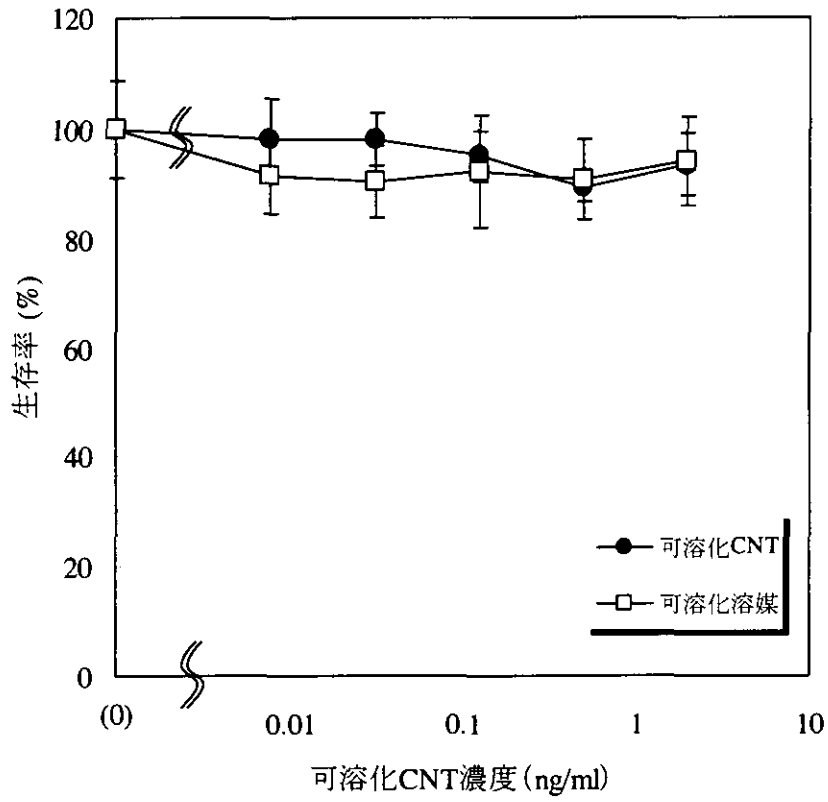


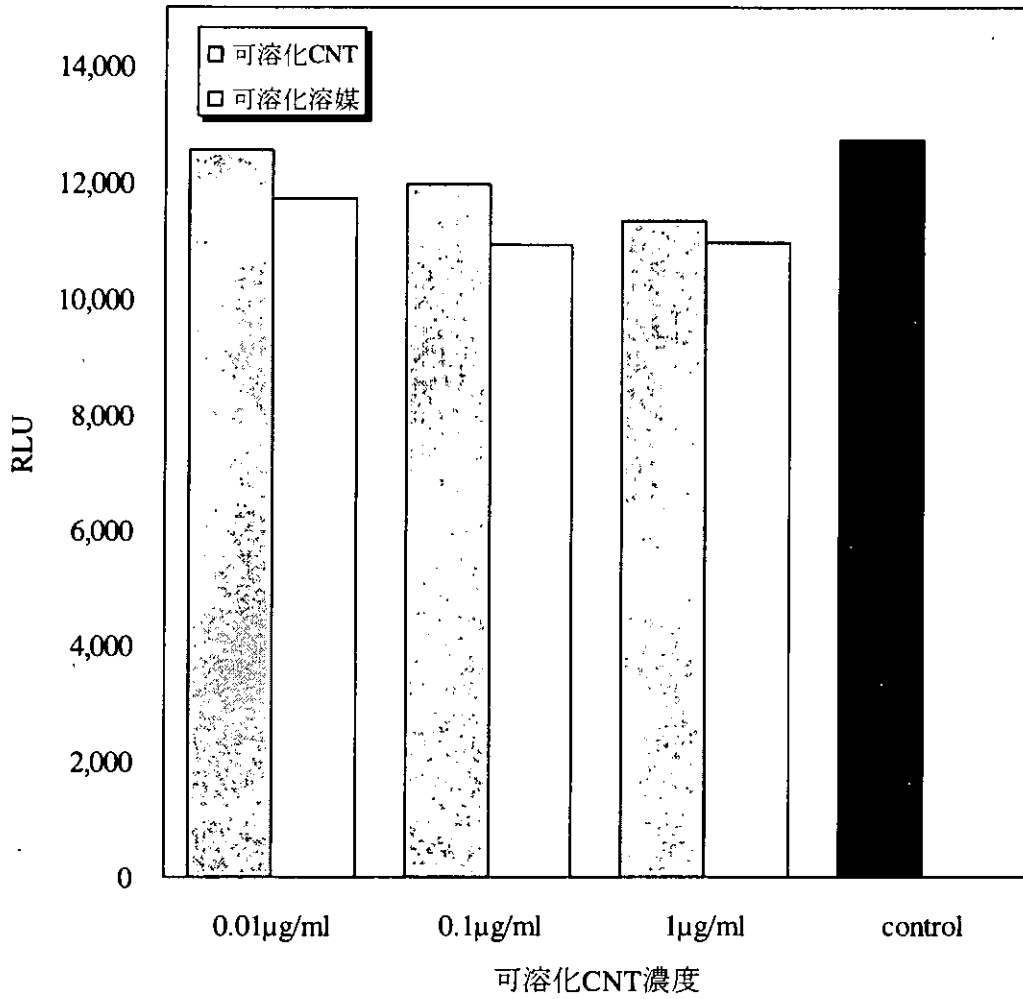
図3



注1) (0)には5%FBSのみを含む培地で培養した際の値を示した。

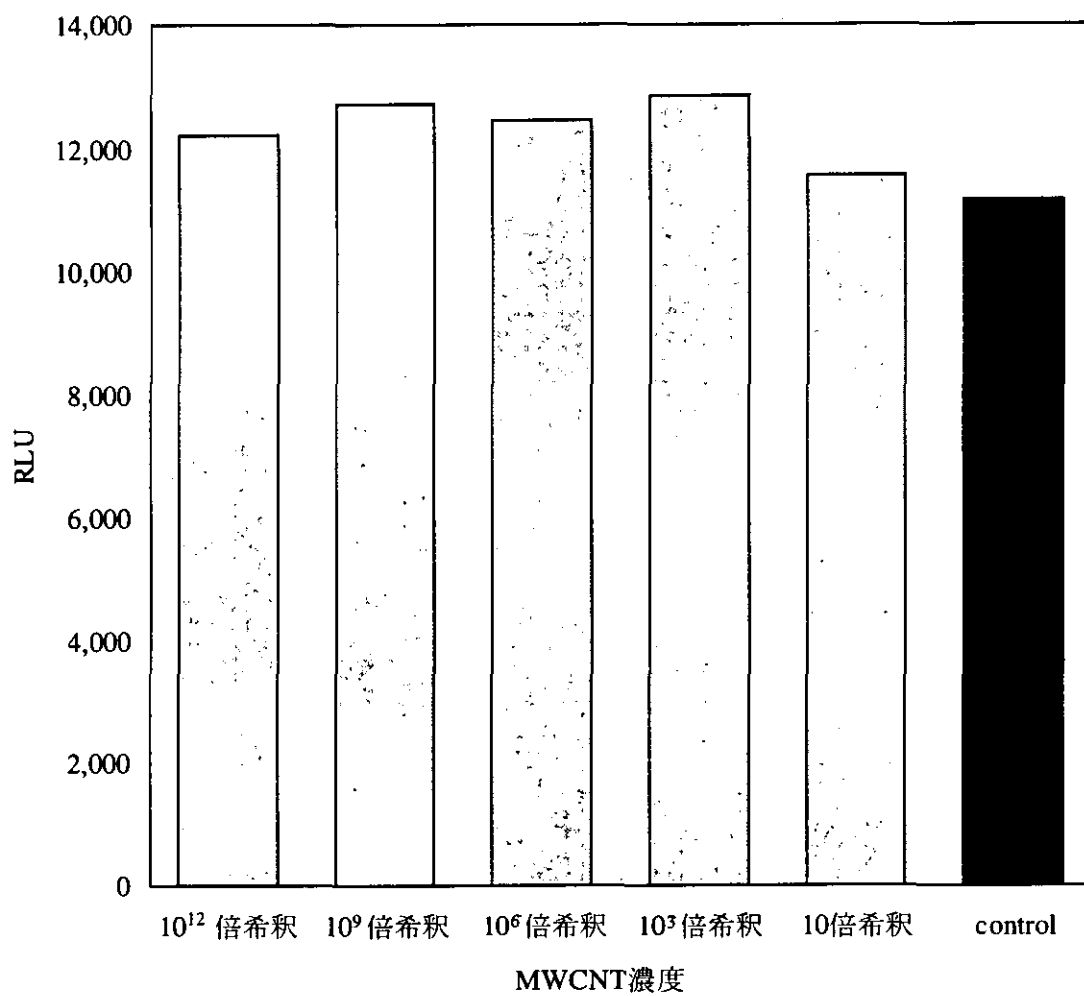
注2) 各プロットの上には標準偏差を示した。

図4



注1) 但し、可溶化溶媒の発光度は、各濃度の可溶化 CNT 調製時と同様の希釈倍率のものを比較として示した。

図 5



注1) 但し、MWCNT濃度は未知のため、希釈倍率を示した。

カーボンナノチューブを用いた複合材料の作製と評価 およびカーボンナノカプセルの生体為害性の調査に関する研究

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カーボンナノチューブ (CNT) と金属またはポリマーとの複合材を作製し、繊維強化金属 (FRM) や繊維強化プラスチック (FRP) のような高比強度の生体材料を合成することを目的として研究を行い、Ti/CNT及びアクリルレジン/CNT複合材料を作成し、その強度を検討した。Tiとの複合においてはCNTがTiと反応しTiCを生成して強化に寄与しなかったが、アクリルレジンに添加した場合には曲げ弾性率がやや向上し、CNTによる強化の可能性が示唆された。またカーボンナノカプセルの生体内での挙動や生体為害性を調査し、その生体為害性は現在金属中で生体適合性に最も優れるとされるチタンが微粉末化したものと同程度であり、為害性が十分に低いことが示唆された。

A. 研究目的

カーボンナノチューブと金属またはポリマーとの複合材を作製し、繊維強化金属/プラスチックのような高比強度の生体材料を合成することを目的とした。

またカーボンナノカプセルは希土類炭化物 (CeC₂など) の周囲をグラフェンシートで覆われた直径20~30nmの粒子である (図1)。このナノカプセルは内部に希土類を含有し、周囲をグラフェン構造で覆われるため、希土類をトレーサーとして生体内でのカーボンナノ粒子の挙動を調査するのに好適である。そこでこのナノカプセルをラットに投与し、生体内での蓄積挙動を調査した。

B. 研究方法

【CNT含有複合材料の作製と評価】

多層CNT (Nanolab社製) と水素化チタン (住友チックス製) をCNT含有量が0, 5, 10, 15w

t%となるよう秤量・メノウ乳鉢で混合し、放電プラズマ焼結装置 (SPS) で焼結した後、断面が1mm角の棒材に加工した。焼結条件は表1の通り。得られた試験片は万能試験機 (インストロン: 4204) により支点間距離15mm、クロスヘッドスピード0.5mm/minの条件下で3点曲げ試験を行い、強度を評価した。また断面をSEM (日立: H-2380) にて観察するとともに、XRD (リガク: Multiflex) により結晶相の同定を行った。

【CNT含有複合材料の作製と評価】

BisGMA(bis-phenol-A di-glycidyl-methacrylate)とTEGDMA(Tryethyleneglycol-dimethacrylate)を重量比1:1に混合し、0.3wt%のカンファーキノンと0.7wt%の過酸化ベンゾイルを添加したレジントリックスを作製した。これに多層CNT (Nanolab社製) を0, 0.5, 1.0, 1.5, 2.0wt%となるよう秤量・混合し、内径1.3mmのポリエチレンチューブに充填し、可視光重合器 (モリタ: α Light) により30分光照射を行って表面を重

合せた後、チューブから取り出して、さらに80℃のオーブンで3時間以上加熱重合を行った。得られた試験片は万能試験機（インストロン：4204）により支点間距離15mm、クロスヘッドスピード0.5mm/minの条件下で3点曲げ試験を行い、強度を評価した。

【ナノカプセルの生体内挙動の評価】

Ce含有カーボンナノカプセル（以下Ce-CNC）を無菌的に生理食塩水中に濃度50ppmとなるよう分散し、ラット尾静脈より2回/日で3日間投与した後、屠殺し各臓器を摘出した。各臓器は乾燥後にB坩堝内で800℃×2時間加熱して灰化した後、5N硫酸で溶解、25mlに希釈した後、ICP（日立：P-4010）にてCe量を定量し、臓器へのナノカプセルの集積状態を調査した。

【ナノカプセルの生体内挙動の評価】

Ce含有カーボンナノカプセル（以下Ce-CNC）を混酸中で50℃×5日間処理し、同溶液を水酸化ナトリウム水溶液で中和後、透析を行いナノカプセル懸濁液を作製した。同懸濁液を無菌的に生理食塩水中に濃度0.1～100ppmとなるよう分散し、ラットの肺胞、腹腔マクロファージ及びTHP-1細胞に0.01～20ppmの各濃度で投与して6時間培養し、通法に従ってTNF- α 及びIL1- β の産生量からCe-CNCの有害性を評価した。

C. 研究結果

【CNT含有複合材料の特性】

図2にTi/CNT複合材断面のSEM像を示す。Ti粉末のみをSPSにて焼結した場合は若干の気孔が見られるが概ね緻密に焼結されていることがわかる。CNT含有量が5%の場合でも断面像に大きな変化は見られないが、CNT含有量が10%以上では内部に多くの気孔が見られ、粗造になっていることがわかる。焼結体の密度はTiのみで4.19g/cc、Ti-10%CNTで4.20g/ccであり、大きな差は見られなかった。Tiのみを焼結した場合でも、Ti本来の密度（4.51）に比べて低くなっており、これは図2に見られるような気孔に起因するものと推測された。

図3はX線回析スペクトルを示しており、CNT10%含有複合材ではTiのピーク以外に炭化物であるTiCのピークが明瞭に観察され、CNTに由来す

るピーク（ $2\theta = 26^\circ$ 付近）は観察されず、CNTの大部分がチタンの炭化に消費されていると考えられた。

本実験ではSPSによる焼結性の向上を考えて水素化チタンを使用した。水素化物使用による還元性雰囲気により、炭化が進行しやすくなった可能性があるため、今後材料の検討を要する。

図4はTi/CNT複合材の3点曲げ試験結果を示す。CNT含有量の増加とともに、曲げ強さ、破断歪ともに低下している。この原因としては上述の通りCNTがTiと反応して炭化物を形成したため、図2に示した通り気孔率の高い構造となったことに起因すると推測される。

【CNT含有複合材料の特性】

図5にTi/Bis-GMA複合材の3点曲げ試験における荷重/変形量線図の例を示す。CNTの添加によって僅かに弾性率の向上が見られるが、破断時の変形量や最大荷重はいずれもCNTの添加によって低下している。

図6は曲げ弾性率とCNT濃度の関係を示している。●は無処理のCNTを用いた場合、□は空気酸化後に6N塩酸及び6N硝酸で酸処理して触媒金属を除去し、分散性を改善したものを超音波ホモジナイザーで混合した場合である。無処理のCNTを用いた場合にはCNT濃度が0.5wt%において若干の弾性率の向上が見られるが、それ以上のCNT濃度ではCNTを含まない樹脂とほとんど同程度である。精製処理したCNTの場合にはCNT濃度が1.2wt%でやや弾性係数が高くなり、CNTによる強化効果が現れていると考えられる。

従ってCNTによる複合材の強化にはCNTの樹脂中への分散性が重要であり、超音波ホモジナイザーによる強制分散やCNTの空気酸化・硝酸処理による分散性の改良などでCNT複合樹脂の強度向上を図る必要があることが判明した。

【ナノカプセルの生体内挙動の評価】

Ce-CNCを投与したラットの各臓器内のCe濃度を表2に示す。表中Controlは坩堝のみで灰化・溶解を行った場合のバックグラウンドのCe濃度を示す。また本ICP装置におけるCeの検出限界は0.02～0.03ppm程度と考えられる。表中の○、△、×はバックグラウンドと比較してCe濃度の信頼

性を示しており、○はほぼ信頼に足る、×はバックグラウンドと大差ない。

この結果から一部の臓器（肺、心臓、腎臓）には、ややCeの存在が認められるが最も多く存在するのは血液中であることがわかる。

またここで検出された全Ce量は約5 μ gと推定され、投与したCe-CNCが300 μ g（50ppmCe-CNC 1ml \times 6回）であり、その重量の大部分がCeであることを考えると、検出されたのはごく一部である。他の代謝・蓄積としては血管内皮の食食や尿への放出が考えられるため、今後さらに検討する必要がある。

【ナノカプセルの生体内挙動の評価】

ラットマクロファージ及びTHP-1細胞にCe-CNCを投与した場合のTNF- α 産生量を図7に示す。CNC濃度が1~2ppmを超えた時点からTNF産生量が増大し、細胞への刺激性が増加していると考えられる。本実験で使用したCNCは数ppm以上になると凝集が起こり目視で確認できる程度の二次粒子を形成することから高濃度域での刺激性はこの凝集体によることが考えられる。

図8は図7で示したTNF- α 産生量（CNC1ppm時）を過去の多様な粒子状物質における値と比較したものである。CNCにおけるTNF- α 産生はTi粒子で最も刺激性が低いもの（150 μ m）と同程度であることが分かった。図10にはCNCを取り込んだマクロファージの光学顕微鏡像を示す。

図9はCNCと対照としてTiO₂を1ppmとなるよう添加した場合のTNF- α およびIL-1 β 産生量の比較である。

肺胞、腹腔マクロファージにおいてTNF- α 、IL-1 β 産生量はともにCNC添加の場合はTiO₂の場合に比べて同等かやや低くCNCの細胞刺激性がTiO₂より低いレベルであると考えられる。またTHP-1細胞ではあまり変化は見られず、これは細胞自体の感受性によると思われる。

D. 考察

CNTと金属及び樹脂との複合材料についてはチタンを用いた場合には炭素との反応性が極めて高いチタンの特性からCNTが残留せずチタンと反応して炭化物（TiC）を生成するため、CNT分散に

よる強化がおこらず、むしろ反応により消失したCNTが空隙を作り強度を低下させたと考えられる。これを防ぐには炭素と反応しにくい（炭化物を形成しにくい）金属を用いることが考えられる。アクリル系の樹脂との複合に関しては用いたCNT原料が純度80%であり、アモルファスカーボンや合成時の触媒金属を含んでいることから樹脂中への分散性が悪く、均一な複合体を作ることが困難であった。そこでCNTを空気酸化処理してアモルファスカーボンを除去し、酸洗浄によって触媒金属を除去すると共に、溶剤に対する分散性を向上させる目的で6N塩酸及び6N硝酸で処理を行った。精製処理したCNTでは樹脂への分散性が改善しており、さらに超音波ホモジナイザーを用いて強攪拌したことから、目視的に均一な複合体を作成できた。同複合体では若干の曲げ弾性率および曲げ強さの向上が見られた。今後の改良によっては更に強度の向上が期待される。

カーボンナノカプセルについては動物への血中投与によって生体内で一部臓器に蓄積する可能性が示唆されたが、現段階では濃度が極めて低く、また分析手法も確立していないため確定は出来ない。培養細胞を用いた刺激性試験ではチタン微粉末と同レベルの刺激性であることが分かった。チタンは金属材料の中では最も生体適合性に優れるものの一つであり、チタンと同レベルの刺激性であれば実用上の生体為害性は無視し得ると考えられた。

E. 結論

カーボンナノチューブ（CNT）と金属またはポリマーとの複合材を作製し、繊維強化金属／プラスチックのような高比強度の生体材料を合成することを目的として研究を行い、Ti/CNT及びアクリルレジン/CNT複合材料を作成し、その強度を検討した。Tiとの複合においてはCNTがTiと反応しTiCを精製して強化に寄与しなかったが、アクリルレジンに添加した場合には曲げ弾性率がやや向上し、CNTによる強化の可能性が示唆された。またカーボンナノカプセルの生体内での挙動や生体為害性を調査し、その生体為害性は現在金属柱で生体適合性に最も優れる

とされるチタンが微粉末化したものと同程度であり、為害性が十分に低いことが示唆された。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

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(歯科用セラミックスの細胞毒性と接着特性)

2) Uo M., Berglund A., Cardenas J., Pohl L., Watari F., Bergman M., Sjöberg S. : Surface analysis of dental amalgams by x-ray photoelectron spectrometry and x-ray diffraction spectrometry, *Dental Materials*, 19, p.639-644, 2003

(X線光電子分光法及びX線回析法による歯科用アマルガムの表面解析)

2. 学会発表

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2) ○ 宇尾基弘・亓理文夫 : X線分析顕微鏡の生体内金属材料及び周囲組織分析への応用, 平成14年度日本電子顕微鏡学会北海道支部学術講演会 (札幌, 2002年2月8日), 同講演予稿集, p. 8, 2003

3) ○ 宇尾基弘・亓理文夫 : 走査型X線分析顕微鏡による生体軟組織中の元素マッピング, 日本顕微鏡学会第59回学術講演会 (札幌, 2002年6月7~9日), 同講演予稿集, p. 9, 2003

表1 Ti/MWCNT複合材のSPS焼結条件

MWCNT含量 (wt%)	圧力 (MPa)	温度 (℃)	保持時間 (分)
0	40	1070	5
5	120	1075	5
10	120	1045	5
15	120	990	5

表2 Ce-CNC投与ラット各臓器内のCe濃度

臓器名	臓器重量 (g:wet)	25ml溶解後 濃度 (ppm)	臓器内濃度 (ppm)
脳	0.35	0.038	0.20 ×
脾臓	0.80	0.039	0.13 ×
肺	2.50	0.060	0.24 △
心臓	1.36	0.060	0.46 △
肝臓	20.16	0.042	0.01 ×
腎臓	3.27	0.057	0.17 △
血液	4.40	0.137	0.58 ○
Control	—	0.035	—

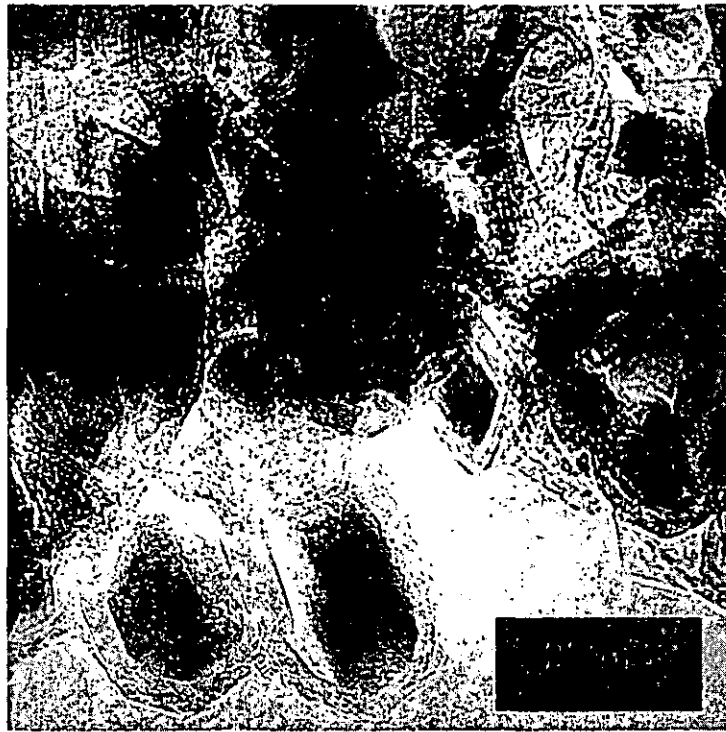


図1 カーボンナノカプセルの透過電子顕微鏡像

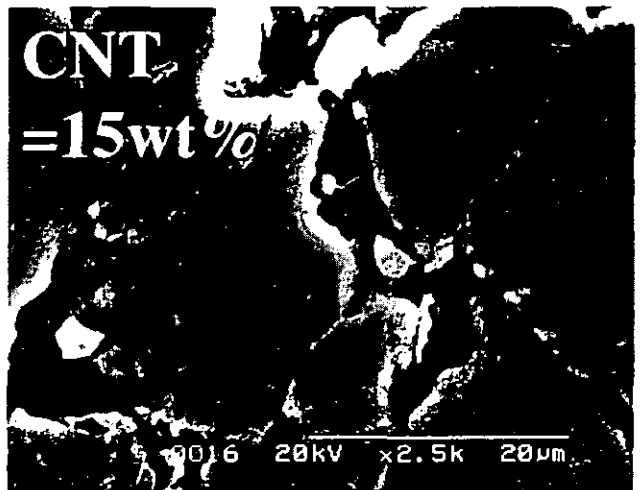
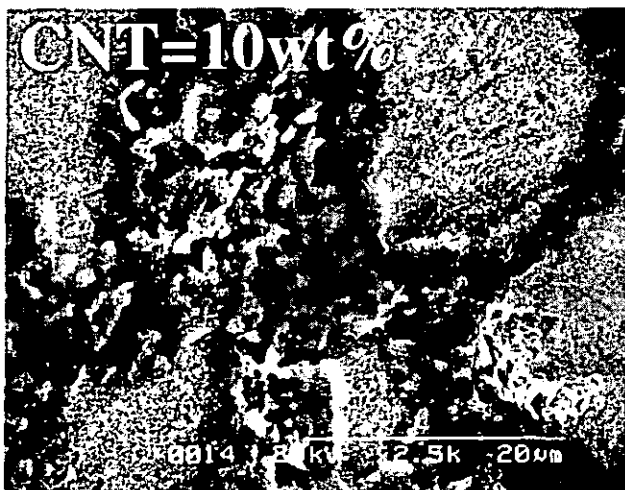
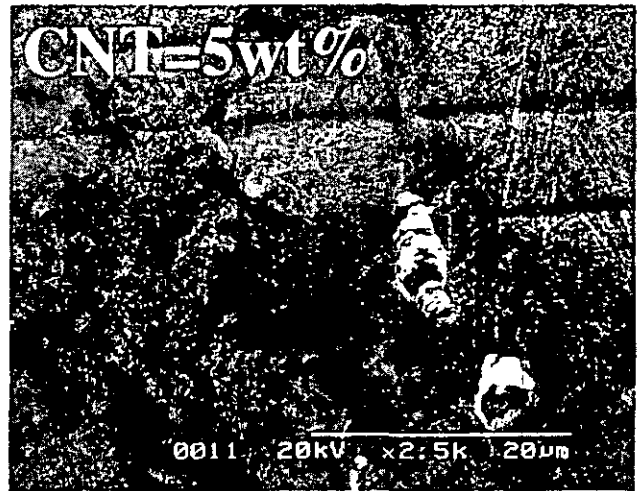


図2 SPSにて焼結したTi/CNT複合材断面のSEM像

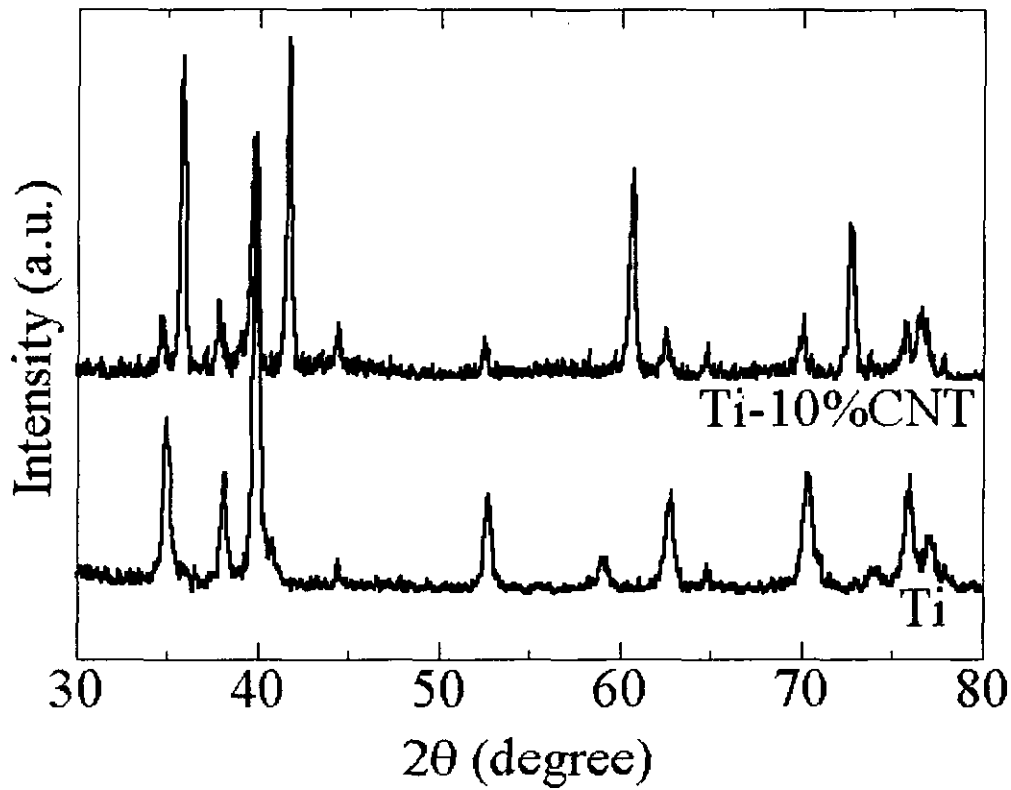


図3 Ti/CNT複合材のX線回析結果

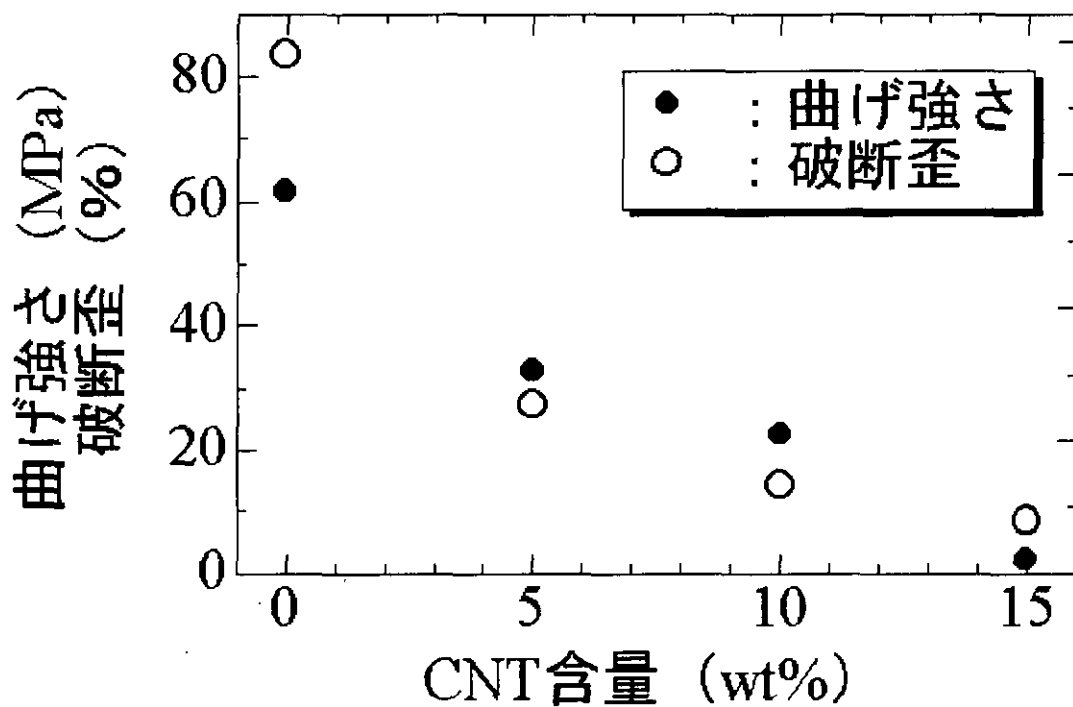


図4 Ti-CNT複合材の3点曲げ試験結果

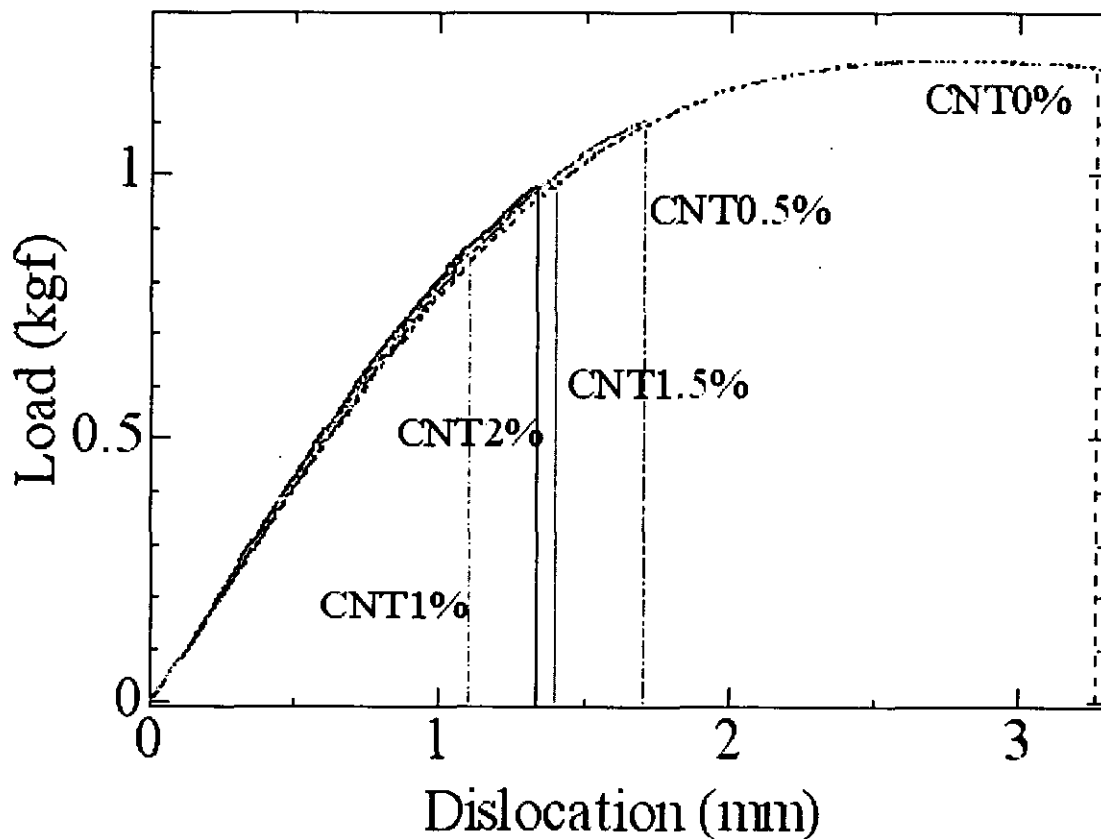


図5 Ti/Bis-GMA複合材の荷重-変形量線図の例

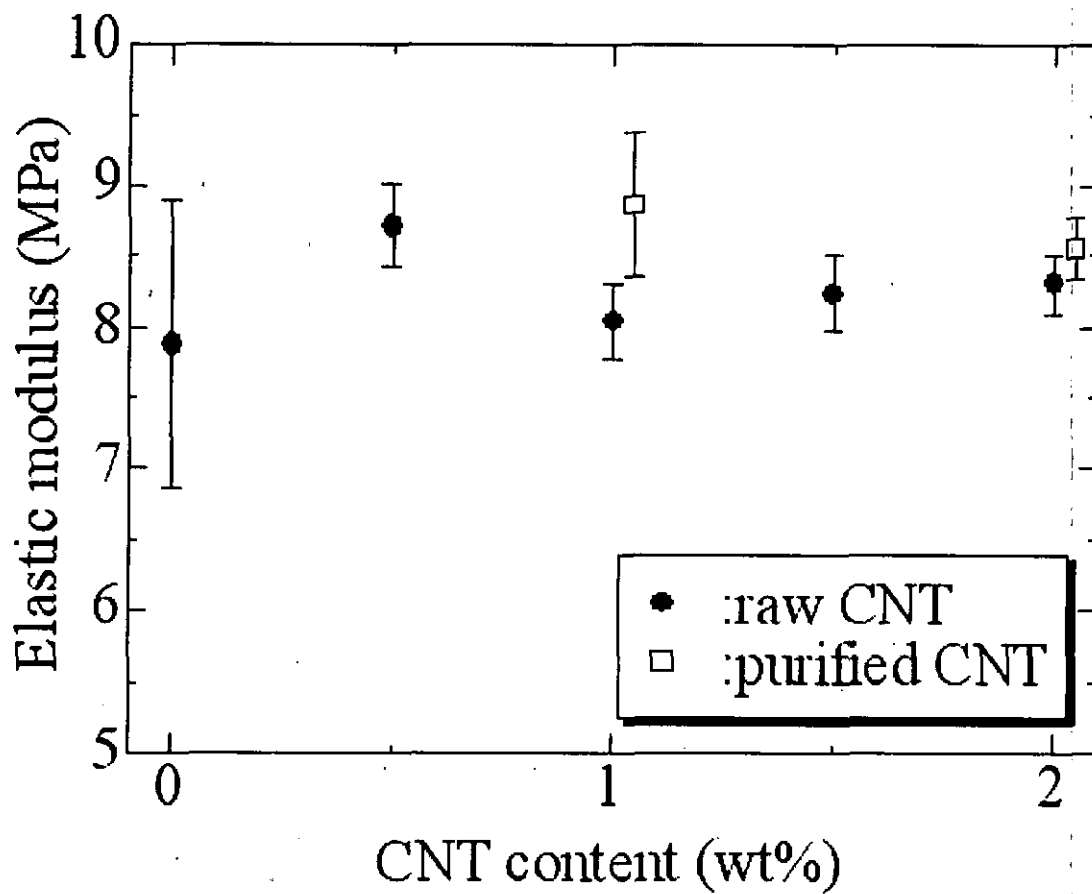


図6 Ti/Bis-GMA複合材の曲げ弾性係数のCNT濃度依存性

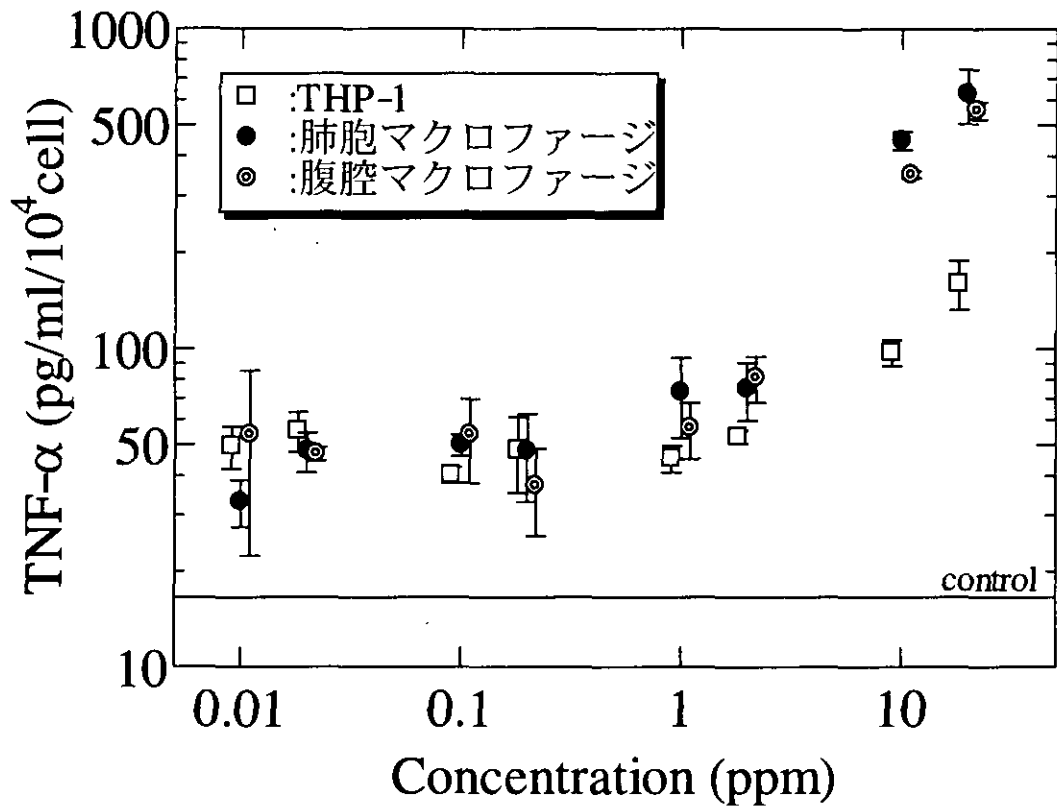


図7 TNF- α 産生量のCNC濃度依存性

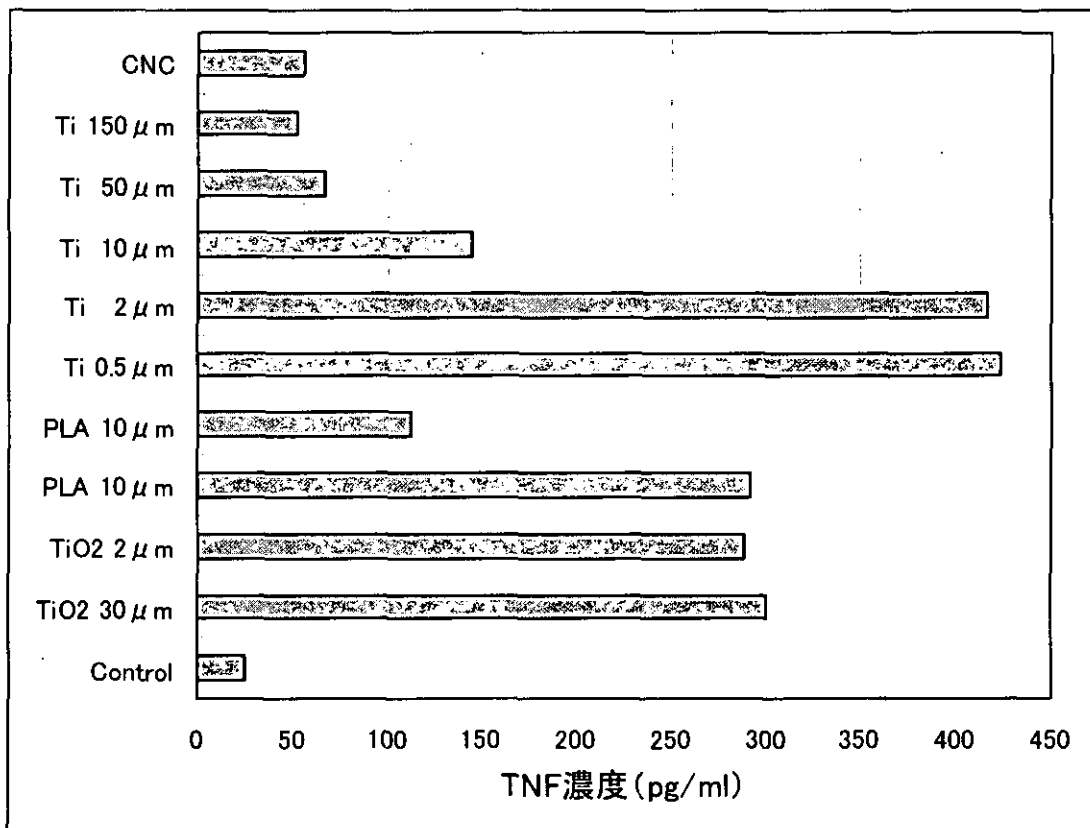


図8 CNC及び他粒子のTNF- α 産生量の比較

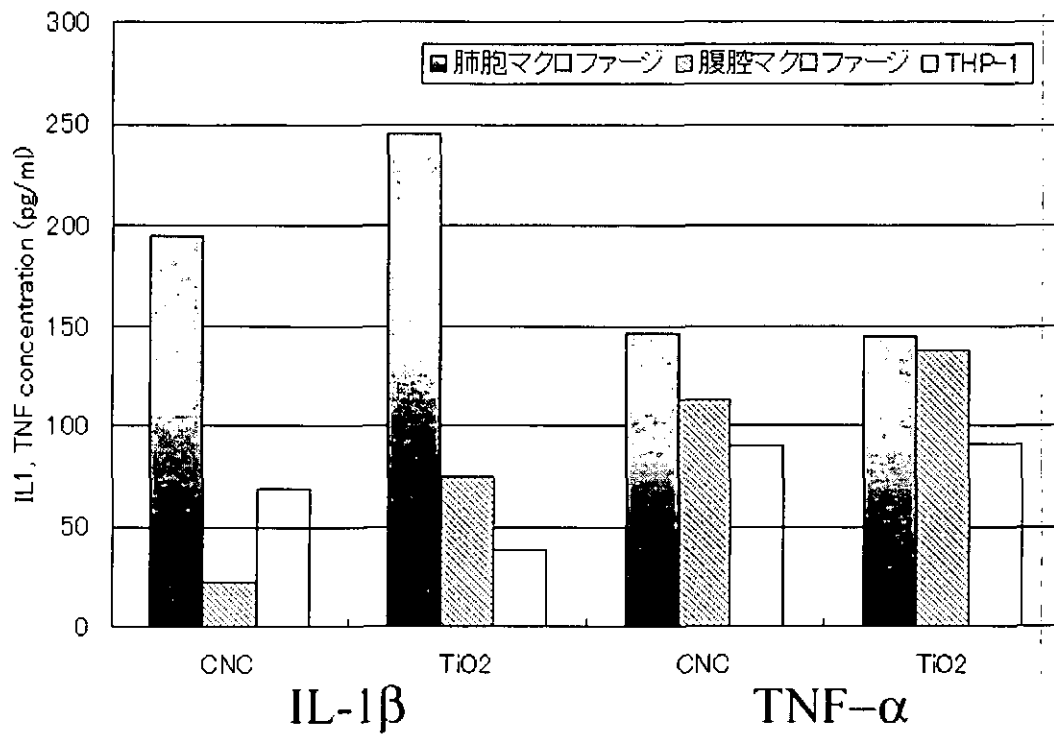


図9 CNC及びTiO₂ (1ppm) のTNF-α、IL-1β 産生量の比較

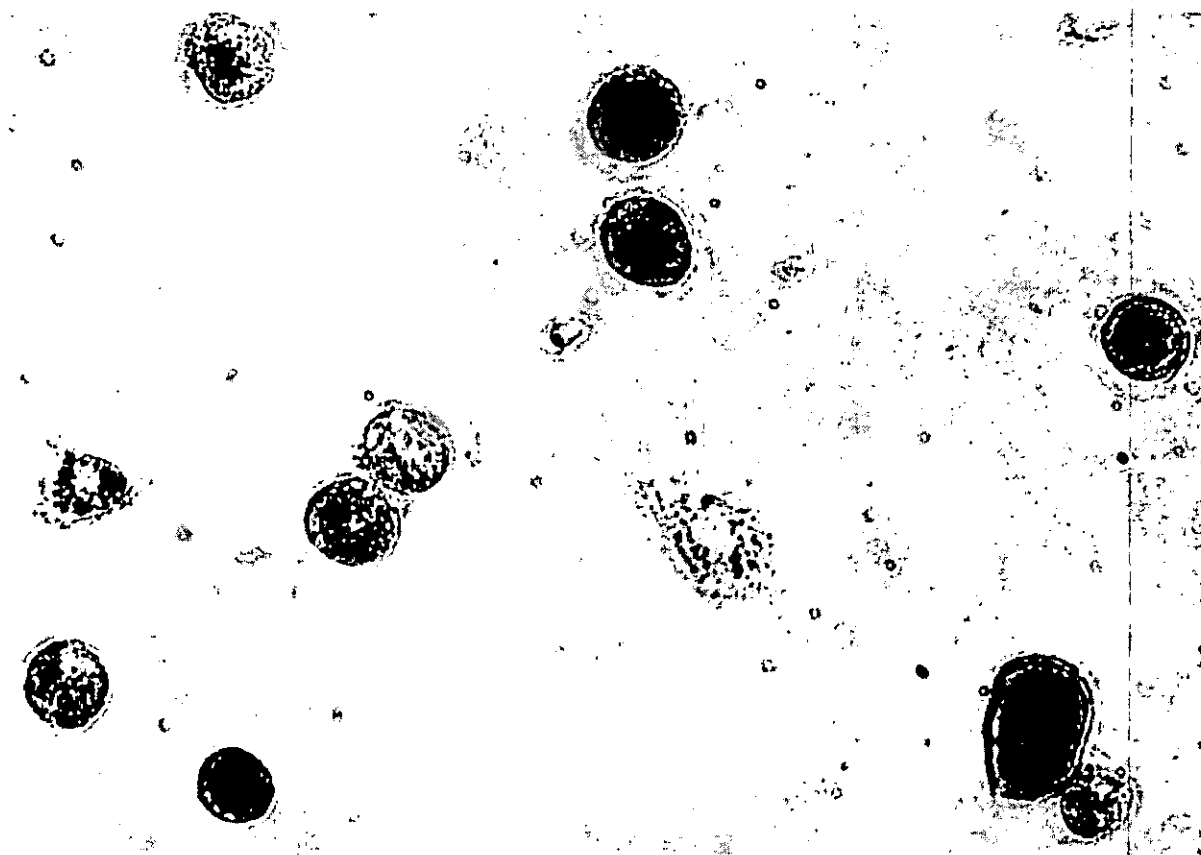


図10 CNCを取り込んだマクロファージの光学顕微鏡像

厚生労働科学研究費補助金
(萌芽的先端医療技術推進研究事業)
分担研究報告書

乳化溶媒蒸発法によるマイクロパーティクル形成

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研究要旨 本研究では、一段または二段乳化溶媒蒸発法によるマイクロパーティクルの形成メカニズムの解明と、マイクロパーティクルへの内包化およびその放出挙動に対し、マイクロパーティクル形成過程の影響を評価した。その結果、急速な溶媒除去の間に内包化された物質は部分的にマイクロパーティクルから漏れ出した。走査型電子顕微鏡とレーザ粒度分布測定装置を用いた観察により、マイクロパーティクルの表面にはナノパーティクル層が存在することが明らかとなった。二段乳化溶媒蒸発法による形成では、内部水相の存在により一段の場合とは異なった形態を持ったマイクロパーティクルが生成した。溶媒が除去される過程において、内部水相の小滴は析出してくる高分子の圧力により合体する。その際、小滴周囲の高分子壁が、小滴より形成される内部空間を壊し、内包される物質の部分的な放出を起こすことが示された。

A. Introduction

The classic emulsification solvent evaporation technique (ESE) elaborated by R. Bodmeier et al. [1], Y. Ogawa et al. [2], H. Jeffery et al. [3], M. Iwata et al. [4], and different recent variations are commonly used for encapsulation of various substances from simple pharmaceutical products to proteins and DNA [5,6].

Although the technique is well defined as methodology, there are only a few studies about the encapsulation and particle formation mechanism [7]. Particle formation mechanism is crucial for size distribution, and morphology,

which in turn determine the delivery system behavior during encapsulation and release.

It is well known that ESE is mainly a two-step process: emulsification of a polymer solution containing the encapsulated substance, followed by solvent evaporation and particle hardening. During emulsification the polymer solution is broken up in microdroplets by the shear stress produced either by homogenizer, sonicator or whirl mixer in the presence of a surface-active agent.

For a given polymer, the performance of the microparticulate delivery system is mainly determined by its size distribution and morphology, which in turn depend on numerous process parameters such as: polymer concentration, surface active agent concentration, phase volumes,

emulsification energies, phase viscosities, etc. Numerous researchers investigated these dependencies, and qualitative and quantitative relationships were established [7-14]. Most of these dependencies are clearly determined by the particle formation and encapsulation mechanism.

In this study the mechanism of microparticle formation for single (O/W_2) and double ($W_1/O/W_2$) emulsion solvent evaporation formulations was elucidated, and its influence on the microparticle encapsulation and release behavior were assessed. Optical microscopy and laser diffractometry revealed different aspects of the microparticle formation, from the initial microdroplet to the final hard microparticle.

B. Materials and methods

B.1. Materials

For this study poly(DL-lactide-co-glycolide) one of the most widely used biodegradable polymer, was chosen. The copolymer, Lactel BP 0100 (PLGA) with lactide, glycolide ratio 50:50, and molecular weight 45000-75000 was purchased from Sigma.

Poly(vinyl alcohol), (PVA) commonly used in emulsification solvent evaporation formulations, with molecular weight 22000, and 86-88% degree of hydrolysis, was obtained from Kanto Chemicals.

Dichloromethane (DCM) one of the most extensively used formulation solvent was purchased from Wako Pure Chemicals.

All chemicals were used without further purification.

B.2. Equipments

High-speed emulsification was achieved by Heidolph DIAX 900 homogenizer using 18G tool for O/W_2 , and 6G tool for W_1/O emulsion. For low speed dispersion Ika Labortechnik mixer with impeller, and baffles was used.

Nikon Optiphot 114 microscope with digital imaging capabilities was used to record the microdroplet evolution into the final microparticle.

The microparticles were sized by laser diffractometry using a Shimadzu SALD 7000 laser particle analyzer.

The morphology of the microparticles was examined by scanning electron microscopy (Hitachi S-4000) after Pd-Pt coating.

B.3. Methods

The investigated single/double emulsification solvent evaporation method follows the classical method [1-4]. For single emulsion formulation a predetermined quantity of PLGA was dissolved in 5ml DCM, forming the oil phase. 500 μ L of 1%w/v PVA solution in deionized water as inner aqueous phase (W_1) was used for the double emulsion formulation. High-speed homogenizer at 20000rpm dispersed W_1 for 2 minutes in the polymer solution, forming the W_1/O emulsion.

The oil phase or the W_1/O emulsion was then dispersed in 30mL of deionized water with different PVA concentrations. For high shear stress mixing, homogenizer at 8000-11000rpm for 2-3 minutes was used. For low shear stress formulations laboratory mixer with impeller and baffles at 500-1400rpm was used. The oil phase or the W_1/O emulsion was added by injection through 0.2mm needle in 15s.

At the end of the high-speed homogenization or after 5 minutes in the case of low-speed formulations, the size distribution of particles was measured, and one drop of emulsion was poured on a microscope slide and sealed with cover glass. The slide was mounted on the microscope and the transformation of the initial microdroplets into the final microparticles was captured by a picture sequence.

Next, the initial O/W_2 or $W_1/O/W_2$ emulsion was poured in 500mL of 0.1%w/v PVA solution, and the solvent was let to evaporate under magnetic stirring (500rpm) for 2 hours.

The microparticles were separated by centrifugation at 3500rpm for 5min, and washed once with 100mL of deionized water. After separation the size distribution of the final particles was measured and the particle morphology was examined by SEM.

C. Results and discussions

C.1. Single emulsion formulation (O/W_2)

Observing regions close to the cover glass edge revealed that the microdroplets shrink very fast, eliminating the solvent and transforming into the final microparticle. Near the cover glass edge DCM concentration in water is close to zero due its fast evaporation through the thin water air interface. Low DCM concentration induces intense solvent diffusion out of the microdroplets.

Figure 1 presents the initial and final images of the transformation of the initial microdroplets into the final microparticles. Far from the cover glass edge where the aqueous

phase is saturated in DCM, solvent elimination from the microdroplets is restricted. Their diameter remains practically unchanged (fig. 1a) until the front of low DCM concentration reaches the droplets vicinity, when they shrink very fast (fig. 1b).

There are two key factors promoting the fast solvent loss: high solvent volatility, and increasing tendency of PGLA to precipitate as the macromolecules become more and more exposed to water. In fact, once the particle shrinkage started, the solvent is literally expelled by the polymer precipitation.

However DCM evaporation under the microscopy was restricted by the presence of the cover glass and the lack of stirring. Actually after the emulsification phase the initial emulsion is poured in much larger quantity of aqueous phase where all microdroplets face, almost simultaneously, the same favorable conditions for rapid solvent elimination.

We defined the theoretical shrinkage factor (α_t) as the ratio of the initial emulsion droplet diameter, and the final solvent-free polymer particle diameter. Knowing the initial polymer concentration, α_t is expressed by the following simple relation:

$$\alpha_t = \frac{d_i}{d_f} = \left(\frac{V_p + V_s}{V_p} \right)^{1/3} = \left(1 + \frac{100 \cdot \rho_p}{C_p} \right)^{1/3}$$

where: d_i – initial microdroplet diameter; d_f – final particle diameter; V_p – polymer volume; V_s – solvent volume; ρ_p – polymer density and C_p – polymer concentration (%w/v). PGLA density measured by picnometer, and calculated using Bicerano's predictive molar volume relation [15], gave 1.31 g/mL.

Table 1 presents the theoretical (α_t) and the measured (α_m) shrinkage factors for different formulation conditions.

Considering the errors in particle diameter measuring, the solvent loss during emulsification and droplet manipulation for microscopy, the measured shrinkage factors are reasonably close to their corresponding theoretical values, sustaining the low residual solvent content of the final microparticles.

Fig. 2 presents the particle size distributions of the initial emulsion, right after the emulsification, and that of the final emulsion after the solvent evaporation is completed. The distributions are practically superposed testifying for the fast solvent elimination. In fact, during the size distribution analysis that takes less than 30 seconds, small volume of the initial saturated emulsion (1mL) is poured in a large amount of

water (250mL) inside the analyzer where DCM is well below its saturation limit, leading to fast solvent elimination.

Almost identical initial and final particle size distribution also means that the microdroplets do not coalesce nor break up after the emulsification step. We found that particle hardening is significantly accelerated if the initial emulsion is poured in a sufficiently large quantity of aqueous phase so that the solvent remains under his solubility limit all over the evaporation. DCM solubility in water at 20°C is 2%w [16].

Considering the above evidences the microparticles are formed by accelerated solvent elimination due to the combined effects of high solvent volatility and polymer precipitation.

However the rate of solvent elimination strongly depends on the initial microdroplet size. As the initial microdroplet diameter increases the available surface for solvent elimination decreases leading to slower solvent elimination.

C.1.1. Microparticle size distribution

The particle size distribution is decided in the short but intense emulsification phase. In order to allow the homogenizer to produce microdroplets with monomodal and narrow size distribution, DCM concentration should be kept as close as possible to its saturation level for a reasonably long time, necessary to reach the equilibrium droplet diameter. This can be achieved by using adequate formulation and design conditions that minimize the solvent loss during emulsification.

In the present study we used an emulsification vessel with high height/diameter ratio (H/D) in order to reduce emulsion-air interface, and with the smallest volume possible in order to maximize the dispersion efficiency and to minimize air bubbles formation. In the case of low shear rate dispersion, baffles were used in order to prevent the formation of the central vortex that increases the emulsion-air interface. Air bubbles are undesirable because they contribute to the surface-active agent depletion and additional solvent loss through the supplementary surface created. Small emulsification volume implies also high organic/aqueous phase ratio leading to smaller particles through increased dispersing efficiency [8].

The size of the microdroplets is mainly determined by the stirring rate. Increasing the stirring rate will reduce the diameter of the microdroplet and that of the corresponding microparticle [8,9].

Increasing the polymer concentration will result in larger particle diameters, partly due to the viscosity effects [9,10,13] and partly to the polymer content of the microdroplet [9].

For PLGA concentration in the 0.5-5%w/v interval, optical microscopy of the emulsions obtained at constant stirring rate showed almost identical microdroplets sizes proving weak viscosity effects. This observation was also sustained by the close values of the calculated average diameter of the initial droplets obtained by the multiplication of the measured average diameter of the final microparticles and the theoretical shrinkage factor. (Table 2)

C.1.2. Microparticle surface morphology

With a few exemptions, when the microparticle surface was found slightly rough [17,18] literature screening evidenced that microparticles are spherical and have smooth surface. Our low magnification SEM micrographs showed the same spherical microparticles with smooth surface (fig. 3a).

High magnification SEM micrographs revealed that a thin layer of nanoparticles cover the microparticles surface (fig. 3b, 4). Especially at high magnification the microparticles are very sensitive to radiation. During high magnification SEM observations, usually the initial shape of the particles is altered (fig. 3b, 4), and in some cases the crust broke-up and drift, proving the existence of a thin layer (fig. 4b).

For some of the formulation conditions laser diffractometry also evidenced the nanoparticles on the microparticle surface by the presence of a secondary peak around 30nm (fig. 5). If the nanoparticles were free, than after the low speed centrifugation (3500rpm) they should be eliminated, but the secondary peak was still present after the centrifugation, proving that the nanoparticles are in fact stuck on the microparticles surface.

These nanoparticles are formed on the microdroplet surface by the combined effects of crust forming due to the polymer precipitation, and crust fragmentation due to the fast shrinkage.

SEM micrographs showed that especially for large shrinkage factors corresponding to low initial polymer concentration, the nanoparticulate layer peels, generating secondary particles (fig. 4c, d)

C.1.3. Encapsulation and release behavior

The encapsulation efficiency is clearly affected by the fast shrinkage because the

encapsulated substance is drained during the solvent elimination. High polymer concentration improves the encapsulation efficiency [9], mainly through smaller shrinkage factor and due to extended macromolecule entanglements, promoting the entrapment of the encapsulated substance. It is well known that microparticles prepared by ESE presents initial burst release due to surface located active principle. Usually O/W₂ emulsification is used for encapsulation of oil soluble substances. At the microdroplet surface, during solvent elimination, not only the polymer tends to precipitate but also the encapsulated substance. In this way the generated nanoparticle layer become enriched with the encapsulated substance, and it reasonable to believe that this enriched thin layer is responsible for the initial burst release.

C.2. Double emulsion formulation (W₁/O/W₂)

The evolution of W₁/O/W₂ microdroplets was assessed in a similar way to the single emulsion method. Optical microscopy revealed that the inner aqueous phase has a strong influence on the particle formation. Figure 6 presents the W₁/O emulsion with microdroplets of aqueous phase ranging from 1µm to 15µm. Figures 7 and 8a are the picture sequences of the initial W₁/O/W₂ microdroplet transformation into the final microparticle.

C2.1. Particle morphology

The solvent elimination process in the presence of the inner aqueous phase produces important particle morphology changes. During shrinkage the inner droplets coalesce under the pressure of the precipitating polymer (fig. 7, I-VI). Since the inner phase is incompressible, the polymer wall around the aqueous droplets breaks, forming holes through which the inner aqueous phase is partially expelled (fig. 7, V-VI).

Optical and scanning electron microscopic observations evidenced that the final emulsion was a mixture of microparticles with honeycomb, capsule and plain structures.

Inside the droplet of the secondary W₁/O/W₂ emulsion the precipitating polymer around the entrapped droplets of inner aqueous phase generates the honeycomb structure (fig 8b). The numerous holes on the particle surface as well on the pore walls prove the loss of the inner phase.

Important coalescence of the inner droplets combined with insufficient oil phase in the

secondary emulsion droplet leads to capsules (fig. 7, 9). Generally the capsules are broken, showing deflated ball like structures on the SEM micrographs (fig 8b, 9b).

The plain particles have no entrapped inner aqueous phase (fig. 9).

We found that the proportion of particles with different structures depends mainly on the stirring rate and the polymer concentration. At low stirring rates and high polymer concentration the honeycomb structure was predominant (fig. 8) [12, 19, 20]. The microparticles were fractured as described in [20]. At high stirring rate (>8000 rpm) the capsule structure predominates (fig 9). As the stirring rate of the secondary emulsion approaches that used for the primary emulsion the proportion of plain particles increases significantly.

3.2.2. Size distribution

Similar to single emulsion ESE, low stirring rate, high polymer concentration, and low the surfactant agent concentration lead to large mean particle diameter [7,10, 11,13], but in this case the dependence is more complex due to the presence of various particle structures in different proportions.

C2.3. Encapsulation and release behavior

The encapsulation efficiency is directly related to the extent of W_1 phase entrapment. Depending on the formulation conditions the inner aqueous phase is partly lost during the solvent elimination process. Furthermore, during the solvent evaporation, the remaining active-principle is continuously partitioning with the external aqueous phase through the surface holes.

For successful encapsulation the W_1 microdroplets should be much smaller than the secondary emulsion droplets [10,14]. Comparable sizes will result in high proportion of broken thin-walled capsules and plain particles with negative influence on the loading efficiency.

Increasing the inner aqueous phase volume will reduce the encapsulation efficiency [12,14] simply because it is less polymer available for encapsulation, thus more W_1 phase will be lost during the shrinkage.

High polymer concentration increase encapsulation efficiency by the reduced shrinkage, especially at low stirring rates when the honeycomb structure is favored.

The holes present on the pore or capsule wall will decisively contribute to the initial burst release. For smaller particles ($\sim 10\mu\text{m}$) obtained at high stirring rate the burst release is more

important [7,11,13] because the broken capsule structure is predominant in this case.

D. Conclusions

In the present study microparticle formation and encapsulation mechanism for single and double emulsification solvent evaporation method was elucidated. The initial O/W_2 , and $W_1/O/W_2$ emulsion microdroplet transformation into the final hard microparticle was pursued by optical microscope and laser diffractometry.

The microparticles are formed by accelerated solvent elimination due to the combined effects of high solvent volatility and polymer precipitation. The fast shrinkage that accompanies the solvent elimination has important influence on the particle morphology.

Because of the high tendency to solvent elimination, the particle size is determined practically during the short but intense emulsification process. For single emulsion ESE, the particle size distributions of the initial emulsion and that of the final hard microparticles were identical, proving that microdroplets do not coalesce nor break-up after the emulsification phase.

During the fast solvent elimination and shrinkage the encapsulated substance is partly drained. High polymer concentration improves the encapsulation efficiency through the low shrinkage.

Scanning electron microscopy and laser diffractometry evidenced the presence of a thin nanoparticulate layer on the microparticles surface. This layer is formed during the solvent elimination by the shrinkage-induced fragmentation of the precipitating polymer crust. It is reasonable to believe that the encapsulated substance will accumulate in this layer contributing to the initial burst release.

The inner aqueous phase (W_1) in the double emulsion formulations has important influence on the mechanism of particle formation. In this case microparticles with different structures are generated. The proportions of microparticles with different structure are determined mainly by the stirring rate and the polymer concentration.

During solvent elimination the droplets of inner aqueous phase coalesce under the precipitating polymer pressure. Due to the incompressibility of the W_1 phase, the polymer wall often breaks resulting in holes through which the inner aqueous phase is partly expelled. Furthermore, after particle hardening the holes will contribute to the encapsulated substances leakage through partitioning with the external aqueous phase, and to the initial burst release.

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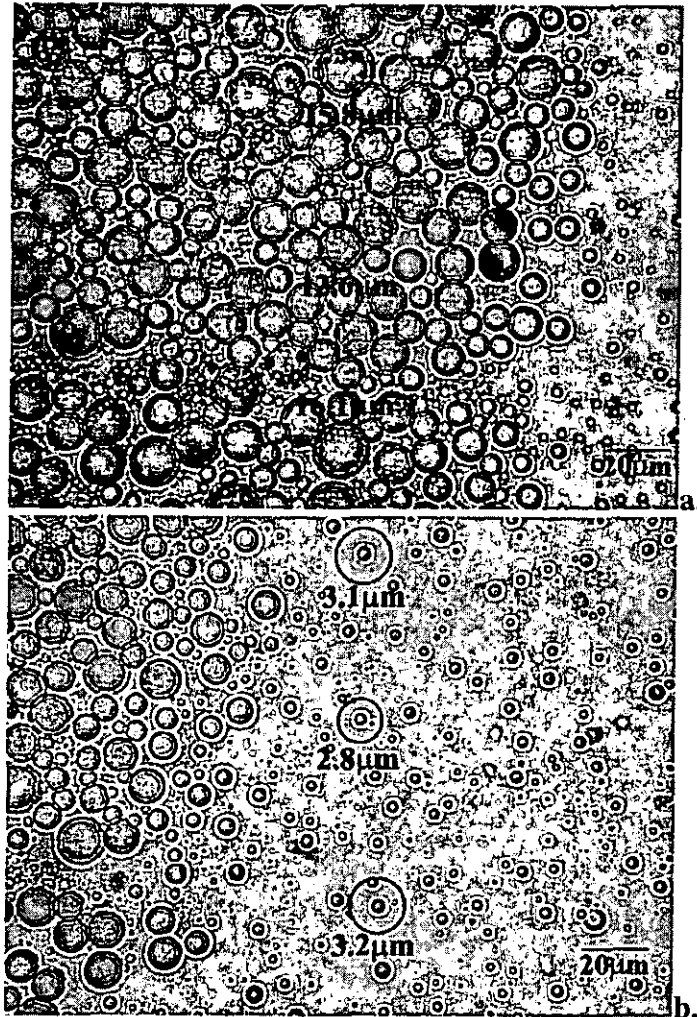


Fig. 1. The selected microdroplets (a) transformation into the corresponding microparticles (b). 1% w/v PLGA; 1% w/v PVA; 8000rpm for 3 minutes.