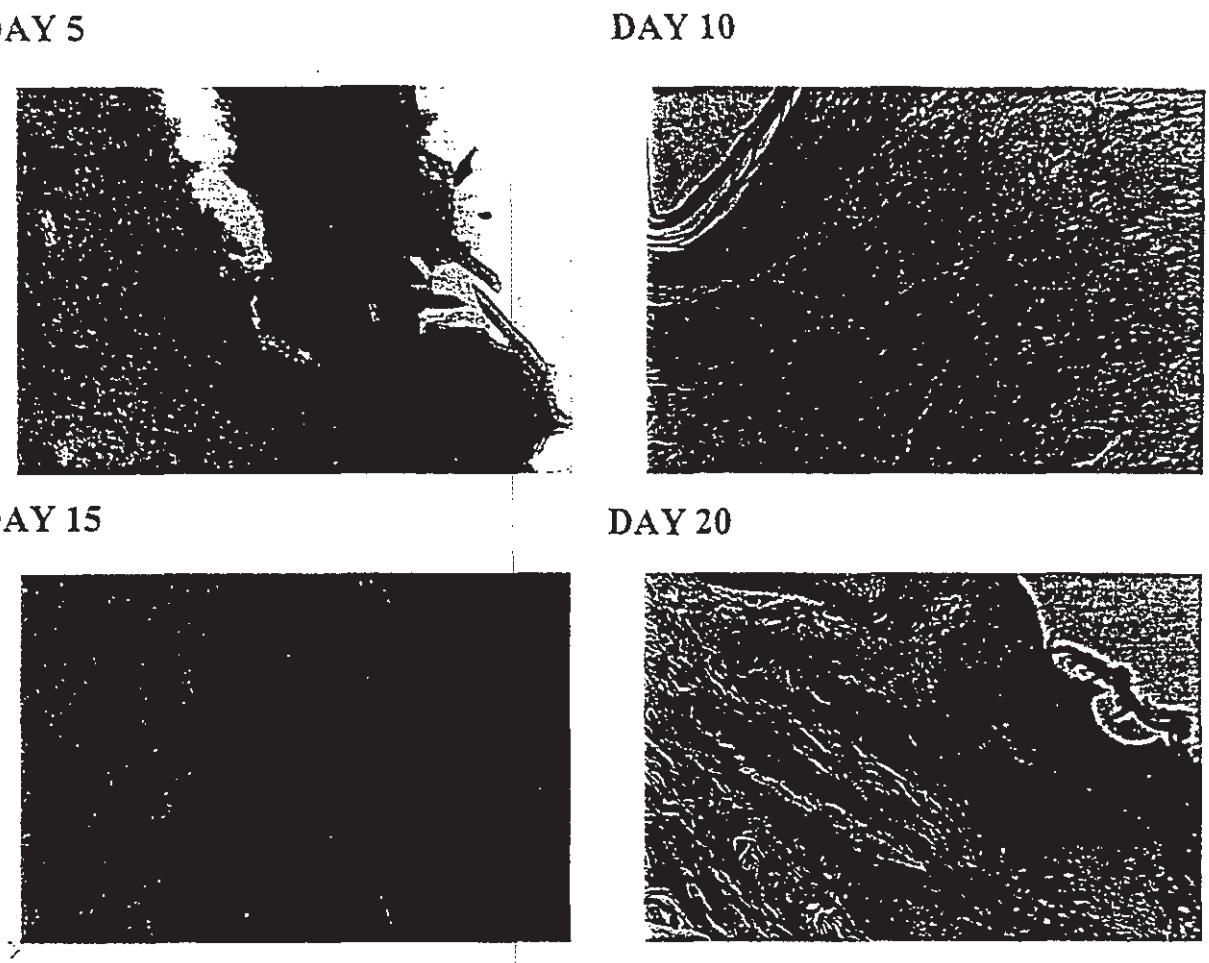

Fig. 3. Photomicrographs of the wound area and its surrounding tissue covered with Beschitin membrane at different time intervals.

The accumulation of polymorphonuclear leukocytes, ıch as neutrophils and eosinophils, in wound areas overed with either Sacchachitin or Beschitin is a sign of n acute inflammatory reaction. However, no bacterial ıfection was observed, indicating that both materials ad a chemotactic effect on the inflammatory cells. On he contrary, the infiltration of a large amount of mono-ytes, i.e. lymphocytes and macrophages, into the wound rea covered with gauze is attributed to a type IV allergic eaction. This is a type of rejection phenomenon involv-g the activation of these chronic inflammatory cells ausing necrosis of the wound tissue or the appearance of isters on the skin. In 1976, it was reported that a mild cute inflammatory reaction of a wound caused by infec-on in the earlier stage of trauma was able to accelerate aling of the wound and to increase the ability to lerate the tension after healing as well [13]. It was also und that numerous polymorphonulcear leukocytes ith some macrophages, which are able to secrete cell okines or growth factors, had accumulated in the inity of the wound. Possible reasons proposed for the celeration of wound healing by the author was at-buted to the action of cell cytokines or growth factors on promoting the differentiation of granuloma granulation tissue in the wound area [13]. This would explain the acceleration of wound healing by Sacchachitin membrane and Beschitin.

When Sacchachitin membrane isolated from the wound area was examined, it appeared that the structure of the mycelia hypha were destroyed and would not stain with PAS (Fig. 5A). On the other hand, the untreated Sacchachitin membranes and that immersed in phosphate buffer solution (Fig. 5B) showed no signs of damage to the structure of the mycelia hypha and they could be stained with PAS. Thus, it appears that the constituents of Sacchachitin membrane that can be stained by PAS were eliminated during the wound healing process.

It has been known that chitin is hydrolyzable by lysozyme [14]. Since chitin is a portion of the Sacchachitin structural unit with some soluble polysaccharides, it appears likely that substances released by the infiltration of body fluid caused dissolution of the chitin to promote wound healing. SEM examination confirmed that the structure of the mycelia hypha was disintegrated and would not stain with PAS. Generally, polysaccharides with 1,6-linkage can be stained by PAS. The

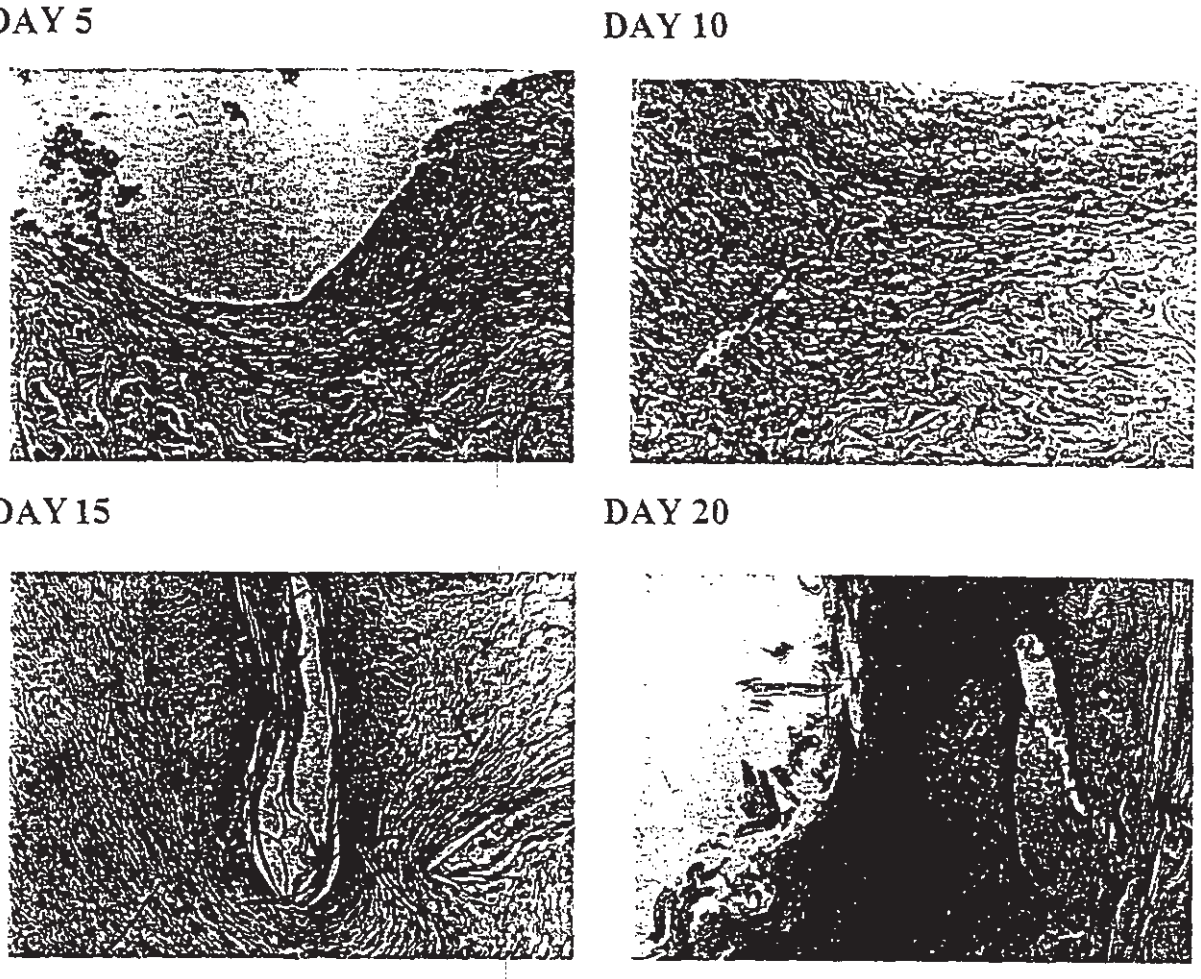

Fig. 4. Photomicrographs of the wound area and its surrounding tissue covered with gauze at different time intervals.

disappearance of the stainability of Sacchachitin membranes covering the wound indicates that this type of polysaccharide had disintegrated and been released. It is possible that the release of these substances can play an important role in the promotion of wound healing.

In order to determine which substances are responsible for the acceleration of wound healing, fibroblast cells, the main component of the dermis, were employed to compare the effect of several materials on their proliferation and migration. The results in Fig. 6 show that the proliferation of fibroblast cells was observed with both of 0.01% w/v Sacchachitin and Beschitin on day 6. Nevertheless, no significant effect on the proliferation of fibroblast cells was noticed until day 9 for 0.01% w/v *N*-acetyl-D-glucosamine and deacetylated Sacchachitin. It demonstrated that there is no difference for 0.01% w/v β-glucan compared to the control. The same results were obtained no matter which method was used for counting the number of cells.

The effect of different concentrations of these soluble materials on the proliferation of fibroblast cells was further examined and the results are plotted in Fig. 7. At a concentration of 0.05% w/v, *N*-acetyl-D-glucosamine and deacetylated Sacchachitin were able to promote the proliferation of fibroblast cells by day 6, whereas this did not occur until day nine for β-glucan (Fig. 7a). When the concentration of β-glucan was increased to 0.1% w/v, the proliferation of fibroblast cells occurred by day three, whereas the proliferation was inhibited with *N*-acetyl-D-glucosamine and deacetylated Sacchachitin (Fig. 7b).

In 1994, Chung et al. [15] reported that chitin isolated from the cell wall of some molds at a concentration of 0.01% w/v was shown to promote the proliferation of fibroblast cells. The same results were observed in this study. In 1987 [16] and 1992 [17], β-glucan was reported to be capable of promoting cell proliferation and macrophage function. However, the promotion of the proliferation of fibroblast cells was not observed until the concentration of β-glucan was increased above 0.05% w/v in this study. On the contrary, increasing the concentration of *N*-acetyl-D-glucosamine and deacetylated Sacchachitin to 0.1% w/v resulted in inhibition of fibroblast cell proliferation. In 1994, the same phenomenon was observed by Richard et al. [15]. Promotion of the

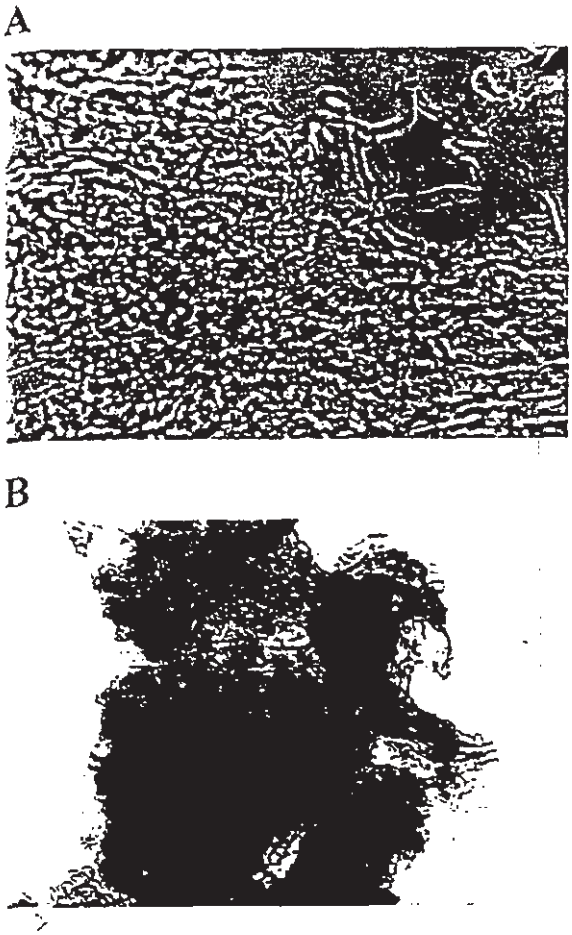

Fig. 5. Photomicrographs of Sacchachitin membranes covering the wound (A) immersed in phosphate buffer solution (B) stained by PAS.

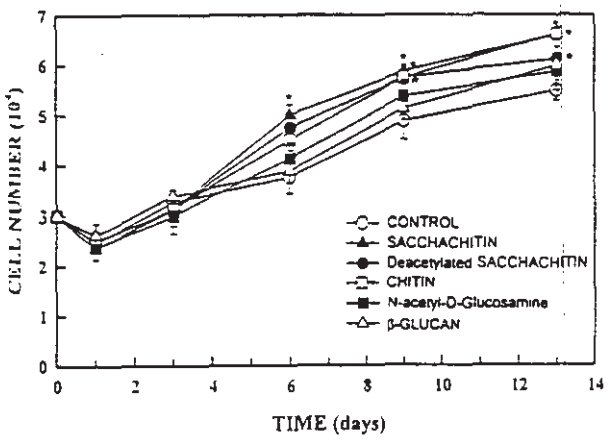

Fig. 6. Comparison of the proliferation of fibroblast cells induced by the addition of several different materials at a concentration of 0.01% w/v in the incubation medium. (*: a paired t-test of significant difference with $P < 0.05$).

Proliferation of fibroblast cells has been attributed to the positive charge carried by chitin increasing the adhesion to the cell surface at lower concentrations. However, the interference of cell membrane function by the adhesion of

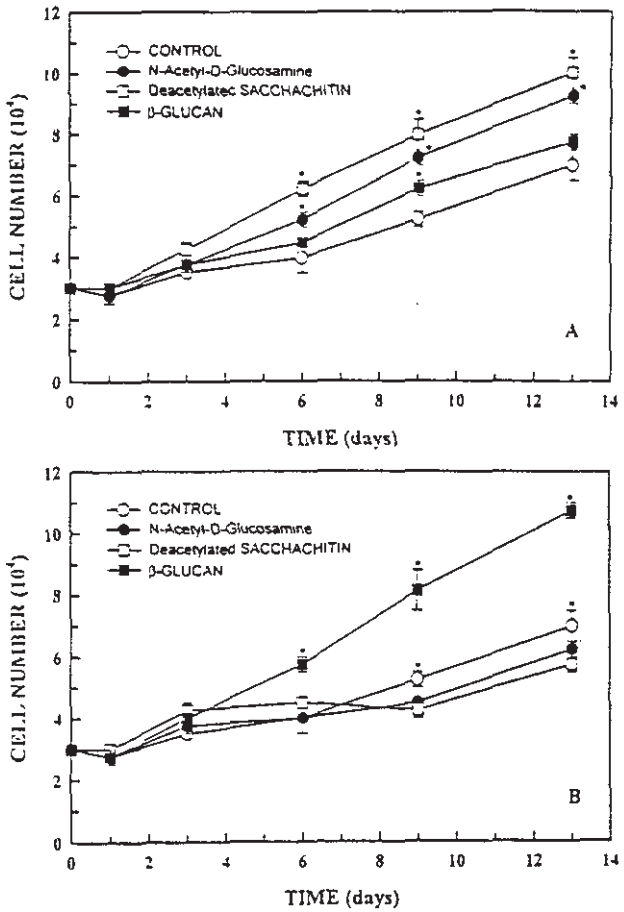

Fig. 7. Comparison of the proliferation of fibroblast cells induced by the addition of soluble materials at a concentration of (A) 0.05% or (B) 0.1% w/v in the incubation medium. (*: a paired t-test of significant difference with $P < 0.05$).

chitin leading to the death of cells was observed at higher concentrations.

Fibroblast cells controlled at the G_0/G_1 phase by aphidicolin were employed in two ways to estimate the extent of migration induced by different materials at a constant concentration of 0.01% w/v. In estimating the extent of migration across the central line in this study, it was found that Sacchachitin and chitin were able to promote extensive migration of fibroblast cells after the 3rd day. No difference in β-glucan from the control was noted. These results are shown in Fig. 8A. Similar results were observed in the migration study as estimated by counting the number of cells growing along the interface between the layers of agarose and the materials from the center hole in the medium (Fig. 8B). The only exception was that the cells migrating along the chitin layer were found to extract their peusdopodium and tended to die. Both results demonstrated that Sacchachitin membrane was able to promote the migration of fibroblast cells and act as a guide for cell growth.

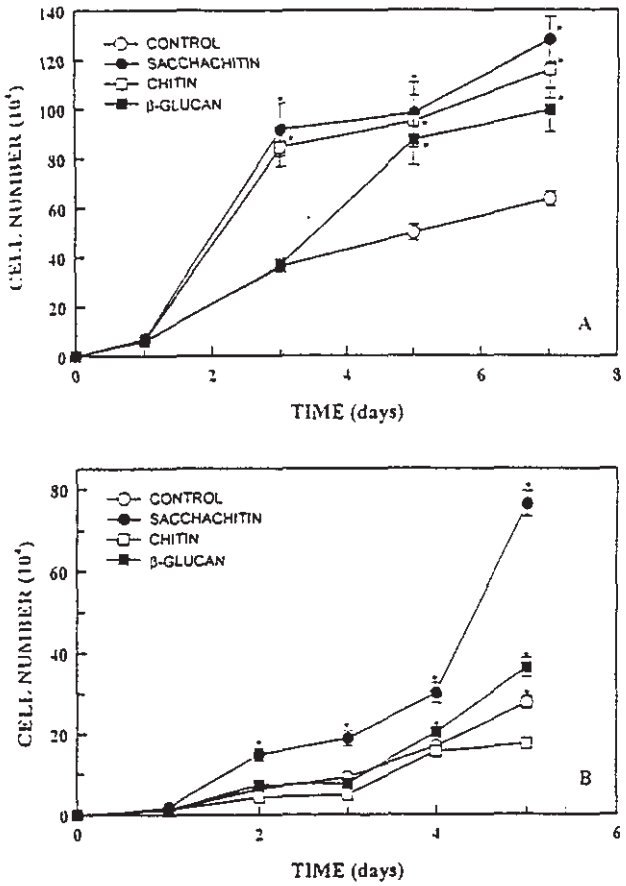

Fig. 8. Comparison of the extent of fibroblast cell migration (A) across the center line of the medium or (B) outside the central hole induced by the addition of soluble materials at a concentration of 0.1% w/v in the incubation medium. (*: a paired t-test of significant difference with $P < 0.05$).

4. Conclusions

Sacchachitin membrane is able to promote wound healing by inducing cell proliferation. A mild acute inflammatory reaction attracted a large number of polymorphonuclear leukocytes and some macrophages to clean away debris and blood clots. Also the secretion of cell cytokines and growth factors by these cells provided an excellent environment for wound healing. The migration of fibroblast cells, which was promoted by Sacchachitin, also plays another important role in the acceleration of wound healing. Optimally, the performance of Sacchachitin membrane as a skin substitute is comparable to the commercial product Beschitin. Further studies on the physical characteristics of the membrane, such as its air permeability and mechanical strength, would be valuable.

Acknowledgements

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Evaluation of Chemical Properties of Membrane from *Ganoderma*

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Ganoderma, an important member of medicinal fungi, has recently been extensively studied for its biological activity and commercially marketed as healthy food. Usually, after removal of the water soluble fraction by hot-water extraction, the remaining water-insoluble part, mycelia fibers, (more than 90%) of *Ganoderma* was discarded.

The structure of mycelia fibers is relatively complicated comparing with most polysaccharides. Mycelia fibers are composed of b-1,3-glucan and poly (N-acetyl-b-D-glucosamine). Both b-1, 3-Glucan and poly (N-acetyl-b-D-glucosamine) can be absorbed and metabolized in our body. Pudden had demonstrated that poly (N-acetyl-b-D-glucosamine) has a beneficial effect in the wound healing. Moreover, oligo (N-acetyl-b-D-glucosamine), the enzymatic hydrolysis intermediate from poly(N-acetyl-b-D-glucosamine), has been shown to have a high adjuvant capacity. Su et al. have previously demonstrated that the skin dressing prepared from mycelia fibers, exerts a strong enhancement effect on wound healing. The properties of biodegradation, attractive biomedical material for medical uses than other synthetic materials. Taken together, these findings suggest that mycelia fibers might be used as the biomedical material for making skin substitute. However, there is not any solvent system suitable to dissolve mycelia fibers so far for the purpose of processing.

Since the chemical property of mycelia fibers is most partially similar to chitin, the same knowledge from our understanding in sacchachitin can be attempted to find the solvent system suitable for processing mycelia fibers. Most of the previously found solvent systems for chitin would induce molecular degradation except lithium salts/amino-containing solvents and calcuim salts/alcohol. In this investigation, we will apply the technique of casting to develop a facile type of sacchachitin membrane from the sacchachitin dope, which will be prepared with mycelia fibers dissolved in metal salt/amine-containing solvent. We believe that the success of this study will provide a very valuable new biomedical material for clinical use.

Key words: Ganoderma, sacchachitin, membrane, biomedical material, binary solvent system

以靈芝子實體殘渣作為人工皮膚取代物的薄膜製作、溶解機制及膜的機械性質探討

Study on Preparation of Membrane from Fungal Ganoderma Residue as Skin Substitute and its Solubility and Mechanical Properties

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靈芝長久以來為藥用真菌之重要成員，近年來更為生理活性之研究對象，並且於市面上以健康食品的方式廣泛販售。然而，在熱水萃取過程中取得水可溶部份之後，其餘至少 90%不可溶部份並無任何用途，成為廢料而被丟棄，由之前研究初步結果顯示，靈芝子實體纖維為主體的廢棄物可加工加以純化，並製成人工薄膜，而經由初步動物實驗證明其對大白鼠的皮膚切除傷口有促進迅速癒合的效果。其主要機轉可能來至靈芝子實體纖維所含的幾丁質及 1,3-β-glucan 複合體所引發的，此符合 1970 年 Pruden 發現 poly-N-acetyl-glucosamine(chitin)為創傷癒合促進的物質，也呼應日本利用螃蟹殼幾丁質作為人工皮膚創傷被覆材之商品例證。

本研究在探討溶劑中金屬鹽類對靈芝子實體纖維溶解行為的影響，進而對靈芝子實體纖維的萃取作最有效的利用。另一方面，利用萃取後的靈芝子實體溶液經過濃縮或和 chitin 溶液混合製成薄膜，除了提供動物試驗所需材料外，也利用 Dynamic Mechanical Analyzer（DMA,動態黏性分析儀）測定膜的機械性質。由所得結果顯示，溶劑中金屬鹽類的濃度及溶解過程中施予昇降溫的步驟可以明顯的增加靈芝子實體的溶解力。其中 5%LiCl/DMAc 溶劑可使靈芝子實體溶解力達到最高，並在溶解的過程中，經由昇降溫四次之後溶解力也有顯著的增加。由 DMA 靜力掃描（static force scan）測定結果中發現純靈芝膜（22.59 MPa）和以 1：1 體積比例加入 chitin 的靈芝膜（23.08 MPa）的斷裂點的單位施力則相差不大。此外，本研究亦利用 NMR 分析以利了解靈芝子實體之結構。

關鍵字：靈芝子實體、溶解力、人工皮膚、動態黏性分析儀

Utilization of Fruiting Bodies from Genus *Ganoderma*

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ABSTRACT

Five fractions by means of stepwise extraction were obtained from fruiting body of Ganoderma. The methods and the uses of these fractions were described for health food production and skin substitution of wound healing. The five fractions were: ethanol extraction rich in triterpenoid, water-soluble polysaccharide, alkaline soluble polysaccharide, SACCHACHITIN and SACCHACHITOSAN.

Introduction

The fruiting bodies from the genus *Ganoderma* have long been used as a herb in traditional oriental medicine and in the recent two decades, through the efforts of many researchers, the extracts of the bitter-tasted, leather-like basidiomes were accepted as an important health food. The present article is to outline the works on the fruiting bodies of *Ganoderma* that had been done in Institute of Biomedical Materials, Taipei Medical University. In addition, the article is also proposed a protocol to use the residual part of the fruiting bodies after water extraction that has been considered as a waste. The complete use of the waste part of the basidiomes not only extends the application as a material to the field of skin wound healing, but also opens an opportunity to obtain a rich source of chitin and chitosan.

Materials and Methods

The fruiting bodies of *Ganoderma* were prepared from cultures from of Culture Collection and Research Center (CCRC), Hsinchu, Taiwan, by the cultivation of sawdust/plastic bag system in laboratory of Institute of Biomedical Materials, Taipei Medical University, Taiwan. Some basidiomes were the gift of Prof. Jin-Ten Peng, Agricultural Research Institute, Wu Fong, Nantou, Taiwan. The specimens used in the present paper were listed as in Table 1.

For experiments for stepwise extractions, fruiting body of *G. tsugae* CCRC-36065 was used through the study. For anti-matrix metallo-protease and Hella cell line tests, fruiting bodies of *G. resinaceum* CCRC-36147 was employed.

The methods of thin-layer chromatography (TLC) (Lai et al, 1995), high performance liquid chromatography (HPLC) (Su et al, 2001), animal model for liver function and cell culture for Sarcoma-180 (Chen 1996), HL-60 (Yang, 1995) and Hella cell lines (Hsieh, 1999) were described as in the literatures. The protocol to

separate different fraction for tests is outlined as in Fig. 1.

Fraction I: Ethanol Extract

Identification of species in the genus of *Ganoderma* by means of triterpenoid pattern

The first fraction separated from the fruiting bodies of *Ganoderma* was the ethanol extract. Usually, 100 ml of methanol or 95% ethanol was used for the extraction of dry fruiting body (2 g) and the extract was concentrated to 1.5 ml for TLC or HPLC analysis (Su et al, 2001). Both TLC and HPLC revealed distinct patterns for each species. TLC patterns of 18 groups (Yang, 1995) were demonstrated as of Fig.2.

Hepato-protective function of triterpenoid fraction from *Ganoderma*

The eighteen types of the extraction of fruiting bodies of *Ganoderma* were tested for CCl_4 induction of liver damage. All the 18-triterpenoid types demonstrated significant decrease of glutamate-oxaloacetate trasaminase (GOT) and glutamate-pyruvate transaminase (GPT) level in peripheral blood of mice. Among them, the ethanol extract from *G. tropicum* showed the most effective one to render GOT and GPT level down to 40%. Pathological examination on liver section also revealed a good repair function of damaged cell around caudal vein in the liver of the mice (Fig.3) (Yang, 1995). This result was in good agreement to our previous report on evaluating hepato-protection function of Ganoderic acids B and C_2 in 1993 (Su, Lai and Jen).

HL-60 cell line inhibition by triterpenoid fraction from *Ganoderma*

The eighteen types of the extraction were also tested for a human leukemia cell, HL-60 cell line, *in vitro*. The extracts from *G. pfeifferi* and *G. resinaceum* were found to inhibit cell growth and enhance apoptosis at a concentration of 5□g/l (Fig, 4 and Fig. 5). The mechanism of the inhibition on HL-60 is unclear and waiting for further investigation (Yang, 1995).

Triterpenoids from *Ganoderma resinaceum* for metallo-pretenase and Hella cell inhibitor

Several triterpenoids from *G. lucidum* were reported to be effective inhibitors of metallo-protenase from HIV and tumor cell lines. Similar results were found in the extract of *G. resinaceum* to inhibit metallo-protenases from kidney and

microorganism (Fig.6). The same compound was also a strong inhibitor (Fig. 7) to Hell cell line in our study and the compound was tentatively named as T-2-1-1 (Hsieh, 1999).

Fraction II: Water Extract

Water soluble polysaccharide

The residual fraction of *G. tsigae* derived from ethanol extraction was further treated with boiling water. The hot water supernatant from the extraction was cut off through an ultra-filtration apparatus (Minitan, Millipore Co., U. S. A.) with a molecular cut off size at 10,000. The yield of this portion was about 1% of the dry weight of the fruiting body after lyophilization. The chemical analysis demonstrated that it contained mainly polysaccharide with glucose, mannose and galactose as major sugar components. Physiological study on its anti-metastasis and anti-nodule formation in mice showed slightly effects in reduction of cell growth and nodule number against S-180 cell line in mice (Fig. 8-9) (Chen, 1996).

Fraction III: Alkaline Extract

Alkaline soluble polysaccharide

The residue from the water extraction was again treated with 1N sodium hydroxide or potassium hydroxide at 85oC for 2.5 hr. Identical procedure to do with water extraction was performed to obtain alkaline soluble polysaccharide. The yield of this portion is higher than that was from water-soluble polysaccharide and anti-metastasis and anti-nodule effects in mice were also slightly higher than the effects of water-soluble polysaccharide (Fig.8-9). The alkaline treatment of the residual also removed protein, nucleic acids, and lipids from the cell of mycelial structure of the fruiting body (Chen, 1996).

Fraction IV: Chitin Rich Polysaccharide

SACCHACHITIN as a skin substitute for wound healing enhancement

The alkaline treated residue was washed very thoroughly by distilled water to remove alkaline in previous treatment and 1% of sodium hypochlride was used to bleach the pulp-like paste in room temperature for approximately 3 hr. The melanin structure was destroyed and the white pulp was washed again to clean up hypochloride in the suspension. The suspension was filtrated, frozen and lyophilized to make into a membrane like sheet and named as SACCHSACHITIN. Chemical analysis revealed a one to one ratio of glucose and N-acetyl glucosamine in SACCHACHITIN (Su et al, 1996, Su et al, 1997).

SACCHACHITIN was compared to a commercial product named BESTCHITIN (Riken Research Co., Japan) derived from crab shell with chitin as its

main component to evaluate wound healing enhancement effect, together with cotton gauge as negative control in rat and guinea pig models (Su et al, 1997, Su et al, 1998, Su et al, 1999). The improvement of the wounds by SACCHACHITIN was evident and the mechanisms of the membrane were proved by enhancements of growth and migration of fibroblasts and keratinocytes from guinea pigs and human foreskin, respectively. Further evaluation of the mechanism including the binding the matrix metallo-protenases *in vitro*, the increment of collagen piling of the wound and microscopic observation to find less inflammatory reaction were observed (Huang, et al, 2001).

Human experiments on patients of bedsore are now under investigation.

Fraction V: SACCHACHITOSAN

SACCHACHITOSAN provided rich source of anti-tumor polysaccharide

SACCHACHITIN contained water insoluble fiber like hyphae of *Ganoderma* fruiting body. The final treatment to the residue was handled the SACCHACHITIN by 45% sodium hydroxide or potassium hydroxide to eliminate N-acetyl group attached on the glucosamine and in some instance the polymer disintegrated into smaller molecule and the suspension system became soluble to water. The high molecule (over 10,000) was harvested through the ultra-filtration system and lyophilization. The yield of this portion was much higher than that from water and alkaline extraction (Table 2) with a more effective potency in decrease metastasis cells and nodule in the lungs of mice when challenged the animals with S-180 cells (Fig. 8-9) (Chen, 1996).

Conclusion

The use of stepwise extraction for the fruiting bodies of *Ganoderma* could provide a total utilization procedure for manufactures of health food or pharmaceutical and thus lowered the cost of production. In view of environmental consideration, the production process might involve some hazardous chemicals such as very corrosive alkaline and bleaching reagent. Our suggestions include neutralizing the reagents with acid and concentrated into fertilizers if potassium hydroxide and potassium hypochloride were used instead of sodium salts. We also recommend that the process in deacetylation of SACCHACHITIN into SACCHSACHITOSAN by means of enzymatic digestion to avoid high concentration alkaline involvement. However, the enzymatic way of treatment is waiting for investigation.

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摘要

靈芝子實體經由逐步萃取可得五種主要部份，本文描述此類成份包括皮膚癒傷材料及健康食品之提取方法及可利用之途徑。此五種主要部份爲：含多量三帖類、之酒精萃取部份、水可溶多醣、鹼可溶多醣、SACCHACHITI 及 SACCHACHITOSAN。

Table 1 Strains of *Ganoderma* used

Species Name	CCRC* Number	Origin of Collection
G. australe	36128	India
G. fornicatum	37048	Taitung, Taiwan
G. lobatum	36245	Canada
G. lucidum	36021	Taichung, Taiwan
	36041	Tainan, Taiwan
	37026	Taipei, Taiwan
	37029	Taipei, Taiwan
	37033	Nantou, Taiwan
	37043	Taitung, Taiwan
	37053	Taitung, Taiwan
	36124	India
	36125	Argentine
	36144	North America
G. mirabile	36152	West Europe
G. neojaponicum	36049	Miaoli, Taiwan
	37042	Taitung, Taiwan
	37051	Miaoli, Taiwan
G. oestedii	36291	Argentine
G. pfeifferi	36159	West Europe
G. resinaceum	36146	West Europe
	36147	West Europe
	36149	West Europe
G. subamboniense var. *laevisporum*	36087	Argentine
G. tsugae	36042	Nantou, Taiwan,
	36065	Nantou, Taiwan
	36090	Nantou, Taiwan
	36203	Nantou, Taiwan
	36204	I-lan, Taiwna
	37063	Japan
G. weiberianum	36145	West Europe
	37049	Taitung, Taiwan
	37081	Taichung, Taiwan
G. boniense	37040	Nantou, Taiwan
	37068	Nantou, Taiwan
G. calidophilum	36205	Nantou, Taiwan
G. curtisii	37064	Nantou, Taiwan
	36064	Nantou, Taiwan
G. fornicatum	37067	Taipei, Taiwan
G. mastoporum	37044	Taitung, Taiwan
G. tropicum	88-1-40	Taipei, Taiwan
	88-1-57	Taichung, Taiwan

*CCRC: Culture Collection and Research Center, Hsing-Chu, Taiwan

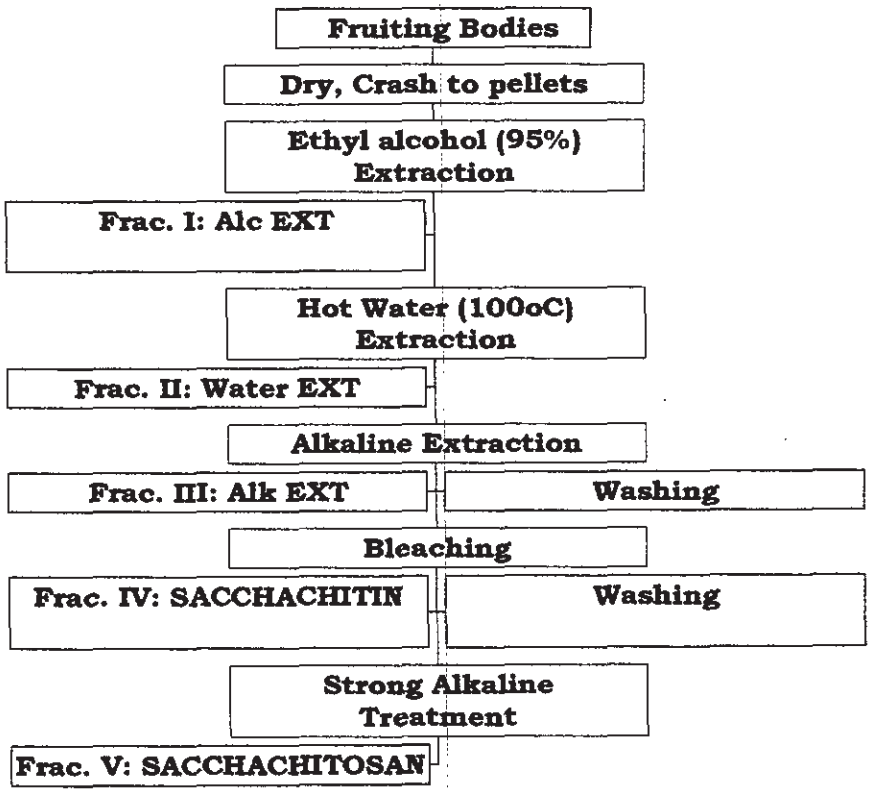

Fig. 1 Protocol for the experiments

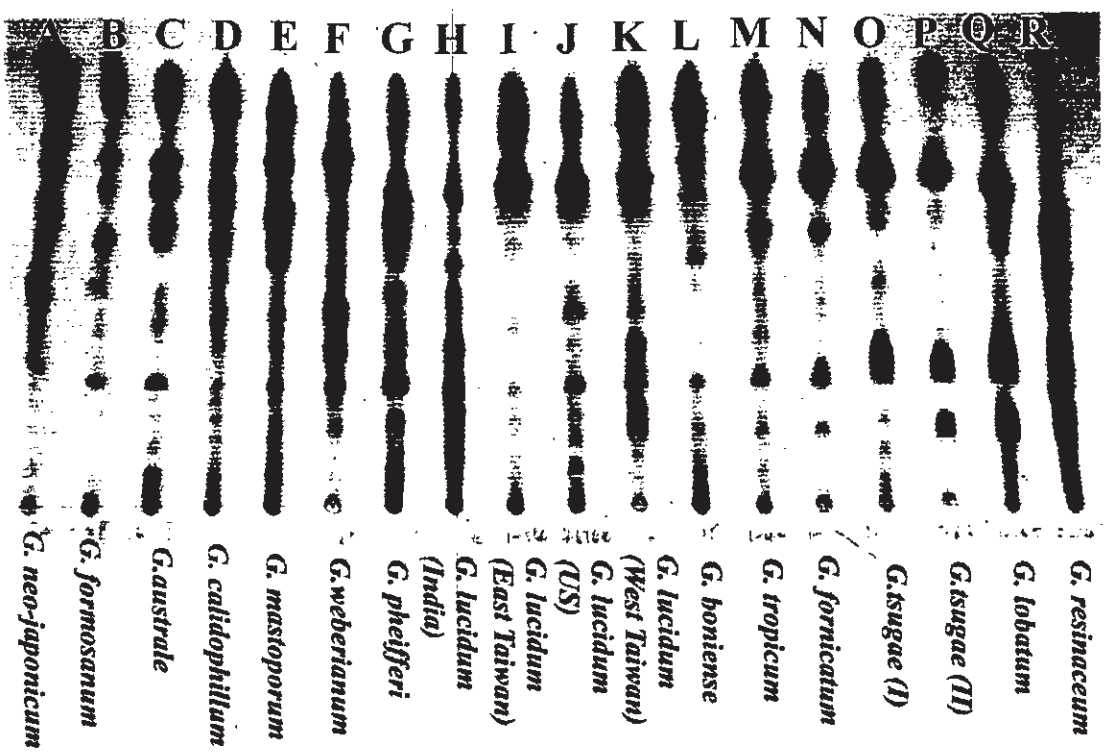

Fig. 2 Thin-layer chromatogram of ethanol fractions from *Ganoderma* fruiting bodies

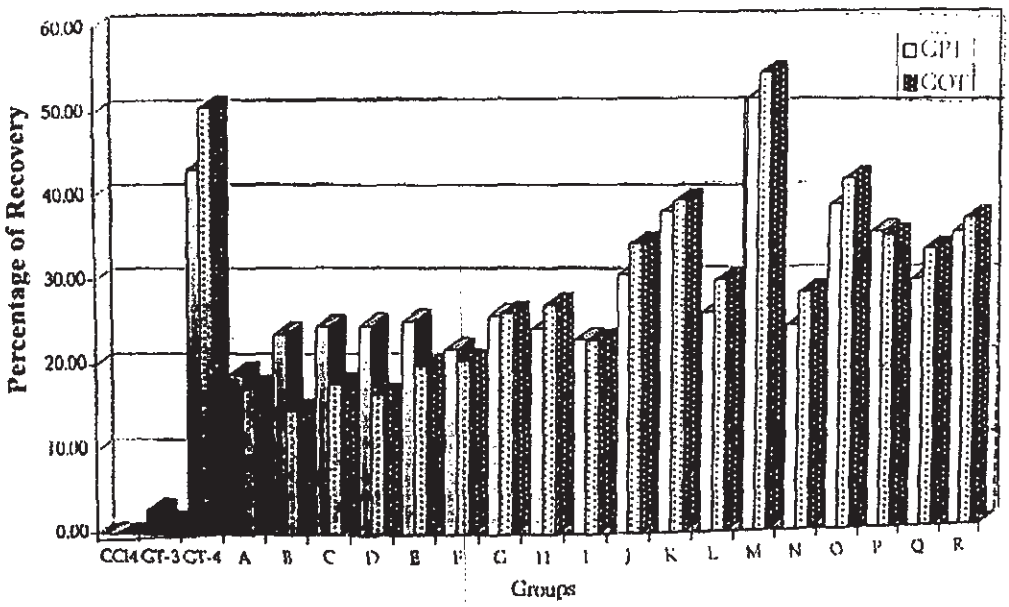

Fig. 3 Hepatoprotective effect of ethanol extracts from fruiting bodies of *Ganoderma*

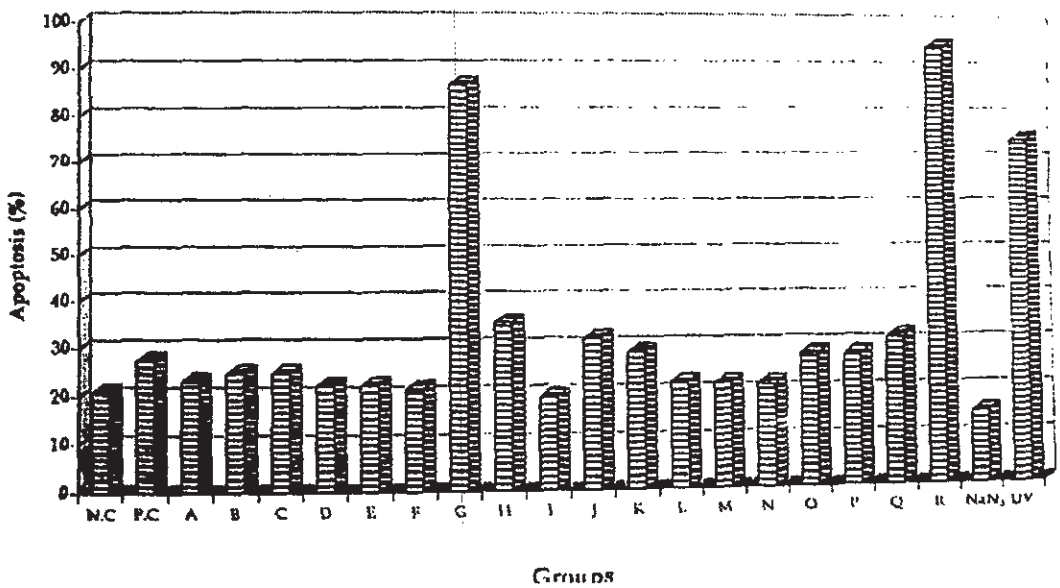

Fig. 4 Induction of HL-60 cell apoptosis by ethanol extract from fruiting bodies of *Ganoderma*

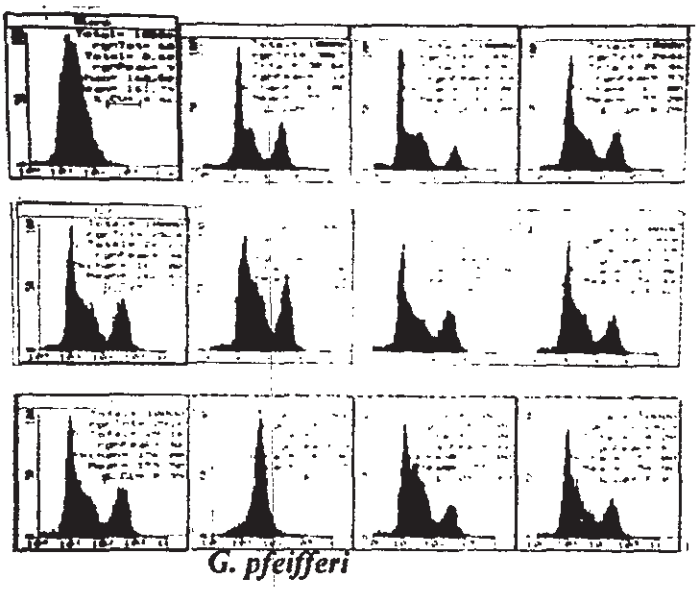

Fig. 5 Flow cytometric diagrams showed DNA fragmentation of ethanol extract of *Ganoderma* on HL-60 cells

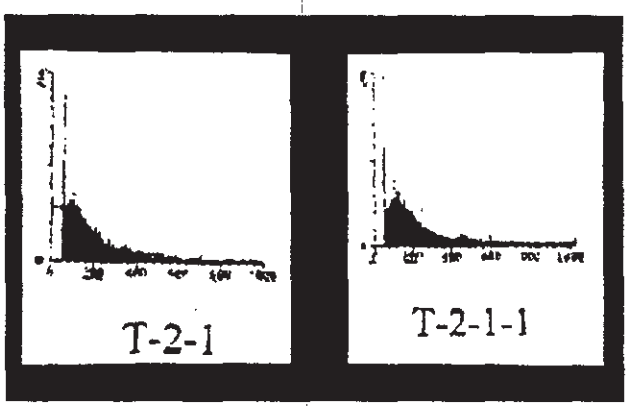

Fig. 6 Flow cytometric analysis of a triterpenoid(T-2-1-1) component from *G. resinaceum* to induce apoptosis of Hella cell line.

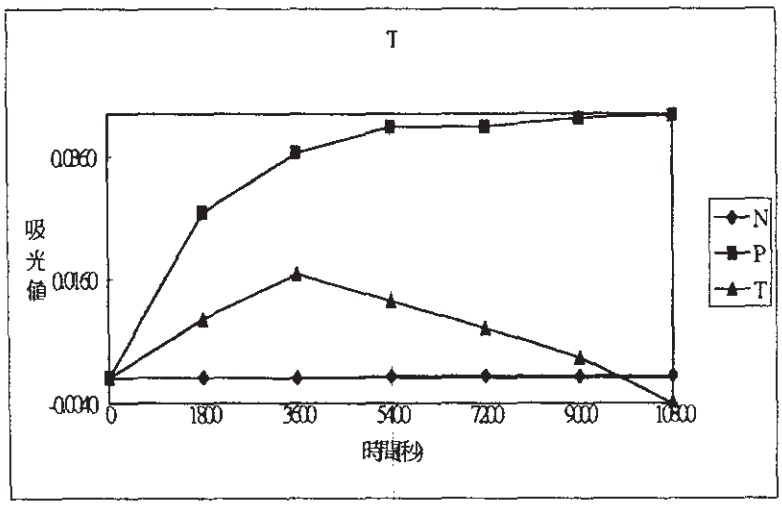

Fig. 7 Inhibition of metalloprotenase inhibited by T-2-1-1.

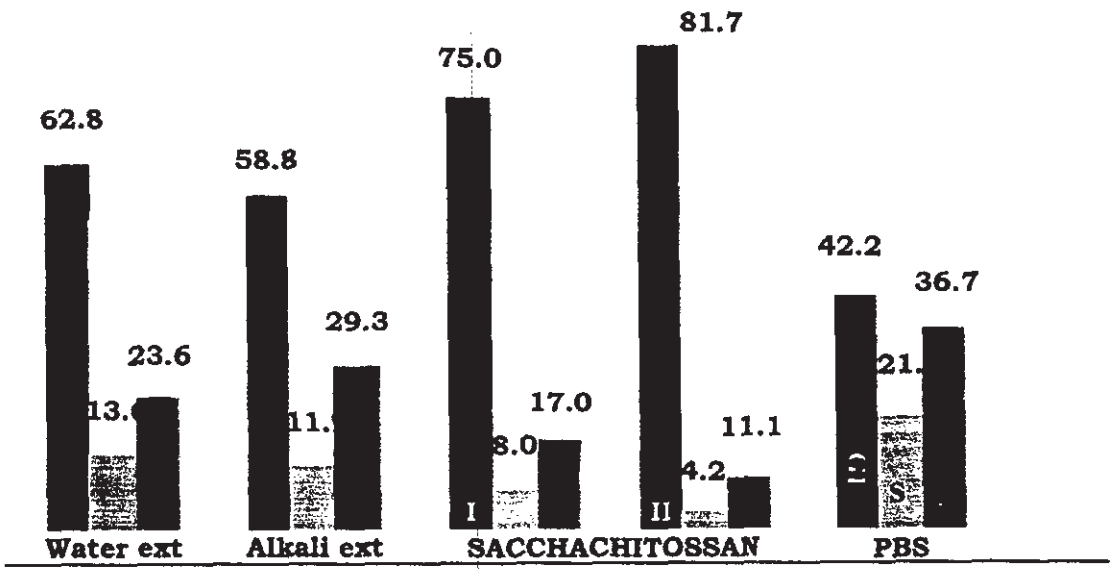

Fig. 8 Flow cytometric analysis on the lung of mice in respect to G1, S and G2/M phases of S-180 cells with the treatment of polysaccharides isolated from *G. tsugae*.

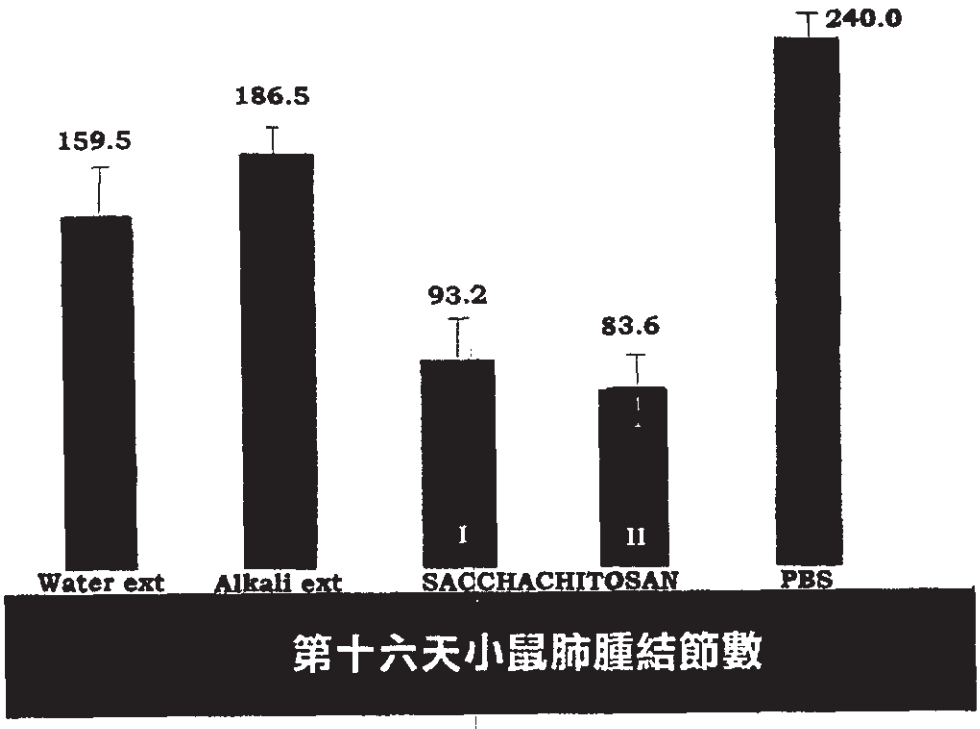

Fig. 9 Nodule number in the lung of mice after challenged by S-180 cell on the 16th day with the treatment of polysaccharides isolated from *G. tsugae*.

Table 2 Yields of polysaccharides isolated from fruiting body of *G. tsugae*

	Yield	I	II
Crude residue (200g)			
Water extract	**1.34g (0.67%)**		
Alkaline extract	**7.20g (3.60%)**		
SACCHACHITOSAN		**21.98 (10.99%)**	**48.00g (24.00%)**

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拾、附錄：

摘要

近年來，許多研究報告紛紛以皮膚角質細胞(keratinocyte)應用在人體傷口癒合機制的探討及人工皮膚的開發上，因此，如何使其能貼附(attachment)並生長在各種不同生物醫學材料上例如 de-epidermized dermis (DED), collagen-glycosamin substrate 和 Chitosan 等，成爲一值得探索的領域。靈芝爲中國傳統的珍貴藥材，文獻上有關靈芝的研究顯示靈芝具有免疫調節、抗腫瘤、抑制血小板凝集、抗發炎和止痛等效果。本實驗室由真菌類松杉靈芝的殘渣所製造出來的靈芝薄膜亦具有促進傷口癒合的效果，因此實驗的目的是延續先前靈芝薄膜對皮膚傷口的癒合作用，近一步探討靈芝薄膜對皮膚角質細胞生長的影響。結果發現本實驗室所製造的靈芝薄膜具有良好的生物相容性(biocompatibility)並不會對皮膚角質細胞產生細胞毒性，且初步發現由靈芝薄膜製成的溶液有促進角質細胞生長的功能，但靈芝薄膜主要是由幾丁質和少部分以 1,3-β所鍵結的聚醣(glucan)所構成，真正影響角質細胞生長的因子及以何種機制影響則有待更進一步探討。

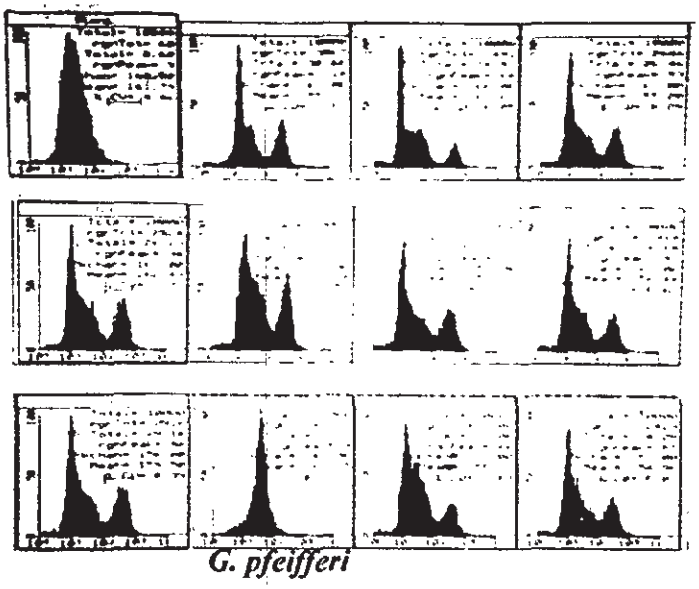

Fig. 5 Flow cytometric diagrams showed DNA fragmentation of ethanol extract of *Ganoderma* on HL-60 cells

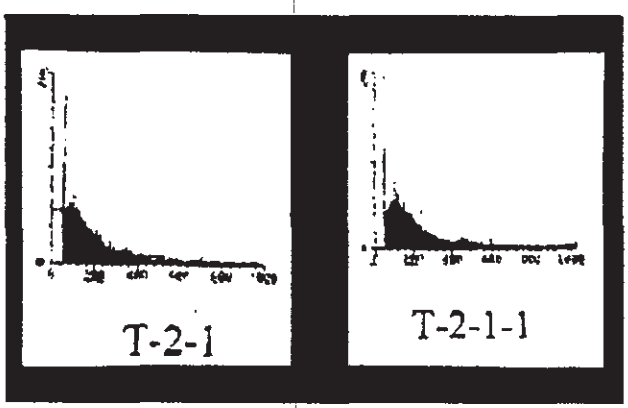

Fig. 6 Flow cytometric analysis of a triterpenoid(T-2-1-1) component from *G. resinaceum* to induce apoptosis of Hella cell line.

(basement membrane)相關的分子包括 fibronectin, laminin, collagen IV 和 heparan sulphate 等在單獨或不同組合的情形存在於培養基會對皮膚角質細胞的貼附與生長產生影響(Morykwas *et al* 1989, Kubo *et al* 1987)，另一方面各種不同的生醫材料如 de-epidermized dermis (DED), collagen-glycosamin substrate 和 Chitosan 等也陸續開發出來(Boyce *et al* 1990, Claire *et al* 2000, Ponec 1992)。

靈芝爲中國傳統的珍貴藥材，文獻上有關靈芝的研究顯示靈芝具有免疫調節、抗腫瘤、抑制血小板凝集、抗發炎和止痛等效果。靈芝菌絲的細胞壁主要是由幾丁質和少部分以 1,3-β所鍵結的聚醣(glucan)所構成，而本實驗室由真菌類松杉靈芝的殘渣所製造出來的靈芝薄膜亦具有促進傷口癒合的效果，因此實驗的目的乃是延續先前靈芝薄膜對皮膚傷口的癒合作用，探討靈芝對表皮最主要細胞—角質細胞生長的影響及靈芝薄膜的生物相容性(biocompatibility)。

二、實驗方法

皮膚角質細胞的初代培養

1. 將從台北醫學大學附設醫院泌尿科取得的新鮮包皮分別用 PBS 緩衝液清洗 3~5 遍後，保存在 4℃的 DMEM 培養基(Gibco co.)中

2. 將包皮切割成適當大小後，置入 4℃分離脢(dispase, 2.5unit/ml))作用 18 小時

3. 以消毒過的鑷子輕輕取下表皮，再以胰蛋白脢(trypsin,0.25%)處理 8~10 分鐘期間並使用滴管加以沖擊，隨即加入大豆抑制劑(soybean inhibitor)停止反應

4. 離心(1000 rpm)10 分鐘留下沉澱物，再以 KSF 培養基(Gibco co.)製成細胞懸浮液注入培養瓶

5. 於 37℃ CO_2 濃度 0.5%下培養，每 2~3 日更換一次培養基

皮膚角質細胞的繼代培養

1. 當細胞於培養瓶中長至 8 分滿時即可準備繼代培養

2. 將原有培養基吸乾再以 PBS 緩衝液清洗 1~2 次，隨即加入胰蛋白脢(0.25%)處理 8~10 分鐘，並用滴管加以輕輕沖擊

3. 加入大豆抑制劑終止反應，離心(1000 rpm)10 分鐘，留下沉澱物再以 KSF 培養基製成細胞懸浮液注入培養瓶

4. 於 37℃ CO_2 濃度 0.5%下培養，每 2~3 日更換一次培養基

靈芝薄膜的生物相容性

1.先將靈芝薄膜剪裁成 2x2 公分平方大小，再經過殺菌釜滅菌後放

入 6-well 培養皿

2.以 KSF 培養基潤濕隔夜後，再以 UV 照射 1 小時

3.吸出培養基，加入培養第三代的角質細胞於 37℃ CO_2 濃度 0.5% 下培養，分別於 24, 72, 168 小時以反相光學顯微鏡觀察，並照相記錄之

靈芝薄膜對角質細胞生長的影響

1.將靈芝薄膜磨碎，加入 PBS 緩衝液製成 10%的水溶液，滅菌

2.將第三代角質細胞分別加入含一定比例靈芝薄膜水溶液之培養基及控制組(不含靈芝薄膜水溶液)，分別於 1, 2, 3, 4, 5 日以反相光學顯微鏡觀察，並血球計數器計算細胞數目紀錄之

三、結果與討論

皮膚角質細胞的初代培養

圖一爲角質細胞培養 24 小時後的照片，可見細胞已經貼附在培養瓶表面，有一些細胞已呈狹長形，圖二爲角質細胞培養 48 小時後的照片，細胞的數目開始增加，圖三爲角質細胞培養 6 日後放大倍數照片，圖四、五爲角質細胞培養 4、6 日後的照片，角質細胞已長滿整個培養瓶，此時就可以準備繼代培養了。

皮膚角質細胞的培養，由於材料取得不易，同時也不易分離培養，所以整個實驗用的培養基及實驗過程都須小心控制。爲了要打散角質細胞而採用0.25%的胰蛋白胨在37℃下處理8~10分鐘，若處理超過10分鐘會對細胞本身產生傷害。一般來說，經過繼代培養之後，角質細胞增殖的能力會漸漸變差，以本實驗的條件下操作可以繼代 8 次，培養時間約兩個月左右。曾有學者指出鈣離子的濃度對於角質細胞的分化扮演一個關鍵的角色；當鈣離子的濃度保持在0.05~0.1mM的時候，角質細胞可以保持在未分化、增殖的狀態，若鈣離子的濃度超過1.2mM的時候，角質細胞就會停止增殖的狀態開始分化，很快的分化成扁平角化的形狀，而在本實驗過程中亦曾提高鈣離子的濃度培養也觀察到相同的現象，學者Rubin(1988)觀察到當鈣離子濃度由低至高的轉變人類角質細胞內與分化相關的一些大分子如角蛋白(keratin)、transglutaminase及involucrin等的數量都會增加。

靈芝薄膜的生物相容性

圖六爲共同培養24小時後，照片左上角爲靈芝薄膜，大部分細胞的形狀呈橢圓形，只有少部分發生改變，圖七、八爲共同培養 72、120 小時後，細胞數量大增，並可見有些角質細胞已經長到靈芝薄膜裡面。當把生醫材料應用於活體時，必須確定其與細胞之間的相容性，所以本實驗以靈芝薄膜的成品與人類角質細胞共同培養的方式進行，觀察靈芝薄膜與人類角質細

胞之間的反應以確認其生物相容性。由圖顯示靈芝薄膜與角質細胞共同培養 24、72、120 小時之後細胞外型皆正常，沒有產生任何變形或死亡的結果，且由於靈芝薄膜是三度空間的結構，有部分的角質細胞還能鑲嵌生長在靈芝薄膜中，可見靈芝薄膜與人類角質細胞之間具有高度之生物相容性。

靈芝薄膜對角質細胞生長的影響

結果如圖九，實驗的細胞接種量爲 $2x10^4$ cell.cm^{-2}，經過 5 天的培養加入靈芝薄膜水溶液組與控制組的細胞濃度分別爲 $6.8\ x10^4$ cell.cm^{-2} 及 $5.5\ x10^4$ cell.cm^{-2} 可知加入靈芝薄膜水溶液有促進角質細胞生長的功能，但靈芝薄膜主要是由幾丁質和少部分以 1,3-β所鍵結的聚醣(glucan)所構成，真正影響角質細胞生長的因子及以何種機制影響則有待更進一步探討。

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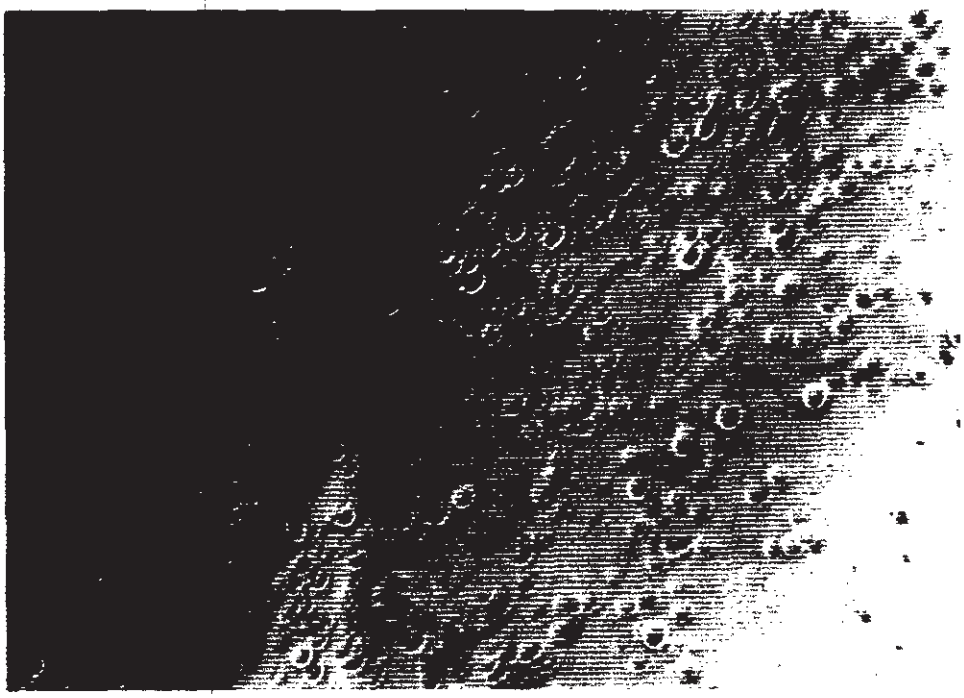
〔圖一〕皮膚角質細胞培養 24 小時(10×10)

〔圖二〕皮膚角質細胞培養 48 小時(10×10)

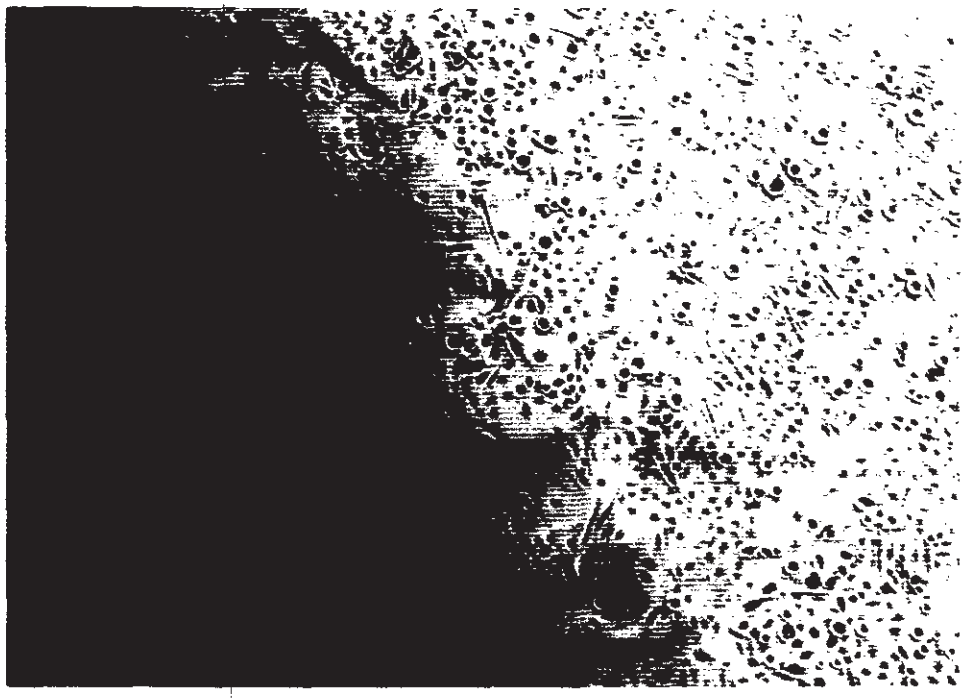
〔圖三〕皮膚角質細胞培養 6 日(20×20)

〔圖四〕皮膚角質細胞培養 4 日(10×10)

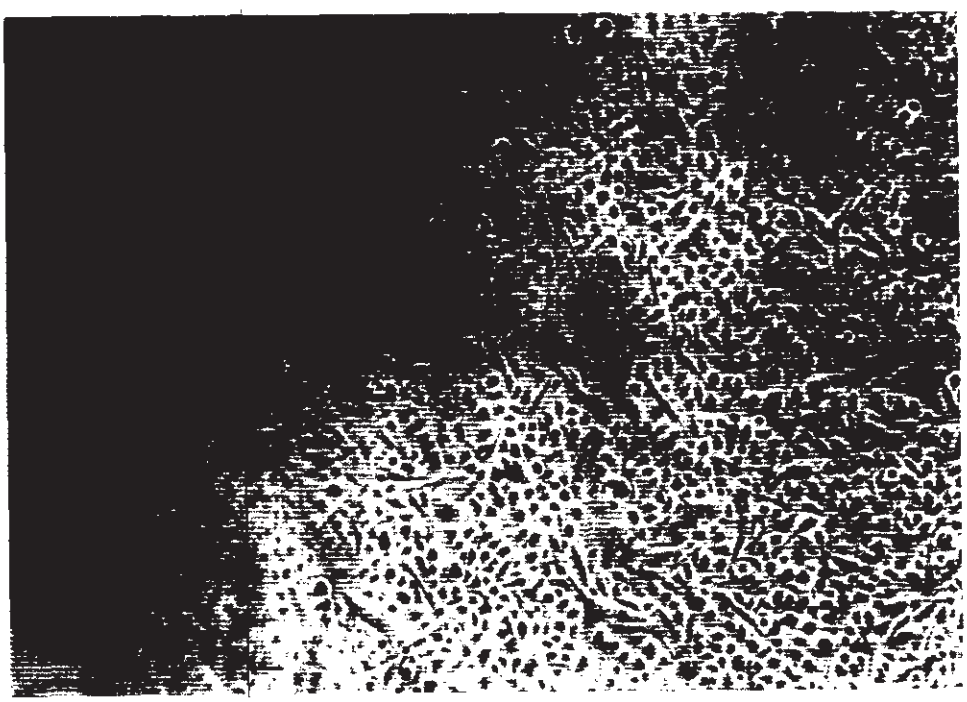
〔圖五〕皮膚角質細胞培養 6 日(10×10)



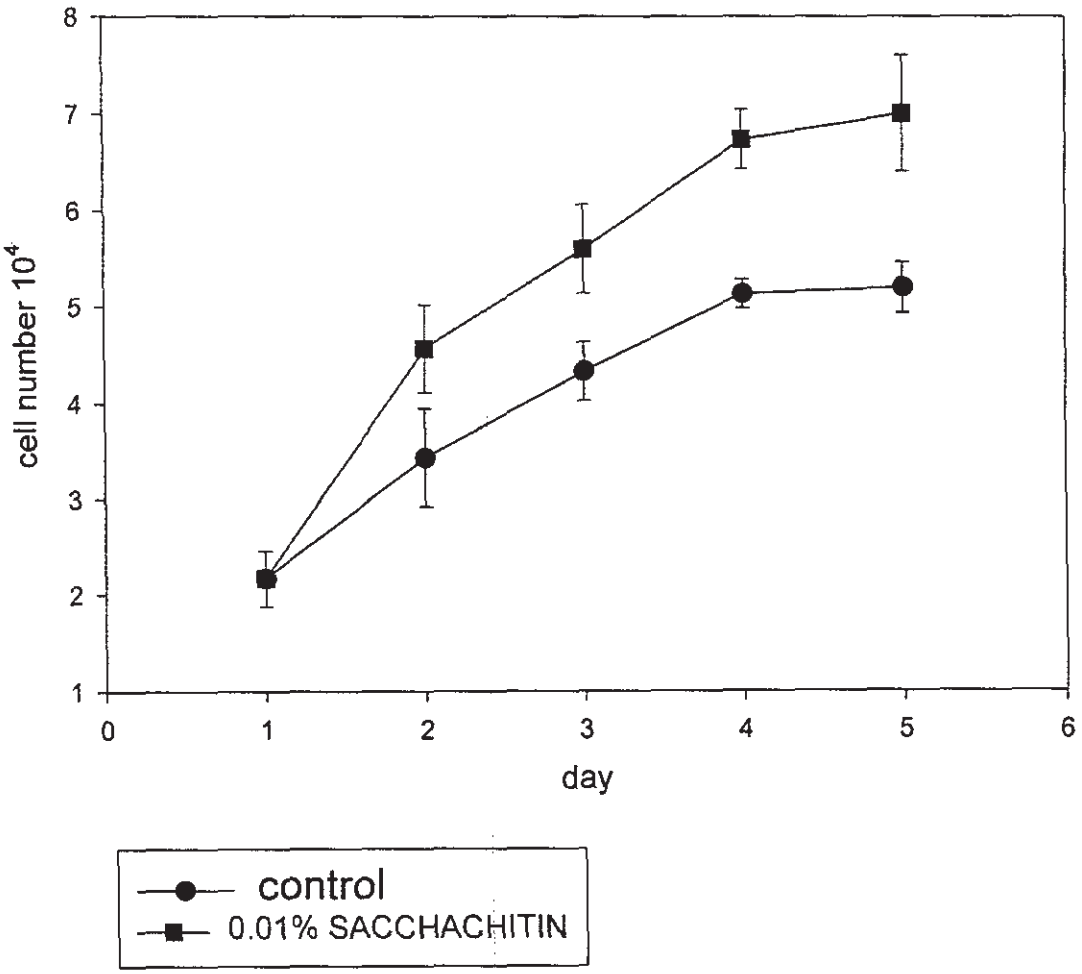
〔圖六〕靈芝薄膜與皮膚角質細胞共同培養 24 小時(10×10)



〔圖七〕靈芝薄膜與皮膚角質細胞共同培養 72 小時(10×10)



〔圖八〕靈芝薄膜與皮膚角質細胞共同培養 120 小時(10×10)



〔圖九〕皮膚角質細胞的生長曲線

中文摘要

爲得到適用於傷口修復的敷料，本研究以靈芝殘渣作不同條件的處理，以得到最佳條件的靈芝薄膜。首先以靈芝子實體萃取過有效成份後的殘渣爲材料，先將殘渣以粉碎機研成碎屑，接著以不同條件的 KOH 爲變因進行鹼處理，鹼處理過後的殘渣以清水沖洗至 pH meter 檢驗呈中性，繼之以不同條件的 $HClO_3$ 爲變因進行脫色，以清水沖洗至 pH meter 檢驗呈中性，且 $AgNO_3$ 檢測無 Cl^- 離子殘留。歷經鹼處理及脫色手續的殘渣，將其以冷凍乾燥乾燥三日，之後取出乾燥過的 Sample，取不同條件下的 Sample，以6 % 的濃度置於二元溶液系 5% LiCl/DMAc (由實驗得知最佳的溶液比例)中溶解三日，完成後依序成膜，再以 (Ethanol/Acetone/Water)各 120、15、60 sec 迅速浸泡後撈起，以清水放流 24 hours 並平鋪在 Teflon 紙上，待其風乾，即可得到初步的靈芝膜。靈芝膜分別以 DMA 測強度，NMR、IR、X-ray 測其結構且加以分析，並將之前溶解的靈芝溶液以黏度計測分子量，最後綜合 Data 找出最合適的成膜條件，大量製造後並計劃與生體相關的實驗。

關鍵字:靈芝，LiCl/DMAc，DMA，NMR，IR，X-ray，黏度計。

Abstract

In order to obtain the material for wound repairing, this research is trying to use different method treating on Ganoderma residues, to prepare the optimal membrane . First, to whet the Ganoderma extracted active ingradient , then various kinds or concentration of KOH was treat on the whetted residues of Ganoderma. The treated residues of Ganoderma were washed by water till the pH was nearly nature. The various concentration of HClO3 was used as blenching agent. And Ganoderma residues were washed again till the pH was nearly nature after HClO3 treated it. Then AgNO3 was used to test if Cl^- was retained in the treated Ganoderma residues. After those steps, treated Ganoderma residues were dried 3 days by lyophilized. 6% of those dried samples were dissolved in 5% LiCl/DMAc which was the binary solvent system for 3 days. The solutions were gradually prepared to become membrane. Those membranes were put from ethanol 120 seconds; through acetone 15 seconds; then through water 60 seconds. The membranes were put in flowing water 24 hours and then cast on Teflon paper. All of the primary membranes were dried naturally. The DMA was used to measure the strength of those membranes; NMR, IR, X-ray were used to detect the structures; then the viscosity of the dissolving sample was measured to get its molecular weight. After those tests, the optimal condition was used to produce large amount of membranes for taking the in vivo tests.

Key word: Ganoderma lucidum，LiCl/DMAc，DMA，NMR，IR，X-ray，viscosity meter。

第一章　緒論

1.1 前言

關於靈芝在醫療上的功效，近來被廣泛的研究與發現。靈芝在外觀結構上，可分爲子實體和菌柄，而子實體又分爲菌蓋和子實層。在分類上，中國古代的「本草綱目」將靈芝分爲青芝、赤芝、黃芝、白芝、黑芝和紫芝等六種；而西洋近代之分類則以西元 1979 年 Alexopolus 建立的真菌分類系統，將靈芝列爲：真菌界/無鞭毛菌門/擔子菌綱/無蕈褶目/多孔菌科的靈芝屬。在文獻中，我們得知靈芝的療效成份主爲其中的三帖類[1]，而靈芝所含的特殊三帖類一般稱「靈芝酸」[2]。三帖類除了是導致靈芝具苦味的原因，也被研究和抑制癌細胞生長、防止過敏、促進肝功能、促進血小板凝集[3]、降血脂，甚至癌症方面的治療.等有關。而在結構上，支持靈芝子實體的多醣類，主爲幾丁質 (Chitin)及少量的 Chitosan。幾丁質目前已知存在蝦蟹殼、昆蟲外骨骼、烏賊類的軟骨、真菌類的細胞壁等結構性組織中，而關於幾丁質(Chitin)的去乙醯基衍生物(Chitosan)，也被初步證明具有促進傷口恢復的療效。此實驗即是收集靈芝萃取三帖類後的殘渣，取其子實體結構部分綜合研究之 [4] ，希望藉由化學處理的步驟，將靈芝子實體作部分的處理，並以適當的二元溶劑系 LiCl/DMAc 加以溶解，得到一幾丁質

(Chitin)與其去乙醯基衍生物(Chitosan)比例最適宜的混合溶液，並將混合溶液平鋪於 Teflon 上製成初步的靈芝膜[5][6]，再作相關的物性探討，模擬最接近皮膚的狀況，希望將來能提供皮膚外傷患者一個既能爲身體本身所降解，又能促進傷口癒合的敷料，並廣泛應用於臨床治療上。

幾丁質(Chitin)的結構是一種由 D-N-acetylglucosamine，以β-(1,4)所相連而成的高分子聚合物，而目前在產業上的幾丁質來源，主要取自甲殼動物頭足綱的軟骨組織。幾丁質目前被廣泛應用在健康食品及減肥用途上[7]，它能夠被人體所自然降解，進而吸收或代謝。而幾丁質(Chitin)既然能做爲許多甲殼動物以及真菌類的支持組織主成分，那麼其結構必定有一定的強度及韌性，這和我們一般常見的人工皮膚有著相似的要求，因此以幾丁質(Chitin)爲主成分的靈芝子實體，即成爲我們欲作爲皮膚外傷敷料的主要原料。

關於幾丁質(Chitin)的去乙醯基衍生物(Chitosan)，被廣爲研究與應用，舉凡加速傷口修復[8]、組織工程的多孔性架構、藥物賦型劑等，都說明了這種材質在生物醫學材料發展上的多元性，它和幾丁質最大的不同，在於結構上是一種β-D-N-glucosamine，少了乙醯基的結果使 Chitosan 具有水溶性，這使得 Chitosan 相對於幾丁質(Chitosan)而言有著較不安定的特性，對於作爲一種外傷的敷料而言，這顯然是較爲不利的條件，但我們又無法捨

棄 Chitosan 多種對傷口復原的正面影響[9]，因此一種以結構的強度爲主要考量，又不失療效的生物醫學材料，在我們的研究中有了初步的構想。

當我們初步取得靈芝的子實體後，可以不同條件的鹼處理，一方面去除不需要的蛋白質及三帖類成分，另一方面則藉由鹼處理的去乙醯作用，將部分的幾丁質(Chitin)轉爲 Chitosan，並藉由往後一連串的分析，找出最適宜所需的條件，以利大量製備爲日後生體相關實驗之用。

目前在皮膚外傷的治療上，主要有利用移植皮膚、矽膠處理等方法。在移植的手術上，恐有排斥性或是感染發炎之虞；而包括矽膠等人工複合材又無法爲人體所吸收，僅能提供一個自體修復的媒介，需要長時間清洗更換。而在已知的文獻上[10]得知：不管是幾丁質(Chitin)或是 Chitosan，它們幾無細胞毒性，人體對它們也不會產生排斥性，更重要的是人體內存有可將它們水解的酵素，使它們具有生體可降解性，免除了更換敷料的不便，也降低了傷口二次感染的機會。

我們實驗中所用的原料—靈芝，其在醫療方面的功效正被一一證實，雖然已知靈芝具有功效的部分主要在三帖類部分，而我們作爲開發生醫材料的部位爲其萃去有效成分後之子實體，但基於靈芝多樣的功效，其子實體在傷口癒合上極可能具有的強化作用[11]，依然相當值得我們期待。

1.2 研究目的

本研究目的將靈芝的子實體部分藉由鹼處理、以及漂白水的初步脫色作用，將其冷凍乾燥爲適宜處理的原料，再以二元溶劑系(LiCl/DMAc)將難溶的幾丁質 Chitin 溶解。以此步驟進行，得之溶液再製成適於作爲敷料之薄膜. [12]，希望藉由一連串的物性分析後，能評估出真正用於臨床的可行性，並開發出一種兼具目前各種處理皮膚外傷方法之優點，而將缺點降至最低的新式生物醫學材料，以提昇傷患復原期間之醫療品質及傷口復原後之完整性。

第二章 文獻回顧

1. 靈芝的應用

根據近代東西方科學整合研究，靈芝的外形爲靈芝子實體，以一年生爲主，大部分具有菌蓋和菌柄。菌蓋呈圓形或腎形；表面具有漆樣光澤，具環紋，有的具放射狀縱皺。靈芝的別稱有靈芝草、不死仙草、瑞草、神草、吉祥草等。而靈芝的產地多分布於陰涼山林之枯木土石上，在台灣、日本、中國大陸及海南島等處，可以找到靈芝的蹤跡。靈芝的主要醫療成分是 Triterpene，這是導致靈芝具有苦味的原因，目前已知 Triterpene 的類別在靈芝種中已發現二百多種，而靈芝特有的 Triterpene 稱之爲「靈芝酸」，它的主要功能爲:抑制癌細胞生長、抑制 Histamine 釋放防止過敏、促進肝功能、促進血小板凝集、降血脂等已被研究的效能。至於結構部分的主成分[13]，也就是我們實驗探討的主軸: Chitin 及 Chitosan 也被廣泛的研究與探討。

2. Chitin 的應用

1799 年，Hachett 將蝦蟹殼利用酸來處理，發現除了產生泡沫之外，接著又出現一種軟膠狀的黃色物質；1811 年，Braconnot 利用稀鹼加熱處理蕈類，發現一種物質，他將此物稱做 Fungine；1823 年 Odier 取昆蟲的外骨骼

及翅鞘部份，經過 NaOH 的處理，萃出一種忌水性的物質，他將其名爲幾丁質(chitin) [14]，希臘文字的意涵有包覆物質的之意 [15] ，這是 Chitin 大致的發現史。

Chitin 在自然界的蘊藏量極爲廣泛，是僅次於纖維素的多醣類，普遍存於無脊椎動物甲殼類和昆蟲類的外骨骼或翅鞘中 [16] ，而烏賊體內透明塑膠狀的軟骨[17]，咸認含有大量且質純的 Chitin，因此目前已爲工業上大量生產 Chitin 的原料之一。而包括靈芝在內的許多蕈類其結構中亦含有大量的 Chitin。然纖維素已經大量地被利用，反觀功效多元的 Chitin 之相關開發就顯得缺乏。其實在許多研究當中，已發現 Chitin 及其去乙醯基之 Chitosan 有許多值得應用之特性.[18]。Chitin 目前在臨床上已應用在人工皮膚、人工韌帶、人工血管、外科縫線等，這些生物醫學材料都是以 Chitin 當作基質，再和其他物質共同形成高分子聚合物，對人體具有親和性同時可降低排斥現象的發生機會。Chitin 也可以提高人體的免疫能力，這是由於 Chitin 能將血液的酸鹼值從酸性轉變成鹼性[19]，間接的促進體內 NK 細胞的形成，並活化 LAK 細胞的淋巴球，降低癌細胞生長或轉移的機會。而也因爲人體內存在著大量的 lysozyme、chitinase、chitosanase 等酵素[20] ，可以切斷 chitin、chitosan 高分子間的鍵結，進而可行降解而由人體吸收，由這個概念爲基礎作爲我們製造人造皮膚敷料的概念[21]，希望能製造出免於替換而能直接爲

人體吸收的材料。Chitosan 被發現其鹽酸鹽[22]衍生物之 C-2 上的氮原子在水溶液中帶正電而形成帶正電的高分子[23]，且此類衍生物遇水後易形成凝膠狀，因此可誘發血小板產生凝血作用，達到止血效果 [24] 。總結而言，chitin、chitosan 所具有的生理活性包括:生體可分解性[25]、傷口治癒促進效果及止血作用。在已知得文獻中[26]，已有動物試驗證明 chitosan 具有臨床上常用的氧化纖維素系列或膠原蛋白系列止血劑具同等的止血效果[27] [28] ，且其吸水性較其他類的止血材料高出數十倍，這都是 Chitin 及 Chitosan 在生物醫學材料上可資應用的利基。

3.Chitin 的構造

幾丁質(chitin)是由數百、數千至數萬個N-乙醯胺基葡萄糖單體(N-Acetyl glucosamine units)以β-1,4 鍵結所形成的一種規則且具連接性的聚合物，其中約每六個 N-乙醯胺基葡萄糖(N-Acetylglucosamine)連接一個葡萄糖胺基(D-glucosamine)；將 Chitin 經由不同的脫乙醯化程度可以得到聚葡萄胺醣(chitosan)，其乙醯化程度可由 65% 到 90 %以上，一般以 70 %-80 %爲主。兩者結構與纖維素相似同樣爲高分子，其差別僅在於 C-2 位置上所接的官能基的差異。自然界中的脊椎動物無法自我生成 Chitin，而無脊椎動物中的甲殼類則多具有生成 Chitin 的能力。

4. Chitin 的脫蛋白作用

由於從自然界中得到的 Chitin 的來源，不管是甲殼類動物或是蕈類，它們的組織中除了 Chitin 外還含有許多蛋白質，因此去除這些物質便成爲精製 Chitin 重要的前置步驟。脫蛋白(deproteination)的步驟在文獻上通常都是用 3-5%的苛性鈉來處理 [29] [30] ，在 60-100°C 下反應 2-6 hours，用以純化出所需的 Chitin。

5. Chitin 的理化性質

幾丁質(chitin)在生物體當中以反向非平行及對稱同向的醣鏈藉氫鍵結合形成。Chitin 的構造經由 X-ray 繞射的研究結果，可依照其雙股螺旋以及對稱軸分子之排列方式的不同而將其劃分爲α、β、γ三種結晶形式。

α型： Chitin 屬於斜方晶系(rhombic)，每個晶格中含有八個 N-乙醯胺基葡萄糖(N-Acetylglucosamine)，其兩股雙螺旋間呈反向平行(antiparallel)排列，醣鏈間彼此以氫鍵結合。此類型結構緻密，質地也較堅硬，是自然界的 Chitin 當中最普遍且最穩定的構形，大部分昆蟲及甲殼類的外骨骼及翅鞘所含之殼糖即屬於此型。

β型：Chitin 屬於單斜晶系(monochinic)，每個晶格中由兩個 N-乙醯胺基葡萄糖(N-Acetylglucosamine)所構成，其兩股雙螺旋呈平行(parallel)排列，組織結構較爲鬆散，與纖維素的結構相似，易被酵素所分解，烏賊軟骨即

屬此型。

γ型：爲α及β型的混合體，藻類或真菌類所含有的 Chitin 即屬於這一類的範疇 [31] [32] 。

6.Chitin 的溶解

Chitin 在性質上是一種難溶於水及一般溶劑的物質。這是因 chitin 的乙醯胺基(acetylamino group)極易產生分子內氫鍵所造成的現象。較早的文獻發現 Chitin 可溶於高濃度的鹽酸、硫酸、硝酸等強酸溶液；但卻會造成 Chitin 的降解與分解，這並非我們欣見的結果，我們不但得溶解 Chitin，且要保持其結構的完整。在近期的研究中發現，Chitin 可以溶於二元系的溶劑，5 % LiCl 溶於 N,N-dimethylacetamide 的二元溶劑系即是 [33] ，它的 Li^+離子會先破壞分子間氫鍵，並同時產生錯合作用，得到的 Li^+/Chitin 錯合物，再以來回數次升降溫的加熱方式使 N,N-dimethylacetamide 溶解之，即能大幅提昇溶解的效果。

7.Chitin 分子量之測定

黏度平均分子量:

將 Chitin 溶於 5 % LiCl /DMAc 溶液，再把所得溶液注入 Scott-Grate 毛

細管黏度計，在 25±0.1°C 的控溫槽中測其黏度，記錄溶液通過 A、B 兩處的時間，每個條件測七次。然後利用下列公式運算：

比黏度 (specific viscosity) $\eta sp = (T/Ts) - 1$

還原黏度 (reduced viscosity) $\eta red = \eta sp / c$

內生性黏度 (intrinsic viscosity) $[\eta] = (\eta red) \rightarrow 0$

T ：Chitin 溶液通過毛細管 A、B 兩處所需時間

Ts ：5 % LiCl/DMAc 溶劑通過毛細管 A、B 兩處所需時間

c ：Chitin 溶液的濃度

將以上所求的內生性黏度[η]代入 Mark-Houwink 方程式中即可求出其黏度平均分子量：

Mark-Houwink 方程式爲 $[\eta] = KMa$

a ： Mark-Houwink exponent

K ： Mark-Houwink exponent parameter

M ： molecular weight

8.Chitosan 之多元應用

Chitosan 是 Chitin 的去乙醯化物，但在醫學材料的應用上，實不亞於 Chitin。Chitosan 具有親水性質，目前包括藥物的賦形劑[34][35] 組織工程的再生骨架等都已應用到了 Chitosan [36] ，而 Chitosan 也具備了生體內降解的特性，且無細胞毒性，所以若 Chitin 和 Chitosan 能夠以適當比例混合且相輔相成，將有卓越的效果。

第三章 研究材料與方法

3.1 實驗材料

1.靈芝萃取後除去三帖類之子實體，the residue of Ganoderma.lucidum without Triterpene

2.α-chitin，研究試藥，FUNAKOSHI，日本

3.HCl，試藥級，SHIMAKYU，日本

4.NaOH，試藥級，NIHON SHIYAKU，日本

5.KOH，試藥級，NIHON SHIYAKU，日本

6.HOCl3，市售級 ，5.25%，The Clorox Company，美國

7.N,N-Dimethylacetamid 99+%，ACROS，美國

8.LiCl，試藥級，NACALAI，日本

9.Ethanol，試藥級，95%，台灣

10.Acetone，試藥級，台灣

11.AgNO3，試藥級，台灣

12.KBr，特級，YAKURI，日本

3.2 實驗儀器

1.粉碎機，RT-04，台灣

2 電子天平，Mettler，AG245，瑞士

3.烘箱，MMM Medcenter GmbH，Venticell 111，德國

4.電磁攪拌器，Corning，pc-420，美國

5.迴旋蒸發器，EYELA，N-N，日本

6.水幫浦，Tokyo Rikakikai Co. LTD.，A-3S，日本

7.真空烘箱：Lingberg，V0914C，ASHEVILLE，美國

8.冷凍乾燥機：KingMech，FD24-2S-6P，台灣

9.離心機，HSIANGIAT，CN-3600，台灣

10.自動毛細管黏度測定儀，SCHOTT，AVS-360，西德

11.毛細管黏度計之恆溫水槽，Firestek Scientific，B801，美國

12.恆溫槽，Firstek Scientific，B401D，台灣

13. 材料機械性質測定儀， Material testing system (MTS)，MTS Corporation ，858 Mini BIONIX，USA

14.X-ray 繞射儀，MaxRC，RIGAKU，日本，V:40 kV，A: 100 mA

酸鹼值測定儀

15.示差掃描式熱分析儀 Differential scanning calorimeter(DSC)，Pyris1，Perkin Elmer，美國

16.震盪器，VM-2000

17.震盪水槽，Shaker bath，Firtek Scientific，B603，美國

18.紅外線光譜測定儀，Fourier Transform Infrared Spectroscopy (FT-IR)，BioRad Laboratories，Perkin Elmer，FTS165，Perkin Elmer 19.核磁共振光譜儀，NMR，Bruker，Avance DRX-500，GmbH

20.偏光顯微鏡

21.實體顯微鏡，華堂光學實驗有限公司

22.DMA ，Perkin Elmer ，Perkin Elmer

23.動態接觸角測定儀，First Ten Amstron，FTA200，USA

3.3 研究方法及進行步驟

我們希望將靈芝的功用發揮，我們將靈芝萃取出 Triterpene 後的剩餘成分，也就是支持結構的部分做適當的處理與應用；在 3.3.1 中先以 95%的 ethanol，煮沸 2 hours 初步洗去靈芝外部的雜質；3.3.2 以不同條件鹼處理，產生不同方向的討論。其一以 1N 以上的強鹼使 sample 中的 Chitin 產生不同程度的去乙醯化效應；其二以 1N 以下的鹼處理，爲的是除去雜質及蛋白成分；3.3.3 以 HClO3 進行脫色，並去除不溶的色素成分，同時 HClO3 的強氧化作用也會切斷高分子間的鍵結而影響到分子量，而分子量的變化，可藉由 3.3.4 以 LiCl/DMAc 溶解 Ganoderma/Chitin 後，3.3.6 測量溶液的黏度並換算得知；3.3.5 以各種條件所製的溶液，製作靈芝膜；在 3.3.7 中，以

Autograph 或 MTS 測量成品的強度，以評估是否能達到取代皮膚的要求；收集所有不同條件、不同處理階段的殘渣，共同以 3.3.8 的 X-ray 測α及β構形之 Chitin 相對比例；以 3.3.9 的 IR 測官能基變化；3.3.10 的 CP MAS ^{13}C NMR 測定質子的分布位置；綜合 3.3.8、3.3.9、3.3.10 對靈芝的結構加以定性，並比較不同方式的處理，對物性所造成的種種影響；最後再以 3.3.11 的 DSC 測樣本熱變化的情形。綜合這些數據結果，作線性的整理並分析之，並擇其中最佳者大量製備，以利往後的研究者更進一步的活體實驗，我們依續對進行的步驟加以詳細說明。

3.3.1 靈芝子實體的初步處理

本實驗由經萃取出 Triterpene 後的靈芝組織結構爲主，取其組織中含有幾丁質(Chitin)及 Chitosan 成分的特性，針對此點設定不同條件，將靈芝子實體殘渣做初步的處理。首先，甫得到的靈芝殘渣需以 95 %的 Ethanol 進行 Extraction 48 hour，而靈芝殘渣在 95% Ethanol 中的重量百分濃度爲 2.5%，之所以要控制在 2.5%的緣故，是考慮到樣品量的多寡，會影響到處理時的均勻性，所以在處理時將量固定，可避免條件外的影響，在接下來的鹼處理和脫色過程，我們同樣將樣品的重量百分濃度定爲 2.5%，道理相同。過濾後，再接著以 100℃的 dil.H2O 煮沸 2hours 後乾燥，以上步驟是爲了初步洗去 chitin 及 chitosan 之外的成分，以利我們接下

來的研究。

3.3.2 樣品的鹼處理

經初步處理過後的靈芝，我們分兩個研究目的進行鹼處理。

其一:爲了得到鹼處理強度對分子量、內部結構及之後成膜強度的影響，我們取 KOH 及 NaOH，分別以 0.1N、0.2N、0.4N、0.6N、0.8N、1N，將重量百分濃度 2.5% 比例的靈芝殘渣在 70℃的條件下煮 2 hour 做鹼處理，而後以透析處理 24 hour，用 pH meter 測之呈中性。在這步驟中的鹼處理是爲了進一步除去靈芝殘渣中的蛋白成分及雜質，純化我們的樣品，所以我們只需取適當的鹼處理濃度，來維持我們所需要的 Chitin 比例，以維持其結構上的優勢，利於做爲皮膚敷料之用。處理過後的成品以冷凍乾燥處理，以冷凍乾燥處理的樣品可均勻的呈現多孔性，在接下來的實驗中可利試劑及樣品間的交互作用，乾燥後的成品置於 N2 中保存，以待下一步進行脫色。

其二:我們爲了觀察鹼處理對靈芝中的 Chitin 成分比例變化影響，我們以 KOH 及 NaOH，分別以 2N、4N、6N、8N、10N 進行鹼處理，欲藉高濃度的強鹼，強行脫去 Chitin 中的 acetyl group，使 Chitosan 的成分比例增加，處理過後的成品以冷凍乾燥處理，取乾燥後的樣品利用元素分析，推測出脫乙醯化的程度，其方法及公式如下[37]:

A：$C_8/203\ X + C_6/161\ Y$ ---1

B：$N_1/203\ X + N_1/161\ Y$ ---2

Degree of deacetylation =Y / X+Y ---3

A = C %，B = N %，Chitin MW = 213，Chitosan MW = 161[38]

藉由不同濃度鹼處理和靈芝內含之 Chitin 脫乙醯化間的關係，可利於我們往後對靈芝的定性以及作醱酵加工處理的參考。

3.3.3 樣品的脫色處理

此步驟延續鹼處理，取市售 5.25% 的漂白水，分別以 1:1、1:2、1:3、1:4、1:7、1:9 的比例稀釋，再將先前經 O.1N KOH 鹼處理及冷凍乾燥過的靈芝殘渣，以 2.5% 的重量百分濃度置於這些不同比例稀釋的漂白水中等速攪拌 15 mins，之後以透析置換 24 hours，以 pH meter 測知呈中性，AgNO3 溶液檢測無 Cl 離子殘存，即可加以冷凍乾燥以待下一步驟。而控制變因取 0.1N KOH 的原因，主要因 0.1N KOH 是此系列實驗中最弱的鹼處理，而這種選擇可將鹼處理可能造成的影響降至最低，以便更專一探討脫色過程對靈芝殘渣中高分子鍵的影響，這可藉由黏度的測量推算出分子量的變化得知其間的關係，而分子量的大小，對於我們成膜的結果優劣，也有直接的

影響。

在不同條件的鹼濃度下，我們選定 1:3 比例漂白水/蒸餾水溶液處理既得樣品，而這種選擇的原因，純粹是粗估 1:3 比例的溶液能在成膜後提供外觀較宜人的色澤，以及維持一定的強度供拉力實驗之故，至於最佳的漂白水/蒸餾水比例，仍需自接下來的實驗中得知。

3.3.4 樣品的溶解

取先前不同條件下處理的樣品，以 6%的重量百分濃度，置於 5%的 LiCl/DMAc 二元溶劑系中，以迴流的裝置進行溶解。首先置於油浴 70℃中，加入攪拌子攪拌以利溶解，保持 70℃溶解 12 hours 後進行第一次降溫，用以降溫的冰浴溫度以 4℃爲準，靜待冰塊融化完畢，再行第二次升溫，如此重覆三次，則在我們設定範圍的所有條件下皆可順利溶解，溶解液再以減壓蒸餾的方法，將多餘的 DMAc 去除，最後再經離心以確認所得部分皆爲 Chitin/Chitosan solution，即能作爲我們接下來成膜的原料。

3.3.5 靈芝膜的製備

取先前所得到的 Chitin/Chitosan 混合溶液，將 5ml 溶液均勻倒在在 15×15 cm^2 玻璃板上，利用成膜器具順向推動溶液，藉此步驟可將溶液平整地鋪平在玻璃板上。接著將玻璃板連靈芝膜浸入 ethanol 溶液 2 minutes，撈

起迅速再將玻璃板連同靈芝膜浸入 Acetone 溶液 30 seconds，使膜形狀固定。最後將所製成膜放在水裡放流約 24 hours，利用 AgNO3 確定水中無 Li 離子殘留，即可將靈芝膜置於 Teflon 上，靜置待其乾燥，及可得到半透明的靈芝膜。我們也可以利用相同原理製成手術用縫線等生醫材，並依需求改變條件，以期多樣化的發展。

3.3.6.黏度測定

在溶解時溫度、時間或是溶劑的濃度，都會影響到脫蛋白的程度這些差異性也會造成高分子間的斷鏈，爲研究當中之差異性，我們以毛細管黏度測定儀進行還原黏度值之測定。首先取 19 ml LiCl / DMAc 溶液 及 1ml 既得樣品溶液加入三角錐瓶內，在 70 °C 下攪拌至均勻溶解。接著將此溶液倒入 0.84 mm 黏度管內，在 25 °C 下進行黏度的測試，每一條件跑 7 次求其平均值，惟在測樣品之前需先以 20ml LiCl/DMAc 先測黏度，且七次的誤差需＜99%，以確認黏度管本身的安定性。記錄所得時間，以公式代入計算比黏度，並求其線性。

比黏度 (specific viscosity) $\eta sp = (T/Ts) - 1$

3.3.7.拉力測試

利用不同條件製成的靈芝膜分別以 DMA 做拉力測試，利用拉力測試找出張力強度強的靈芝膜。首先裁剪膜大約爲 0.5 cm 寬，長度大約是 4 cm 左右。利用模具將 sample 夾緊，設定 Dynamic 及 Static 的測試條件，直到 sample 拉伸到斷裂爲止，將所有數據收集，並找出之間的線性關係。

3.3.8.X-ray 試驗

α-type 和β-type Chitin 因爲構型不同，利用 X-ray 繞射光譜，可以比較 α-chitin 膜、β-Chitin 膜和靈芝膜在結晶度(crystallinity)的差異。因此利用 Rigaku D 公司的 X-ray 繞射儀，將各個條件下的樣品固定，並設定以每分鐘 5°之角度旋轉，在室溫下角度從 10°測定到 60 ，以 Cukα射線λ爲 1.5418，在室溫下對濃度 1 % 之α-chitin 膜和靈芝膜測試，經由測定器將訊號傳到電腦進行分析，及得知靈芝所含 Chitin 的構形爲何。

3.3.9.IR 測定

1. 經初步鹼處理的靈芝殘渣，在透析且冷凍乾燥後，利用 FT-IR 的偵測，觀察官能基的改變狀況。
2. 經脫色處理的靈芝殘渣，同樣利用 FT-IR 的偵測，觀察官能基的改變狀況。

3. 成形後的靈芝膜成品，同樣利用 FT-IR 的 ATR 偵測，觀察官能基的改變狀況。

以上三組條件的測試，其波長皆是在中紅外光 4000 nm 到 500 nm 的範圍下進行 IR 的光譜分析。

3.3.10 NMR 的測定

測定核磁共振光譜可以預測分子的結構式，選用固態及液態 NMR 的測試方式，將不同的強鹼(NaOH)以 12N 的高濃度處理靈芝殘渣，使其中 Chitin 轉換爲 Chitosan，再以 3%的重量百分濃度溶解於質子化溶劑中(D2O)，並取 0.5ml 放入 NMR 測定專用管中測定，主要測定:氫譜(H-NMR；範圍:10~0.5ppm)及碳譜(C-NMR；範圍:200~10ppm)，觀察鹼處理對靈芝殘渣的結構影響，以分析其變化。

3.3.11 DSC 測熱性質

將鹼處理過以及脫色過的靈芝殘渣，取 5~10 mg 置入 sample 盤中，以專用壓片機壓片，在將之置於 DSC 左側 oven，設定條件(temperature:30~220℃，10℃/min；220℃，5mins；50℃/min)開始以電腦測定並記錄數據。

Reference

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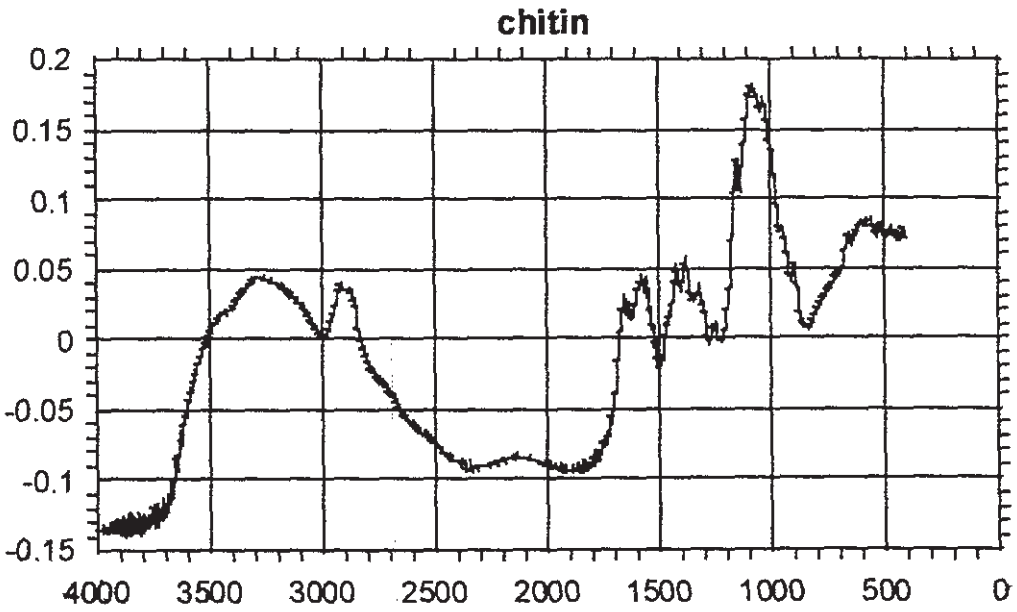
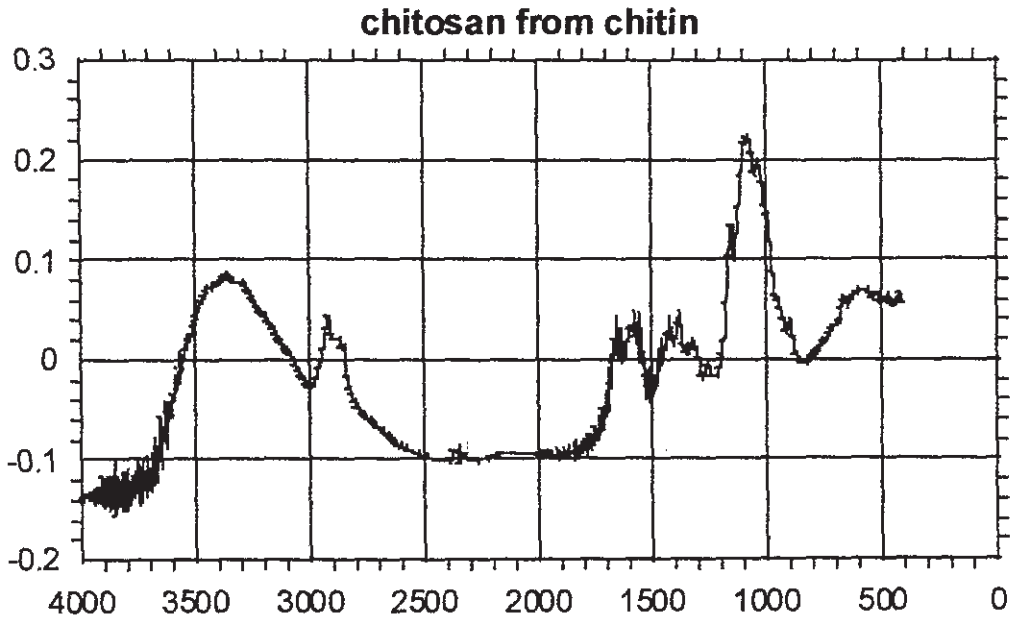
Chitin 及 Chitosan 只有比率上的不同，所以在維持本品的特性上，我們的處理方法是被認可的。

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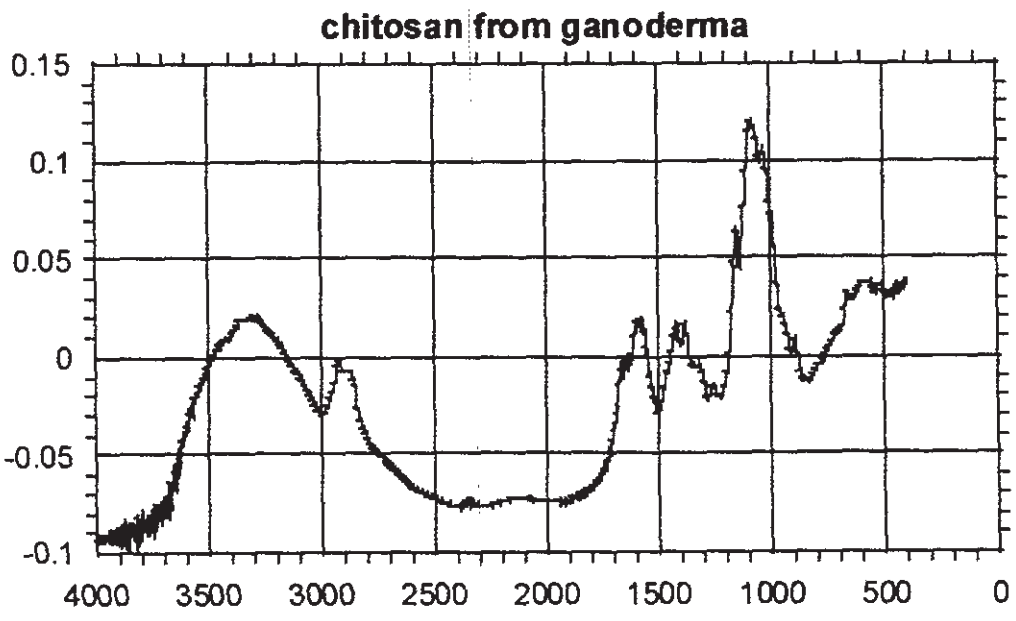
3000
2500
2000
1500
1000
500
0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0.05
0
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-0.1
4000
3500
3000
2500
2000
1500
1000
500
0

NMR 的測試部份:

我們發現由 Ganoderma 處理得來的 Chitosan 與文獻上的 NMR 圖譜無論是固態或是液態 NMR 都有相仿的表現;不同處往往只有一些 peak 的輕微 shift，這些可能是側鏈接上不同的醣類造成的結果;但無礙於我們實驗品項的主要特性。

ORIGINAL

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70.5674

BRUKER

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PPG:
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DATE 19-12-0

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TE	297
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FW	36200
O2	5050.000
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D0	5.000S
D1	3.500U
D2	4.000U
D3	20.000U
D4	1.000U
LB	50.000
GB	0.0
NC	3
CX	21.00
CY	12.00
SR	-1050.91

MEMBRANE

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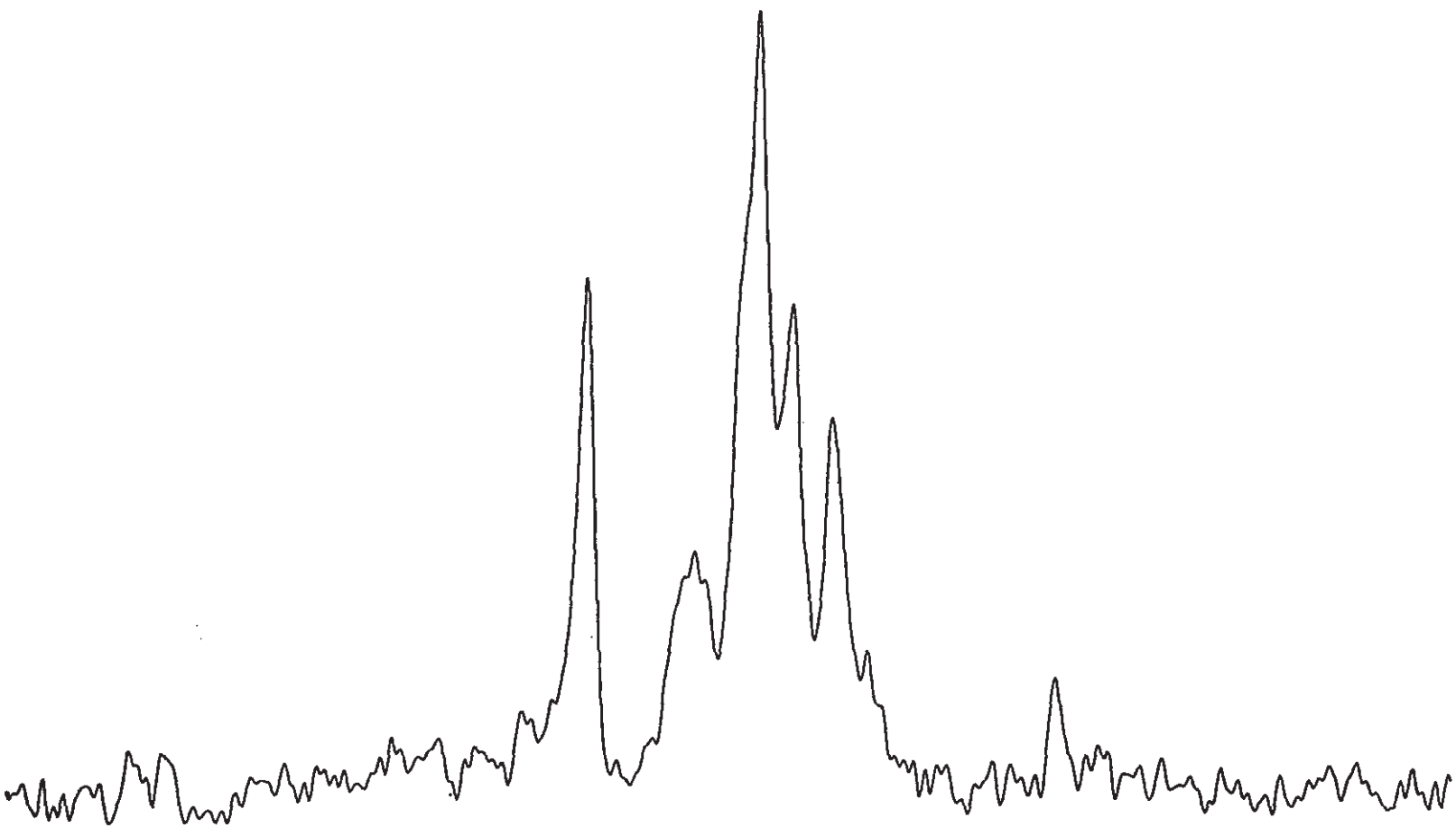

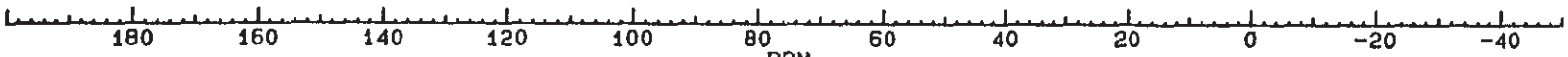

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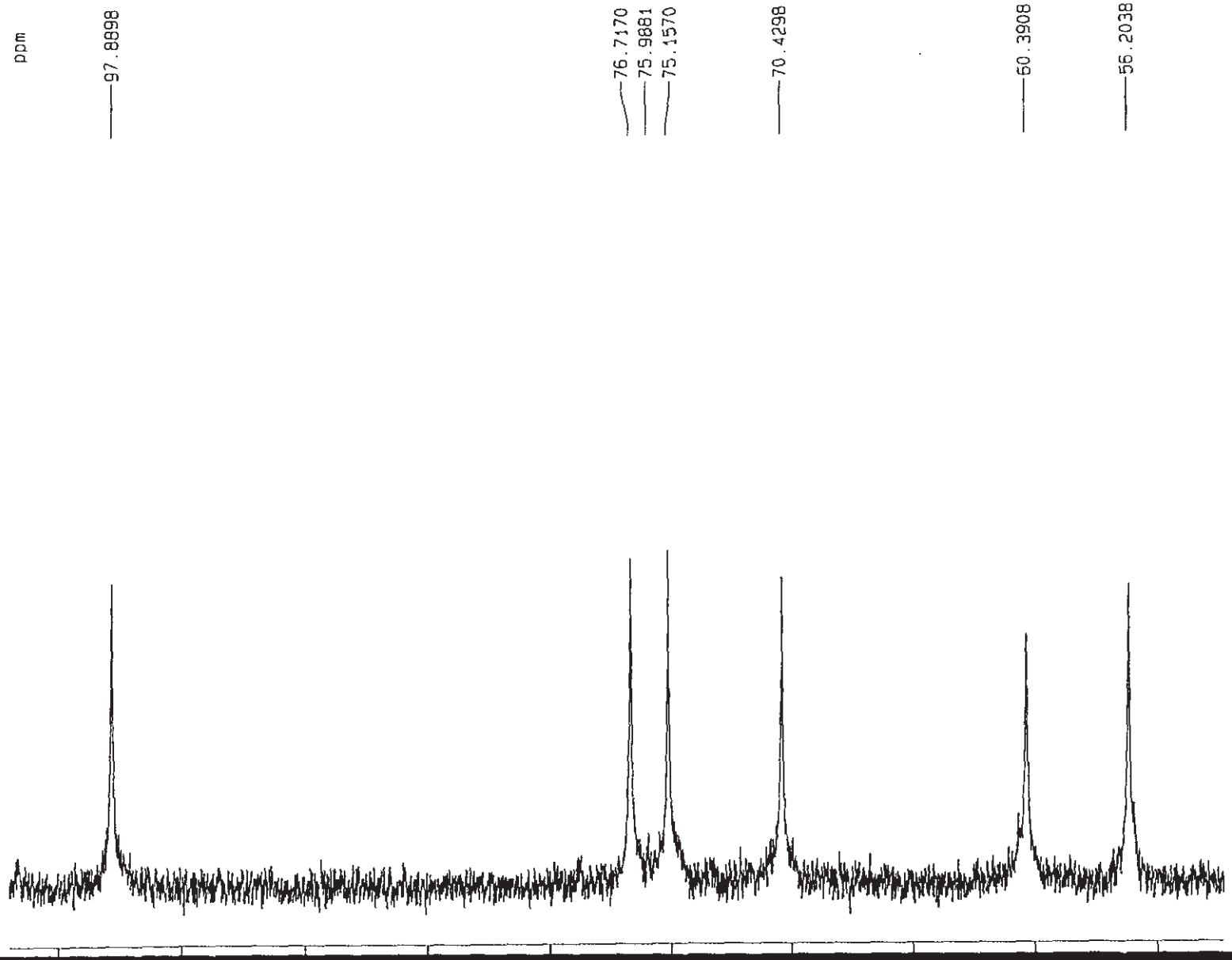
0207-chitosan/2/1/u/th



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FIDRES	0.479836 Hz
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RG	8192
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DE	6.50 usec
TE	300.0 K
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PL13	120.00 dB
D1	1.50000000 sec
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PCPD2	90.00 usec
SFO2	500.1320005 MHz
NUC2	1H
PL2	-4.00 dB
PL12	15.00 dB
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NUC1	13C
PL1	3.00 dB
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F2 - Processing parameters

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PC	1.40

1D NMR plot parameters

CX	20.00 cm
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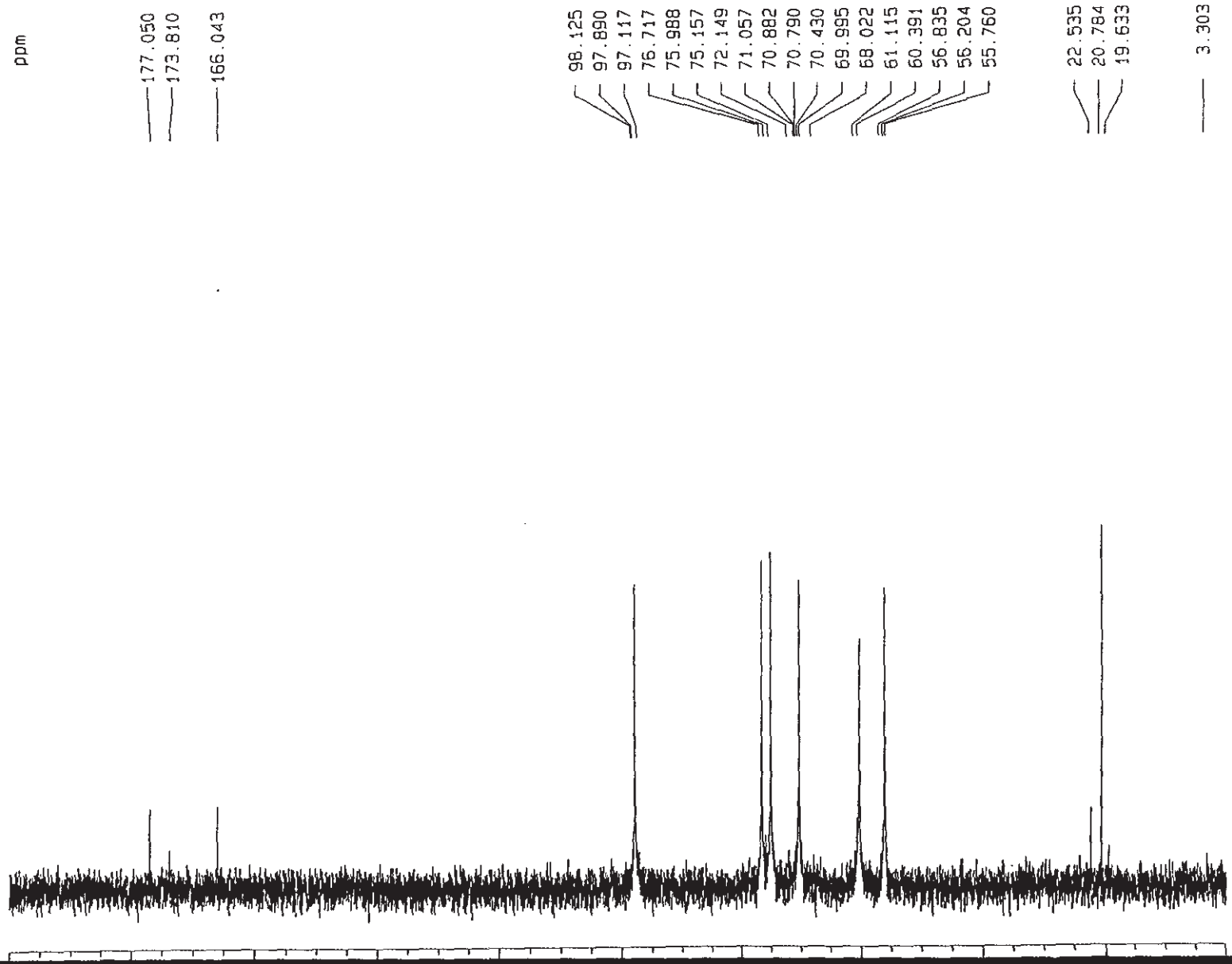
chitosan/H2O+D2O/C13
0207-chitosan/2/1/u/th



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FIDRES	0.479836 Hz
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DW	15.900 usec
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TE	300.0 K
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PL13	120.00 dB
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CPDPRG2	waltz16
PCPD2	90.00 usec
SFO2	500.1320005 MHz
NUC2	1H
PL2	-4.00 dB
PL12	15.00 dB
P1	12.50 usec
SFO1	125.7715724 MHz
NUC1	13C
PL1	3.00 dB
D11	0.03000000 sec

F2 - Processing parameters

SI	32768
SF	125.7577390 MHz
WDW	EM
SSB	0
LB	1.00 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	20.00 cm
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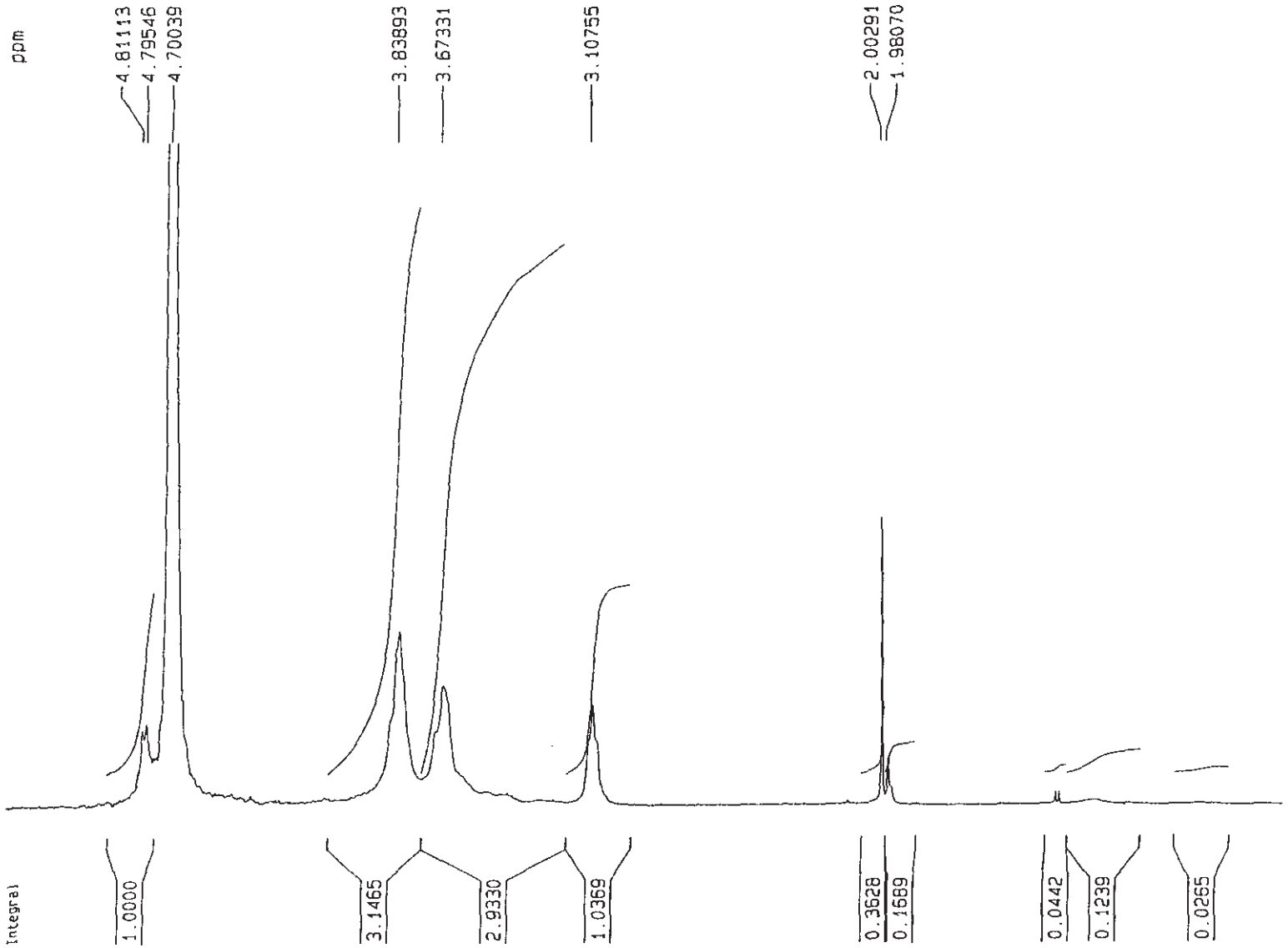
F1	25151.55 Hz
F2P	0.000 ppm
F2	0.00 Hz

chitosan/H2O+D2O/1D-H
0207-chitosan/1/1/u/th



Current Data Parameters

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PROCNO	1

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PL1	-4.00 dB

F2 - Processing parameters

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LB	0.30 Hz
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PC	1.40

1D NMR plot parameters

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F2P	0.470 ppm
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