

SnCl<sub>2</sub> was used as the catalyst for the synthesis of P(LA-CA)<sub>25</sub> 10000 and P(LA-CA)<sub>50</sub> 18000, but the residual amounts of Sn in these polymers were negligible.

#### 4. References

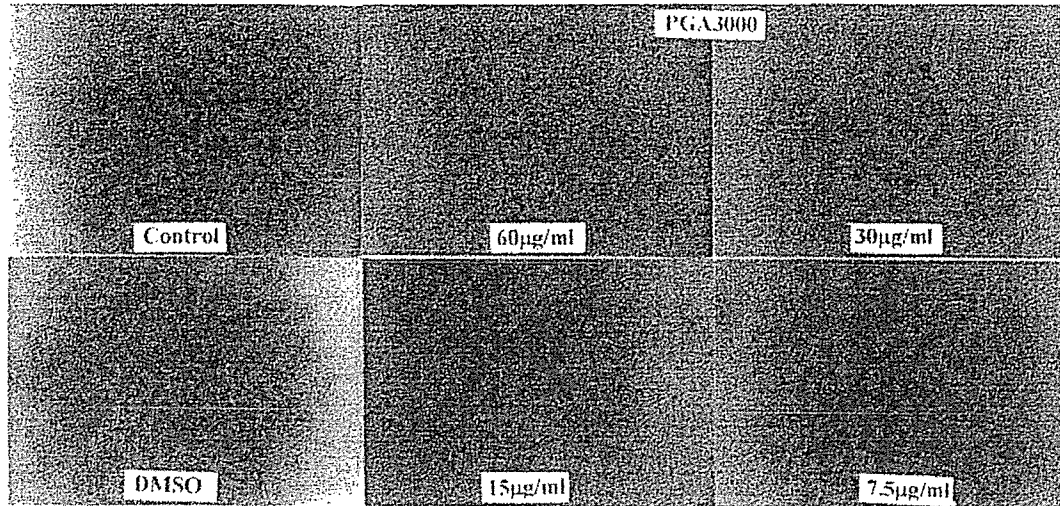
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Table 1. Biological activities of five chemicals in the neuroal cell differentiation of rat midbrain cells.

Chemicals	Biological activities*	
	Cell differentiation (% of inhibition)	Cell proliferation (% of inhibition)
PLLA5000	↓ (17.5%)	↓ (18.5%)
P(LA-CL) <sub>25</sub> 10000	↓↓↓ (94.9%)	↓↓ (66.5%)
P(LA-CL) <sub>50</sub> 18000	↓ (15.9%)	↓↓ (45.1%)
PGA3000	↓↓ (41.6%)	↓↓ (46.2%)
SnCl <sub>2</sub>	↓↓↓ (100%)	↓↓↓ (98.7%)

- \* : Biological activities were evaluated at the concentration of 60 µg/ml.  
 ↓ ↓ ↓ : Extremely strong inhibition (70 ≤ % of inhibition < 100)  
 ↓ ↓ : Strong inhibition (30 ≤ % of inhibition < 70)  
 ↓ : Inhibition (10 ≤ % of inhibition < 30)

Appearance of hematoxylin staining cultures  
of rat midbrain cells. (x 7.5)



Appearance of hematoxylin staining cultures  
of rat midbrain cells. (x 40)

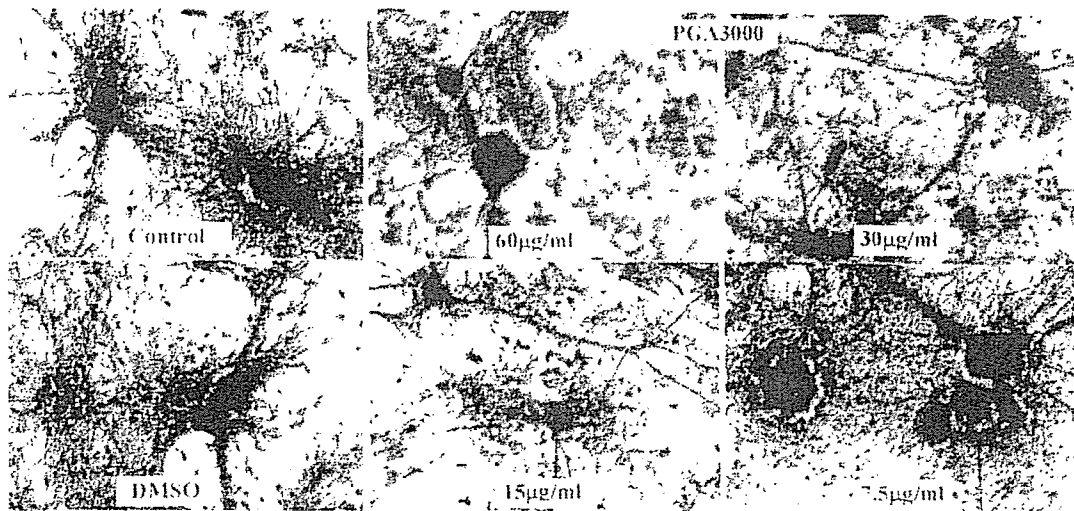


Figure 1. Appearance of hematoxylin staining cultures of rat midbrain cells after incubation with or without PGA 3000 solubilized with DMSO. Control; rat MB cells were cultured in F12-10% FCS. DMSO; vehicle control.

# 原子間力顕微鏡 (AFM) による 蛋白質のイメージング

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## Protein imaging by atomic force microscopy

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### Abstract

Atomic force microscopy (AFM) has been used for imaging of non-conductive surface using a cantilever with a sharp probe to mediate the atomic force interaction between the probe and substrate. The application of AFM for the imaging of protein including transmembrane protein has been studied and revealed their single molecular structure on a nanometer scale. Especially for the transmembrane proteins that lack of 3D structural information obtained by X-ray crystallography, AFM imaging has significant advantages. Since the imaging is capable in the aqueous solution, the obtained images are expected to provide information that reflects structures found in the living cells. Additionally, the force curve measurement for intra- or inter-molecular non-covalent interaction such as protein folding or ligand-receptor interaction will be explained.

**Key words:** atomic force microscopy, protein imaging, recombinant P2X<sub>2</sub> receptor

### 1. 蛋白質の構造解析

生体の主要構成成分の一つである蛋白質は、生体内での有機化合物の代謝(酵素など)、情報伝達(受容体など)、組織骨格形成などにかかわる機能性分子であり、その機能に関連する構造の解析は現在の化学の分野で最もホットな分野の一つである。一般にアミノ酸配列を決定する一次構造解析は、繁用法があり、その結果から、 $\alpha$ -ヘリックスや $\beta$ -シートなどの二次構造が推定される。しかし生体内での蛋白質の機能に大

きくかかわっている三次元構造の決定は困難な点が多い。

現在行われている蛋白質三次構造決定法としては、X線結晶構造解析法と核磁気共鳴(nuclear magnetic resonance: NMR)分光法などがあげられる。X線解析法は解析対象とする蛋白質の単結晶を調製し、X線の照射回折を行い、三次元的画像を得るもので、解像度が非常に高い(Åレベル)という利点がある。特に近年の放射光を用いた測定法の開発により、解析される蛋白質の数は飛躍的に伸びた。しかし、本法は単

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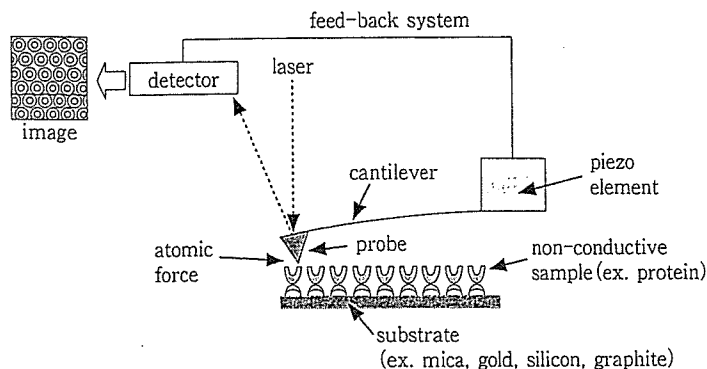


図1 原子間力顕微鏡(AFM)の模式図

結晶の調製が困難である難溶性の蛋白質の解析にはあまり向いておらず、また、得られる構造が結晶格子内における分子構造であるため、動的な構造ではなく、活性型の蛋白質の構造との相関には不明な点が残る。後者のNMR法は溶液中での測定法であり、生体内の蛋白質の挙動に近い情報を求めることが期待され、また、核オーバーハウザー効果(nuclear overhauser effect: NOE)などの手法を用いると、核の間の空間的距離の情報が得られ立体的構造に関する解析も可能である。ただし、蛋白質をNMRで測定して得られるスペクトルは、非常に複雑で解析が難しく、 $^{15}\text{N}$ -enrichした試料の調製が必要になる場合もある。また、試料としては高濃度溶液が必要であるため、難溶性あるいは微量しか発現していない蛋白質の解析にはあまり向いていない。

## 2. なぜ原子間力顕微鏡(AFM)で蛋白質の画像化を行うのか?

原子間力顕微鏡(AFM)を用いた蛋白質の解析の目的は蛋白質の大まかな構造と機能を知ることである。現在のところ、AFMの解像度はX線解析に遠く及ばない(大まかに言って1/10程度である)が、AFMを用いることの利点を以下にあげる。

- (1) 試料の量が微量で済む。
- (2) 高濃度溶液や単結晶の調製が不要である。
- (3) 溶液中測定も可能であり、より生体内環境に近い条件下での測定が可能である。

(4) 経時的測定モードによりダイナミックな構造情報が得られる。

(5) 単一分子測定が可能である。

## 3. AFM からどんな情報が得られるのか?

### a. 装置の概略とイメージング

AFMが最初に発表されたのは、1986年にPhysical Review Letters誌に掲載されたIBM-チューリヒの研究者達の報告<sup>1)</sup>においてである。同研究グループはその数年前に、走査型トンネル顕微鏡(scanning tunneling microscope: STM)を発表し、原子像の画像化に成功しているが(1986年ノーベル物理学賞受賞)、トンネル電流を利用したSTM<sup>2)</sup>が電導性のサンプルの測定のみ適用されるのに対し、AFMは非電導性のサンプルの測定も可能であり、DNAや蛋白質などの生体高分子の新しい解析手法として、威力を発揮することが期待されている。

機器の基本的な構造は、主に以下の4つの部分からなる(図1)。

- (1) 試料の表面を走査する微細な探針(probe)
  - (2) 探針と試料表面との間に働く原子間力を増幅する微小な板ばね(cantilever)
  - (3) 探針および板ばねの高さや位置を調節する圧電(ピエゾ)素子
  - (4) 板ばねの変位を測定するレーザー光と、その反射を検出するフォトダイオード検出器
- 試料と原子間力を介して直接相互作用するのは板ばねに結合した探針の先端で、その位置制御は微細な圧電素子の動きによって行われ

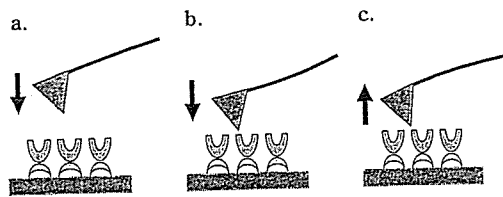


図2 AFM 探針の試料の相互作用と板ばねのそり

- a: 試料へ接近. b: 試料へ接触と, 板ばねのそり.  
c: 試料からの離脱と原子間力による板ばねのそり.

る(図2). 試料表面にナノメートルレベルの距離で接近した板ばねは探針と試料との間に生じる原子間力に応じてそり(deflection)などの変位を起こす. そこにレーザー光を照射し, 生じる反射光を四分割フォトダイオードディテクターで検出し, その結果に基づきサンプルのトポグラフィカルな情報を画像化するのである. 具体的な蛋白質のイメージングの例は, 4. 測定法の項に後述する.

#### b. アンフォールディング

蛋白質の機能に大きくかわる三次元構造は, 蛋白質のフォールディング(折りたたみ)により形成されるが, その機構に関しては未知の部分が多い. しかし, BSEに代表されるように蛋白質のミスフォールディングが疾患に関連している例が知られるようになり, フォールディングに関する基礎的知見は, 疾患の検出あるいは診断に重要な役割を果たすことが期待される.

ミュンヘン大学のGaubらのグループは, 膜貫通型蛋白質の一つバクテリオロドプシンを用いて, AFMによるアンフォールディングの実験を行った<sup>3)</sup>. バクテリオロドプシン分子が大量に発現した*Halobacterium salinarum*の紫膜を劈開したばかりのマイカ表面上にマウントし, そこへAFM探針を接触させた後引っぱり上げると, フォースカーブに繰り返しパターンがみられた(図3). フォースカーブ上にピークが生じた距離と, フォールディングしているアミノ酸残基の長さは一致しており, ピークは蛋白質のフォールディングパターンを示していると考えられた.

#### c. 分子間相互作用の測定 (chemical force microscopy)

ハーバード大学のLieberらは, AFM探針先端を特定の有機化合物で修飾し, 対象とする試料表面の官能基を選択的に検出しようとするchemical force microscopyを報告した<sup>4)</sup>. 彼らは, 金で表面をコートしたシリコンナイトライド探針上に親水基(-COOH)あるいは疎水基(-CH<sub>3</sub>)を末端に有するアルキルチオール分子で自己組織化膜(self assembled monolayer: SAM)を形成させる方法でAFM探針の修飾を行い, フォースカーブ測定を行った(図4). その結果, CH<sub>3</sub>/CH<sub>3</sub>, CH<sub>3</sub>/COOHおよびCOOH/COOH対での相互作用の違いをフォースカーブで検出することに成功した.

同様に, SAMで修飾したAFM探針を用いて, 分子のキラリティーの判別<sup>5)</sup>, 超分子ホストゲストコンプレックスの結合能の測定<sup>6)</sup>などが報告されている. Gaubらのグループは, このchemical force microscopyをレセプター対リガンドの結合能測定に応用し, ストレプトアビジンとビオチンの結合能をフォースカーブとして測定した. この手法を種々のレセプター-リガンド対に応用することで, 将来的にはごく微量の蛋白質を用いたドラッグスクリーニング法として有用となる可能性も考えられる(図5).

### 4. 測定法(各論)

#### a. 試料の調製法

AFM測定において, 最も要となるところが, 試料の調製法である. 測定結果の良し悪しは, この試料調製法によるといっても過言でない. AFMは基本的にナノメートルレベルでの表面解析である. したがって, 測定する試料はできるだけ平坦なものである必要がある. 調製した試料の凸凹が激しいと, 単分子レベルでの測定を行う解像度が望めなくなるからである.

基板としては, マイカ, 単結晶金, グラファイトなどの原子レベルで平坦なものを用いる. マイカ, グラファイトは, 劈開したばかりのものを用い, 金は蒸着後高温でアニールし単結晶表面を形成したものを用いる. ここに目的の蛋

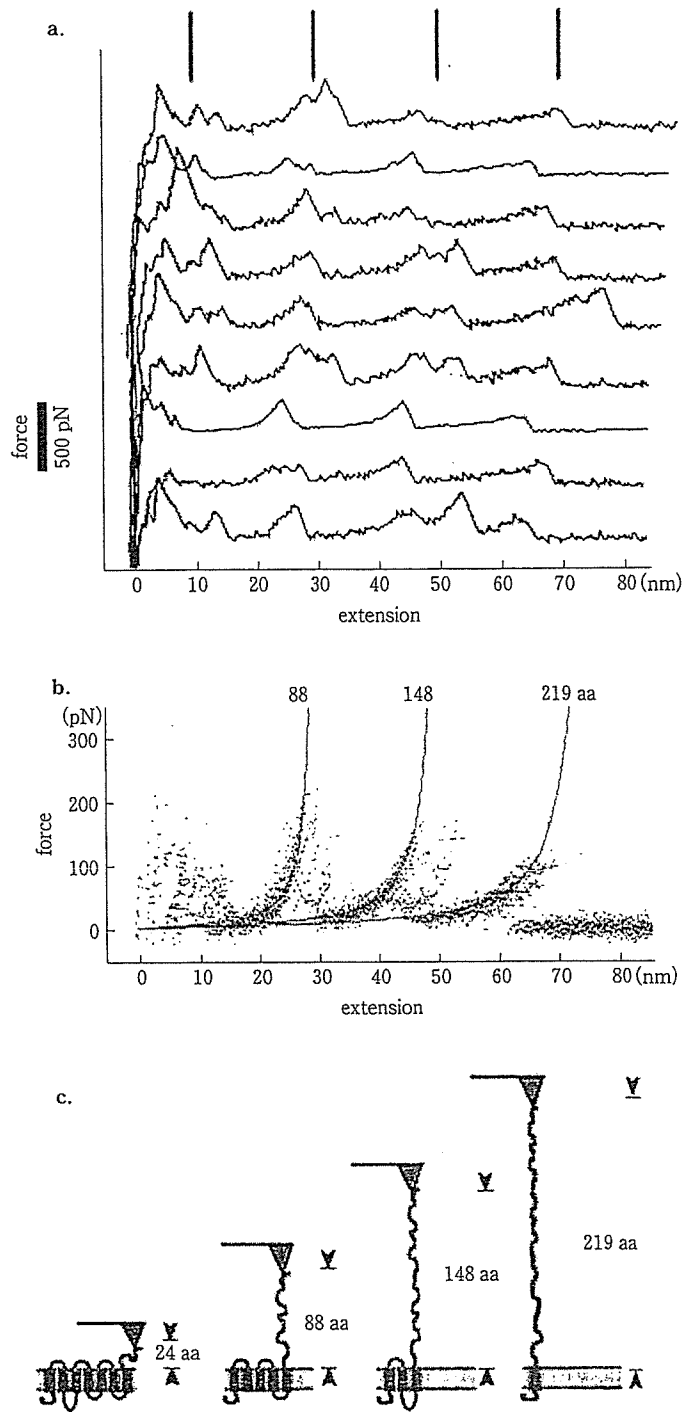


図3 AFMを用いた蛋白質アンフォールディングの実験

細胞膜中の膜蛋白質にAFM探針を接触(-1nN; 1秒間)させ、C末端をAFM探針の先端に接着させる。それを引っ張ると、フォースカーブが生じ、カーブ上のピークまでの距離は、予測されているフォールディングパターンのアミノ酸残基の長さとも一致する。

(文献<sup>9)</sup>より引用)

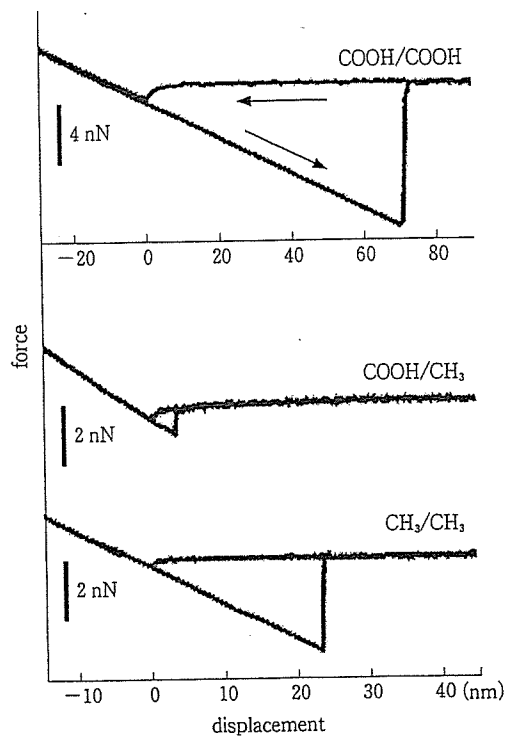


図4 末端に親水基あるいは疎水基を有する分子の自己組織化膜で修飾したAFM探針によるフォースカーブの測定 (文献<sup>9)</sup>より引用)

白質を単分子膜として不動化させる。測定する蛋白質は、nativeなものを用いる場合もあれば、精製したものを用いる場合もある。膜結合型蛋白質では、細胞膜上に蛋白質が大量に発現していれば、それを直接測定することも可能であるが、精製した蛋白質を基板上に不動化したうえで測定する場合もある。測定は、溶媒を乾燥させたうえで行う場合と、溶液中でそのまま行う場合がある。いずれにしても超純粋な溶媒を用い、ほこりなどが基板に吸着しないような環境下で試料調製を行う必要がある。図6に調製法の異なる試料のイメージングの例を示す。乾燥した試料の大気中測定では蛋白質の凝集がみられたが、水溶液中での測定では、分散した蛋白質が単分子レベルで観測された。緩衝溶液中での測定では、蛋白質の再構築がよく観察され、チャンネル孔らしき構造も観測された<sup>7)</sup>。

## b. 測定

### 1) 測定モード

基本測定モードには、コンタクトモード、タッピングモード、ノンコンタクトモードの3種類があり(図7)、測定する試料に応じてモードを選択する必要がある。コンタクトモードは、探針の先端と試料を接触させながら測定する方法で、探針と試料の接触により生じる板ばねのそりをフィードバックシステムで一定に保つように設定し、電気的信号をもとに画像化する。スキャン速度を上げることができる、比較的凹凸の大きい試料の測定にも適しているなどの利点があるが、直接接触することにより試料に損傷が生じる可能性があるため、ソフトな試料(特に生物学的試料)の測定にはあまり適していない。これに対し、タッピングモードと、ノンコンタクトモードは一定の周波数で振動させた板ばねを用いた測定法である。タッピングモードでは、探針が試料に最も近づくときに軽く試料に接触するが、ノンコンタクトモードでは、全く接触しない。試料と探針が接近すると、相互作用により振動幅に変化が生じるが、これらのモードではフィードバックシステムにより振動幅を一定に保つよう調節し、その調節に必要な電気的信号をもとに画像化を行う。双方とも試料と探針の接触が少なく、ソフトな試料の測定に適している。

### 2) カンチレバー

AFMの解像度は探針の先端に依存する。すなわち、高分解能な測定のためには、探針の先端がなるべく微細であることが重要である。この微細加工が可能であること、更に、高い共振周波数を得られるという理由から、シリコン、シリコンナイトライドなどの素材が現在汎用されている。また、前述のchemical force microscopyなどの応用測定に用いられる、金コート済みの探針も市販されている。

更に高い解像度を達成するためには、探針の先端が非常に鋭利であることが求められ、単分子であることが理想である。より微細な探針素材としてはカーボンナノチューブを用いる研究も行われている<sup>8)</sup>。

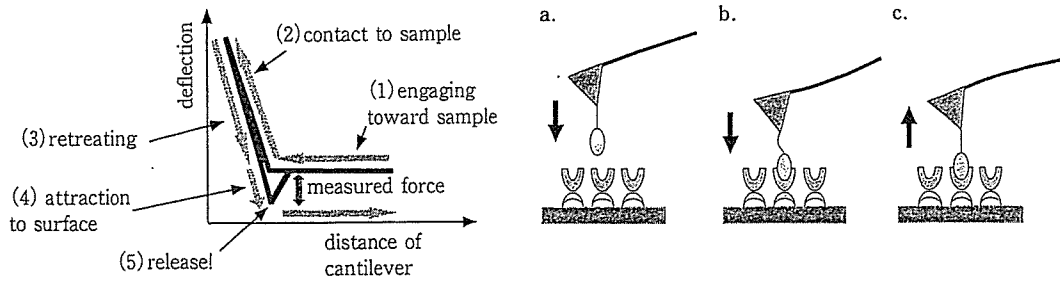


図5 Chemical force spectroscopy を用いたレセプターとリガンド間の分子間相互作用の実験  
a: 試料に接近. b: 試料に接触. c: 相互作用(フォースとして現れる).

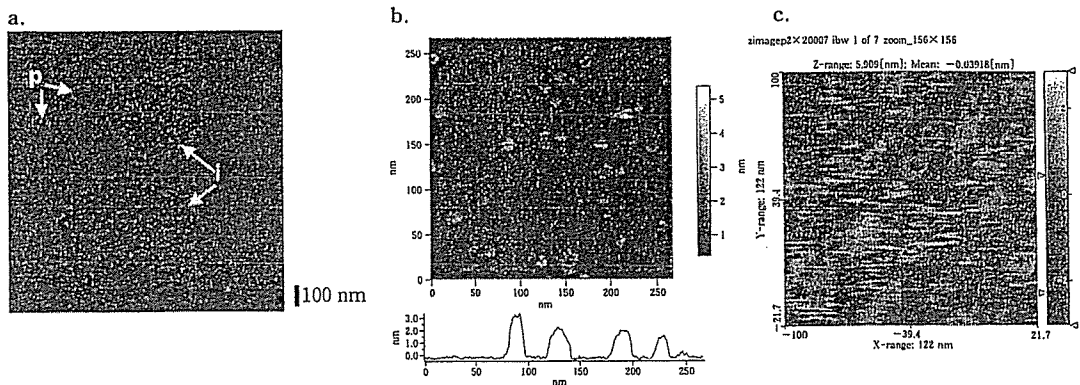


図6 膜蛋白質 P2X<sub>2</sub> 受容体の AFM によるイメージング

- a: 蛋白質水溶液をマイカ上に滴下後、乾燥させたサンプルを測定した画像。蛋白質が島状に凝集している状態が見える。
- b: 蛋白質水溶液をマイカ上に滴下、そのまま水中で測定した画像とその断面図。蛋白質が単分子状に分散している状態が見える。
- c: 蛋白質の緩衝液をマイカ上に滴下、そのまま緩衝液中で測定した画像。蛋白質が再構築し、チャンネル状の構造を取っている状態が見える。

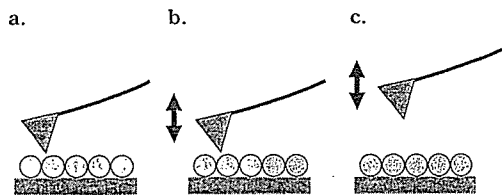


図7 AFM の測定モード

- a: コンタクトモード. b: タッピングモード.
- c: ノンコンタクトモード.

3) 膜結合型蛋白質のイメージングの例

難溶性で単結晶の作成が難しい膜蛋白質のイメージングにおいて、AFM が最も威力を発揮する。ドレスデン工科大学の Müller らの研究グループは、膜蛋白質の AFM イメージングを

精力的に行っている。その成果の例を図 8 に示す。

図 8 は、Cx26 Hela 細胞に多量に発現したコネキシン 26 分子を精製後、マイカ上にマウントして緩衝液中で AFM 測定を行ったものである<sup>9)</sup>。コネキシン分子の細胞外部分がサブ分子レベルで観測されている。コネキシン分子が六量体でチャンネル孔を有する様子がわかる。図 8-a はカルシウムイオン非存在下、b は存在下での測定で、カルシウムイオンの添加により蛋白質のコンフォメーションが変化し、チャンネルの入り口の直径が 1.5 nm から 0.6 nm へと小さくなる事が観測された。

図 9 は脂質二重膜中に埋包させた膜蛋白質



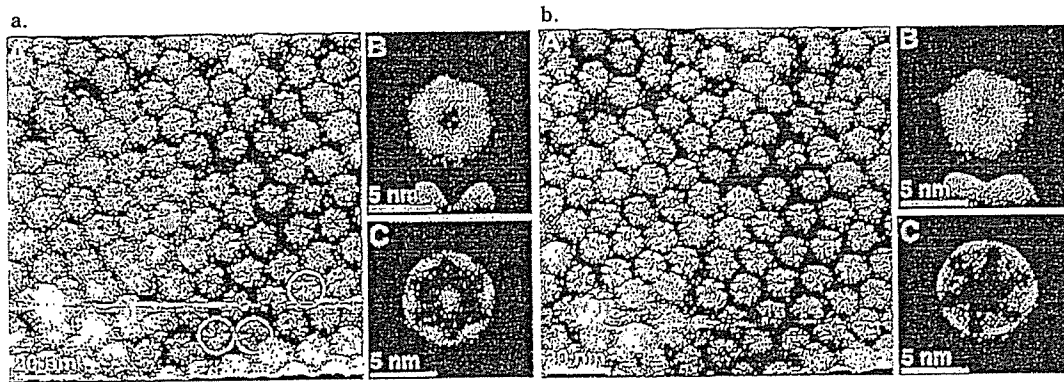


図8 コネキシン分子のAFMイメージ

a:  $\text{Ca}^{2+}$ 非存在下での画像. b:  $\text{Ca}^{2+}$ 存在下での画像. コンフォーメーションの変化がみられる.  
(文献<sup>9)</sup>より引用)

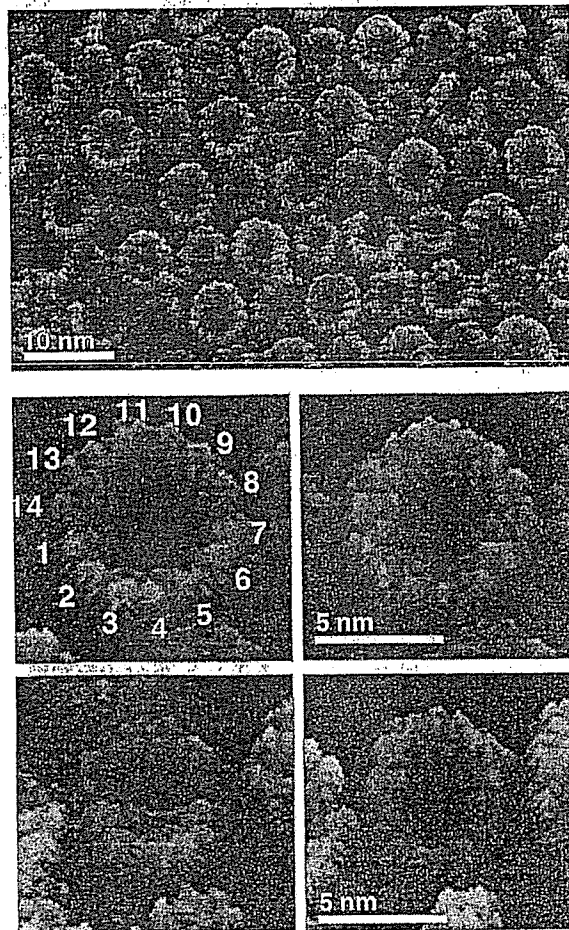


図9 精製後脂質二重膜中で再構築させた spinach chloroplast ATP synthase のAFMイメージ  
(文献<sup>10)</sup>より引用)

のAFMイメージの例である<sup>10,11</sup>。精製したATP synthaseをdodecyl maltosideの存在下、フォスファチジルコリンおよびフォスファチジン酸と混合することで埋包させマイカ上で測定を行った。

### 5. 今後の進展

以上に紹介したように、AFMの生物試料への応用は主として膜蛋白質の解析に利用されている。これは、膜蛋白質の基板上での配向が均一であり、解析が行いやすいためである。膜蛋白質のX線結晶構造解析が酵素などの水溶性蛋白質より困難であることを考慮すれば、解像度は落ちるとはいえAFMの形状解析がこの種の蛋白質の研究に今後も有用であると予想される。また、X線による構造解析が既になされている

蛋白質についても、AFMによる水溶液中の形状解析が動的構造について新たな知見をもたらす可能性が考えられる。

本稿では、単一蛋白質のイメージングの例を紹介したが、2種以上の蛋白質の水溶液中での会合状態を観察することも可能である。例えば、受容体とこれに直接的に働くエフェクター分子(G蛋白質など)との会合状態を観察することにより、生化学的応答の機構の可視化が可能となるかもしれない。

分子間相互作用の測定については、受容体とそのリガンド、酵素とその基質などの分子相互作用を1分子レベルで解析できれば、生体の分子メカニズムについて、これを力学的に解析するという全く新しい分野を切り開くことになるであろう。

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## Two-dimensional electrophoresis of protein from cultured postimplantation rat embryos for developmental toxicity studies

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### Abstract

A simple method for two-dimensional electrophoresis (2-DE) of rat embryonic protein was described. Rat embryos cultured for 24 h from day 10.5 of gestation were used as protein samples. Protein samples were lysed in rehydration buffer and separated by isoelectric focusing with immobilized pH gradient for the first dimension and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the second dimension. The use of the DeStreak Reagent as an antioxidant in the lysis buffer and electrode pads in the isoelectric focusing greatly improved the 2-DE pattern. When an embryo was used as a protein sample, about 800 protein spots were detected by silver staining in a 2-DE gel of the standard format. Eighty-one protein spots were identified by mass spectrometry for a primary 2-DE map. The same method could be applied to yolk sac membranes from the cultured embryos. The present method was considered to be suitable for a concomitant 2-DE analysis in *in vitro* developmental toxicity studies.

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**Keywords:** Two-dimensional electrophoresis; Rat; Embryo; Yolk sac; Developmental toxicity; Proteome

### 1. Introduction

Recent advances in two-dimensional electrophoresis (2-DE) made it possible to analyze the changes in protein expression pattern caused by exogenous stimulations in various tissues, such as the liver (Fountoulakis et al., 2000), kidney (Xu et al., 2005) and blood components (Piubelli et al., 2005). In developmental toxicity studies, the analysis of embryonic protein expression pattern by 2-DE is considered to be useful for the mechanism-based evaluation of developmental toxicants.

On the other hand, it is expected that the cultured rat embryos can be better protein samples for the analysis of

protein expression pattern in developmental toxicity studies. Postimplantation rat embryo culture is now widely used in developmental toxicity studies, by which embryos can be exposed to test chemicals under controlled conditions with a small number of animals and a small amount of test chemicals (Schmid et al., 1997).

However, there have been no suitable 2-DE methods for the analysis of embryonic protein expression pattern since most methods are for radio-labeled proteins but not for total proteins (Baumgartner et al., 1994; Praxmayer et al., 1992). The standard 2-DE methods that we tried could not be applicable because of a high salt concentration and poor solubility of embryonic samples. A method reported for the proteome analysis of mouse embryos is not considered suitable for routine analysis in developmental toxicity studies because of troublesome pretreatment of embryo samples, i.e., water-wash and dry-ice freezing (Greene et al., 2002).

In the present study, we described a simple 2-DE method for the analysis of cultured postimplantation rat embryos. By our method about 800 protein spots were detected in a

**Abbreviations:** 2-DE, two-dimensional electrophoresis; IEF, isoelectric focusing; IPG, immobilized pH gradient; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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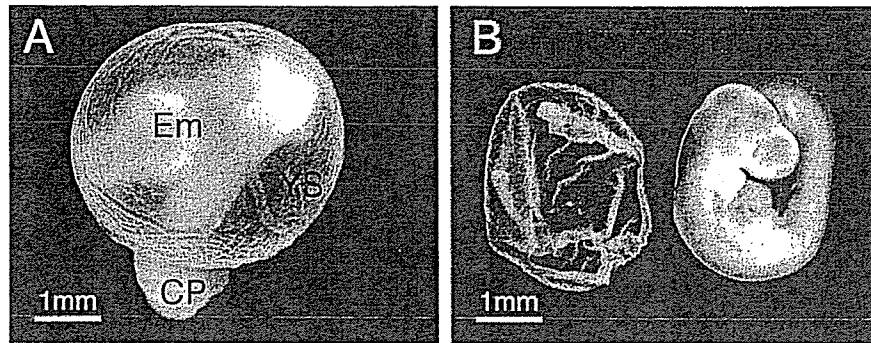


Fig. 1. Appearance of a rat embryo cultured for 24 h from day 10.5 of gestation: (A) At the end of culture. Em, embryo; YS, yolk sac; CP, chorio-allantoic placenta. (B) After the separation of embryonic membranes. Left: yolk sac membrane, Right: embryo.

single gel. The protein spots were identified by mass spectrometry for the construction of a primary 2-DE map of cultured postimplantation rat embryos. The same method could be applied to the 2-DE analysis of yolk sac membranes.

## 2. Materials and methods

### 2.1. Embryo culture

Rat embryos were cultured for 24 h by the roller bottle method as previously described (Usami and Ohno, 1996). Embryos were explanted from pregnant Wistar rats (Crlj: WI, Charles River Laboratories Japan, Inc., Kanagawa, Japan) at day 10.5 of gestation (plug day = day 0.5) under ether anesthesia. Explanted embryos were placed in a culture bottle at one embryo per 0.8–1 ml of rat serum and rotated at 35 rpm for 24 h at 37–38 °C. After the culture, the embryos and yolk sac membranes were washed three times with ice-cold buffer (0.01 M Tris–HCl, pH 7.0, 0.15 M NaCl), and placed in 1.5-ml eppendorf tubes individually with a minimum amount of the buffer for storage at –80 °C.

### 2.2. Preparation of embryo or yolk sac membrane samples

For 2-DE, frozen embryos or yolk sac membranes were lysed in 300 µl of rehydration buffer consisting of 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v) SB-10, 0.5% (v/v) IPG buffer (Amersham Biosciences, Piscataway, NJ) and 0.12% (v/v) DeStreak Reagent (Amersham Biosciences), by pulsed sonication with a 7-mm Ø tip immediately after the addition of the buffer. Care was taken to avoid heating and forming during the sonication. Embryo or yolk sac membrane lysates were kept at 20 °C and their protein concentration was determined with the 2D Quant Kit (Amersham Biosciences).

### 2.3. Isoelectric focusing (IEF) for the first dimension of 2-DE

IEF was carried out on 13-cm immobilized pH gradient (IPG) strips (Immobiline DryStrip pH 3–10 NL, Amer-

sham Biosciences) with the IPGphor system (Amersham Biosciences). IPG strips were rehydrated with the rehydration buffer containing 50 µg protein of embryo or yolk sac membrane lysate for at least 12 h at 20 °C under a cover with silicon oil. Before the start of IEF, 3-mm-wide IEF electrode strips (Amersham Biosciences) dampened were inserted between the IPG strip and both electrodes as electrode pads. Electrophoresis conditions were – Step 1: Step-n-hold at 500 V for 1 h, Step 2: Gradient at 1000 V for 1 h, Step 3: Gradient 8000 V for 2.5 h with the current limit of 50 µA per IPG strip. When electrophoresis for the second dimension was not carried out immediately after IEF, the IPG strips were stored at –80 °C.

### 2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for the second dimension of 2-DE

SDS–PAGE was performed according to the method of Laemmli (Laemmli, 1970) except that no stacking gel was used. After IEF, IPG strips were equilibrated with 5 ml of SDS equilibration buffer consisting of 0.05 M Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.025% (w/v) bromophenol blue. For the first equilibration, IPG strips were placed in a tube containing equilibration buffer with dithiothreitol (50 mg/5 ml) and rocked for 20 min. For the second equilibration, IPG strips were placed in a tube containing equilibration buffer with iodoacetamide (125 mg/5 ml) and rocked for 20 min. Equilibrated IPG strips were applied onto polyacrylamide gels (12.5% T, 2.6% C, 14 × 6 cm) and sealed with 0.5% agarose in electrode buffer. Electrophoresis was carried at a constant current of 10 mA

Table 1  
Size and protein content of cultured rat embryos

Item	Mean	Standard deviation
Yolk sac diameter (mm)	4.37	0.10
Crown-rump length (mm)	3.99	0.16
Number of somite pairs	26.0	0.67
Embryo protein (µg)	298.2	31.0
Yolk sac protein (µg)	153.0	20.2

Values for 10 embryos are shown.

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per gel for 15 min and thereafter 20 mA per gel until the dye front reached the edge of the gel.

### 2.5. Stain of 2-DE gel and protein identification

After SDS-PAGE, 2-DE gels were stained with the Plus One Silver Staining Kit Protein (Amersham Biosciences) using the Multi Processor (Amersham Biosciences) according to the manufacturer's instruction. Stained 2-DE gels were scanned and analyzed with the PD Quest 2D analysis software (Bio-Rad, Hercules, CA).

For the identification of protein spots by mass spectrometry, 2-DE gels were stained with the mass-analysis compatible Proteo Silver staining kit (Sigma, St. Louis, MO) and protein spots were excised and cut into 1-mm cubes with a scalpel. Proteins in the gel cubes were digested with trypsin (Shevchenko et al., 1996), cleaned-up with Zip-Tip C18 $\mu$  (Millipore, Bedford, MA) and analyzed by mass spectrometry using the 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). The proteins were identified by the use of mass spectrometry data for the search of primary

sequence databases with the MS/MS ion search mode of the Mascot search engine (Matrix Science, Boston, MA).

## 3. Results

### 3.1. Embryo culture

Fig. 1A shows a cultured rat embryo at the end of the 24-h culture period. Embryos and yolk sac membranes were separated as samples for 2-DE analysis (Fig. 1B). The amnion and chorio-allantoic placenta were removed and discarded. The size and protein content of the embryos and yolk sac membranes of the cultured rat embryos are shown in Table 1. Protein content in the individual embryo or yolk sac membrane was sufficient for 2-DE analysis.

### 3.2. 2-DE of embryo protein

Proteins of cultured embryos were well separated by 2-DE with the present method (Fig. 2). About 800 protein spots were consistently detected in replicate gels each

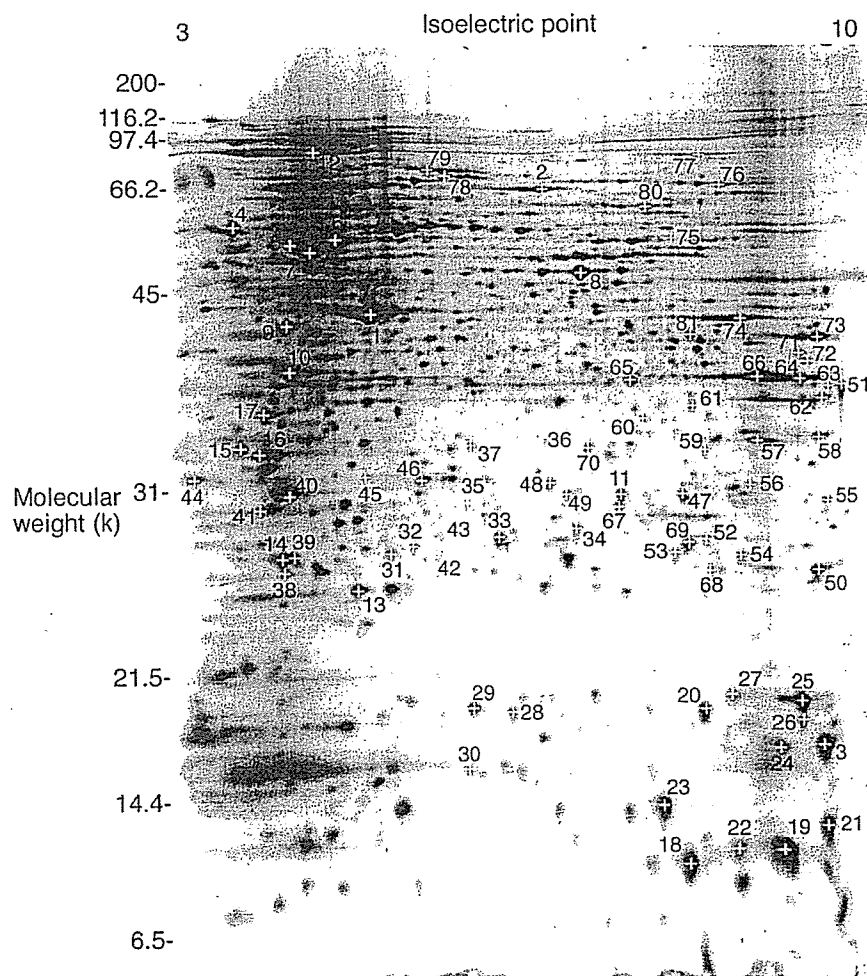


Fig. 2. 2-DE pattern of embryo proteins from a cultured rat embryo equivalent to day 11.5 of gestation. Proteins (50  $\mu$ g) were separated by IEF with 3–10NL IPG strip (13 cm) and by SDS-PAGE with 12.5% gel (14  $\times$  6 cm), and were stained with silver. Identified protein spots were numbered and listed in Table 2.

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Table 2  
Protein spots identified in the 2-DE of embryo protein

Spot no.	Accession no.	Protein name	Nominal mass	Calculated pI
1	gi 71620	Actin beta	42,066	5.29
2	gi 19705431	Albumin	70,670	6.09
3	gi 37748460	Peptidylprolyl isomerase A	18,091	8.34
4	gi 11693172	Calreticulin	48,137	4.33
5	gi 202549	Iodothyronine 5' monodeiodinase	54,375	4.87
6	gi 38014578	Tubulin, beta, 2	50,225	4.79
7	gi 54792127	ATP synthase, H <sup>+</sup> transporting, mitochondrial F1 complex, beta subunit	56,318	5.19
8	gi 38649320	Eno1 protein	51,736	6.70
9	gi 38014840	Laminin receptor 1	32,917	4.80
10	gi 112077	Nucleolar phosphoprotein B23.1	32,711	4.62
11	gi 12844989	Unnamed protein product	28,799	6.67
12	gi 51859516	Heat shock 90 kDa protein 1, beta	83,631	4.97
13	gi 8394432	Peroxiredoxin 2	21,941	5.34
14	gi 6678437	Tumor protein, translationally-controlled 1	19,564	4.76
15	gi 48675371	Complement component 1, q subcomponent binding protein	31,320	4.77
16	gi 34876714	Predicted: similar to Eukaryotic translation elongation factor 1 beta 2	24,831	4.55
17	gi 7242171	Proliferating cell nuclear antigen	29,108	4.66
18	gi 27668426	Predicted: similar to hemoglobin: Subunit = zeta	16,124	6.75
19	gi 3367724	Epsilon 1 globin	16,151	7.90
20	gi 55926145	Expressed in non-metastatic cells 2	17,386	6.92
21	gi 1628436	Profilin	15,149	8.46
22	gi 3367724	Epsilon 1 globin	16,151	7.90
23	gi 40254577	Ribosomal protein S12	14,905	6.81
24	gi 37748460	Peptidylprolyl isomerase A	18,091	8.34
25	gi 509201	Cofilin	18,749	8.22
26	gi 7441446	Destrin	18,661	7.78
27	gi 6671746	Cofilin 2, muscle	18,812	7.66
28	gi 19924089	Expressed in non-metastatic cells 1, protein (NM23A) (nucleoside diphosphate kinase)	17,296	5.96
29	gi 38328242	Stmn1 protein	17,278	5.76
30	gi 12850597	Unnamed protein product	17,138	5.95
31	gi 57006	Unnamed protein product	22,320	5.55
32	gi 202945	Apolipoprotein A-I precursor	30,100	5.52
33	gi 3688521	Thiol-specific antioxidant protein	24,860	5.64
34	gi 8394082	Proteasome (prosome, macropain) subunit, beta type 3	23,235	6.15
35	gi 20071222	NADH dehydrogenase (ubiquinone) Fe-S protein 3	30,358	6.40
36	gi 1381643	Cysteine protease p32-beta	30,097	5.68
37	gi 30410794	Proteasome activator subunit 3 isoform 1	29,602	5.69
38	gi 21312044	Eukaryotic translation initiation factor 3, subunit 12	25,356	4.81
39	gi 6671696	Chromobox homolog 1 (Drosophila HPI beta)	21,519	4.85
40	gi 27664664	Predicted: similar to 25 kDa FK506-binding protein	25,220	9.29
41	gi 6381991	Integrin beta 4 binding protein	27,007	4.63
42	gi 202945	Apolipoprotein A-I precursor	30,100	5.52
43	gi 2897818	Huntingtin interacting protein-2	22,503	5.33
44	gi 6978499	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	28,718	3.99
45	gi 47169488	TPA: proteasome subunit alpha type 3-like	28,621	5.19
46	gi 62664759	Predicted: prohibitin	27,757	5.44
47	gi 297111	Phosphoglyceromutase	28,908	8.85
48	gi 16758298	Proteasome (prosome, macropain) subunit, beta type 7	30,250	8.13
49	gi 16758848	Endoplasmic reticulum protein 29	28,614	6.23
50	gi 56789700	Peroxiredoxin 1	22,323	8.27
51	gi 38648863	Malate dehydrogenase, mitochondrial	36,117	8.93
52	gi 16758182	RAN, member RAS oncogene family	24,579	7.01
53	gi 5420030	Glutathione transferase	25,550	6.77
54	gi 25453420	Glutathione S-transferase, pi	23,652	6.89
55	gi 56550075	Proteasome (prosome, macropain) subunit, alpha type 7	28,010	8.60
56	gi 8394069	Proteasome (prosome, macropain) subunit, alpha type 4	29,764	7.59
57	gi 18543331	Guanine nucleotide binding protein, beta polypeptide 2-like 1	35,529	7.60
58	gi 38051979	Vdac1 protein	32,060	8.35
59	gi 1906812	Inducible carbonyl reductase	30,920	7.64
60	gi 62661724	Predicted: similar to esterase D/formylglutathione hydrolase	37,322	6.45

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Table 2 (continued)

Spot no.	Accession no.	Protein name	Nominal mass	Calculated pI
61	gi 57527447	Ribose-phosphate pyrophosphokinase I -like	35,297	6.51
62	gi 54261548	Lactate dehydrogenase A	36,712	8.45
63	gi 7949053	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform 1	36,028	8.67
64	gi 62653546	Predicted: similar to glyceraldehyde-3-phosphate dehydrogenase	36,045	8.44
65	gi 6978491	Aldehyde reductase 1	36,230	6.26
66	gi 62653546	Predicted: similar to glyceraldehyde-3-phosphate dehydrogenase	36,045	8.44
67	gi 76647405	Predicted: similar to proteasome subunit alpha type 6 (proteasome iota chain) (macropain iota chain)	27,838	6.34
68	gi 8394079	Proteasome (prosome, macropain) subunit, beta type 2	23,069	6.96
69	gi 8394063	Proteasome (prosome, macropain) subunit, alpha type 2	26,024	6.92
70	gi 62655706	Predicted: similar to RIKEN cdna 1110025F24	26,482	5.90
71	gi 61889115	Phosphoserine aminotransferase 1	40,943	7.57
72	gi 66911068	Pcbp2_predicted protein	35,666	8.17
73	gi 202837	Aldolase A	39,691	8.31
74	gi 38649310	Phosphoglycerate kinase 1	44,909	8.02
75	gi 584875	Adenylyl cyclase-associated protein 1 (CAP 1)	51,857	7.16
76	gi 74144333	Unnamed protein product	67,573	7.18
77	gi 19424312	KH-type splicing regulatory protein	74,466	6.38
78	gi 228784	Alpha fetoprotein	70,167	5.71
79	gi 228784	Alpha fetoprotein	70,167	5.71
80	gi 2511703	p60 protein	63,158	6.40
81	gi 2644966	hnRNP-E1 protein	37,987	6.66

Proteins names with their NCBI accession numbers were shown for the protein spots in Fig. 1.

from one embryo. Selected protein spots were analyzed by mass spectrometry and 81 protein spots were identified as shown in Table 2. These protein spots also served as landmarks for the matching of protein spots among the gels.

### 3.3. 2-DE of yolk sac membrane protein

Proteins of yolk sac membranes were separated as well as those of embryos by 2-DE with the present method (Fig. 3). The 2-DE pattern of yolk sac membranes was fairly different from that of embryos. Most protein spots were common but were quantitatively different between the yolk sac membranes and the embryos. In addition, there were embryo specific and yolk sac membrane specific protein spots.

## 4. Discussion

The present method is simple and can be carried out concomitantly with in vitro developmental toxicity studies using rat embryo culture. The whole 2-DE procedures are completed within four days. IPG strips with narrower pH ranges would be useful for precise and convenient analysis of specific protein spots. For screening purposes or focused proteomics, the same procedures could be applied to the mini-gel format with shorter IPG strip and mini SDS-PAGE gel.

The primary 2-DE map constructed in the present study is useful for interlaboratory comparison of 2-DE patterns. Abundant and characteristic protein spots can be used as landmarks for matching of the 2-DE pattern. We are planning to identify more protein spots for a more precise and

accurate 2-DE map of cultured rat embryos in future, giving priority to protein spots of interest that are differentially expressed due to the effects of developmental toxicants. Identification of very faint protein spots may require a separate 2-DE experiment with an increased amount of applied protein samples.

It is expected that 2-DE analysis by the present method enables informative experiments for developmental toxicity studies. Because protein from less than one embryo is sufficient for 2-DE analysis in the present method, toxic effects observed in individual embryos may be related to the changes in protein expression pattern. Alternatively, analysis of specific parts isolated from single embryos may improve the efficiency of differential analysis. It is also noted that the present method is valid for 2-DE analysis of yolk sac membranes as well as embryos. Simultaneous analysis of yolk sac membranes with embryos would be useful to investigate the action sites of developmental toxicants.

The critical point of the present method is the use of the DeStreak Reagent as an antioxidant and the electrode pads. Both of which greatly improved the 2-DE pattern by decreasing horizontal streaks in the present method. We initially used dithiothreitol or tributylphosphine as a reducing agent to avoid the oxidation of proteins but could not decrease the streaks sufficiently. High concentration of redox agents such as glutathione in the embryo or yolk sac membrane samples (Harris et al., 1995) might affect redox status of proteins when dithiothreitol or tributylphosphine was used.

As far as we tested, addition of protease inhibitors to the lysis/rehydration buffer had no effects on the 2-DE pattern. It is considered that the constituents of the lysis/rehydration buffer were effective for inactivation of proteases in the

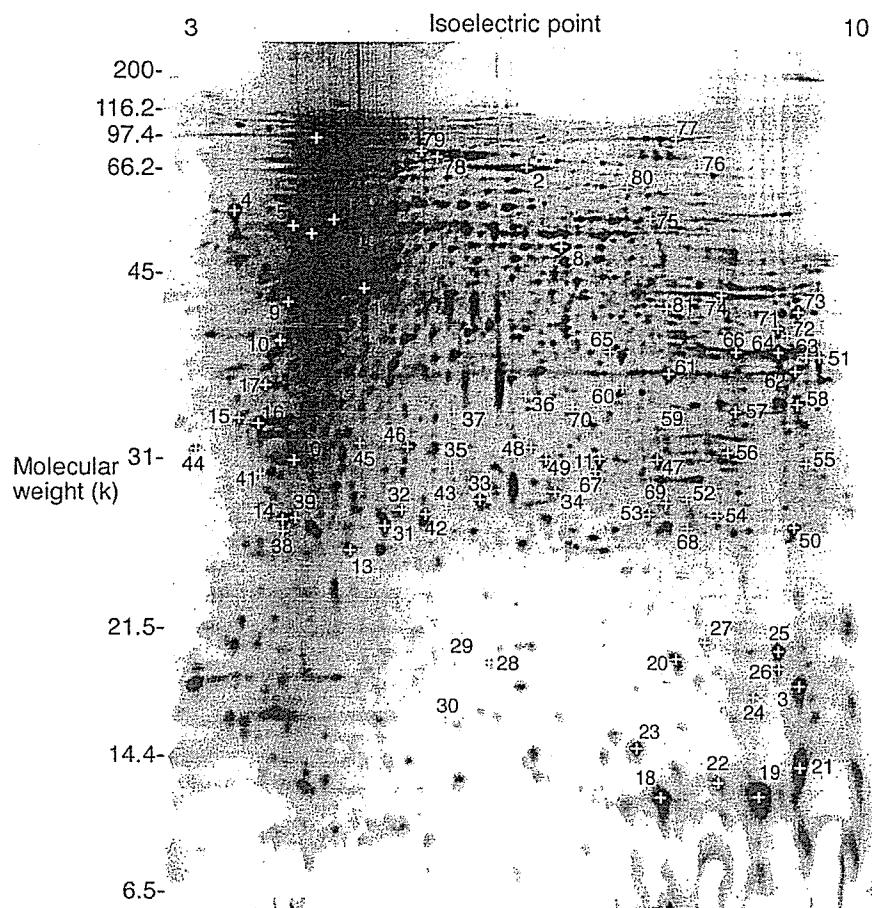


Fig. 3. 2-DE pattern of yolk sac membrane proteins from a cultured rat embryo equivalent to day 11.5 of gestation. Proteins (50  $\mu$ g) were separated by IEF with 3–10NL IPG strip (13 cm) and by SDS–PAGE with 12.5% gel (14  $\times$  6 cm), and were stained with silver. Proteins identified for the embryo are indicated by the same number as in Fig. 2.

embryo or yolk sac membrane samples. No requirement for protease inhibitors in the present method saves the cost and labor in the 2-DE analysis.

In conclusion, it is considered that the present method is suitable for 2-DE analysis of embryos and yolk sac membranes in developmental toxicity studies using postimplantation rat embryo culture.

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ORIGINAL ARTICLE

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## Synthesis of a novel $\beta$ -tricalcium phosphate/hydroxyapatite biphasic calcium phosphate containing niobium ions and evaluation of its osteogenic properties

**Abstract** To promote the osteogenic properties of osteoblasts, we synthesized a hydroxyapatite (HAp) with  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) biphasic calcium phosphate containing Nb ions (NbTCP/HAp). NbTCP/HAp was prepared by annealing precipitates obtained by coprecipitation of an aqueous solution of  $\text{Ca}(\text{NO}_3)_2$  and a mixture of  $(\text{NH}_4)_2\text{HPO}_4$  and aqueous Nb solution. The precipitates can be regarded as a calcium-deficient HAp, the  $\text{PO}_4$  sites of which are partly occupied by Nb ions. NbTCP/HAp was successfully synthesized by thermal decomposition of the precipitates. NbTCP/HAp enhanced the calcification of normal human osteoblasts (NHObst), and the amount of calcified tissue increased in proportion to the Nb ion concentration in the NbTCP/HAp. The alkaline phosphatase (ALP) activity of NHObst was also enhanced by NbTCP/HAp. Because Nb ions significantly enhance the ALP activity of NHObst, calcification by NbTCP/HAp is considered to be due to enhancement of ALP activity induced by Nb ions dissolved from NbTCP/HAp. These results indicate that NbTCP/HAp can be an effective bone repair material.

**Key words** Tissue engineering · Bone · Osteoblasts · Calcium phosphate · Nb ions

### Introduction

Bone tissue engineering offers a promising alternative strategy for healing severe bone injuries by utilizing the body's natural biological response to tissue damage in conjunction with engineering principles. Osteogenic cells, growth factors, and biomaterial scaffolds form the foundation of the many bone tissue engineering strategies employed to achieve regeneration of damaged bone tissue. An ideal bio-

material scaffold will provide mechanical support to an injured site and also enhance osteogenic differentiation to encourage bone growth.<sup>1</sup> To develop biomaterial scaffolds with optimal performance, understanding the interactions between osteoblasts and scaffolds is extremely important.

Hydroxyapatite [ $\text{HAp}$ ,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ] and related calcium phosphate ceramics, e.g.,  $\beta$ -tricalcium phosphate [ $\beta$ -TCP,  $\beta$ - $\text{Ca}_3(\text{PO}_4)_2$ ], have good biocompatibility with bone tissue because their chemical compositions are very similar to the mineral phase of human bone. It is well known that these calcium phosphate ceramics can be biologically bonded to natural bone. In fact, it has been reported that porous materials composed of HAp,  $\beta$ -TCP, or  $\beta$ -TCP/HAp biphasic calcium phosphate are useful for bone tissue regeneration because of their osteoconductivity.<sup>2-6</sup> It has also been reported that  $\beta$ -TCP/HAp biphasic calcium phosphate shows better osteoconductivity than HAp or  $\beta$ -TCP alone.<sup>7,8</sup> Therefore, this material has been actively studied for use as a scaffold for bone tissue regeneration.

In a previous study, Nb ions were reported to lower cytotoxicity<sup>9</sup> ( $\text{IC}_{50}$  of Nb ions for L929 fibroblasts is  $3.63 \times 10^3$ ), and we reported that Nb ions significantly promoted the calcification of normal human osteoblasts (NHObst).<sup>10</sup> Furthermore, we succeeded in synthesizing a hydroxyapatite containing Nb ions (NbHAp) and showed that NbHAp has the potential to promote alkaline phosphatase (ALP) activity, an important factor in the generation of new bone, in NHObst.<sup>11</sup> In this study, to further promote the cell activity of osteoblasts, we synthesized  $\beta$ -TCP/HAp biphasic calcium phosphate containing Nb ions and investigated interactions between  $\beta$ -TCP/HAp biphasic calcium phosphate and NHObst in vitro.

### Materials and methods

Synthesis and characterization of  $\beta$ -TCP/HAp biphasic calcium phosphate containing Nb ions

Reagent grade  $\text{Ca}(\text{NO}_3)_2$ ,  $(\text{NH}_4)_2\text{HPO}_4$ , and  $\text{NbCl}_5$  (Wako, Osaka, Japan) were used without purification. NbTCP/HAp

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samples were prepared by annealing precipitates obtained from coprecipitation of an aqueous solution of  $\text{Ca}(\text{NO}_3)_2$  with a mixture of  $(\text{NH}_4)_2\text{HPO}_4$  and an aqueous solution of Nb as described below.  $\text{Ca}(\text{NO}_3)_2$  and  $(\text{NH}_4)_2\text{HPO}_4$  were completely dissolved in distilled water. The aqueous Nb solution was prepared by mixing distilled water and  $\text{NbCl}_5$  dissolved in 5% hydroxyacetone and 5% 2-aminoethanol.<sup>12</sup> A 0.2 M  $(\text{NH}_4)_2\text{HPO}_4$  aqueous solution was combined with 0.01 M  $\text{NbCl}_5$  and stirred with a magnetic bar at Nb/(Nb + P) molar ratios of 0.0000, 0.0167, or 0.1667. The pH of the mixture was adjusted to 10 using 1 N NaOH throughout the reaction, and 0.2 M  $\text{Ca}(\text{NO}_3)_2$  was slowly dropped into the mixture (20 ml/min). The amount of 0.2 M  $\text{Ca}(\text{NO}_3)_2$  solution was adjusted to a Ca/(Nb + P) molar ratio of 1.6 in order to synthesize  $\beta$ -TCP/HAp biphasic calcium phosphate, followed by stirring the suspension for 24 h at room temperature. The precipitates were centrifuged at 3600 rpm for 5 min and washed with distilled water. The resulting precipitates of Nb/(Nb + P) with molar ratios of 0.0000, 0.0167, and 0.1667 were named NbHAp-0, NbHAp-I, and NbHAp-II, respectively. These precipitates were then annealed at 800°C for 2 h (temperature increase: 5°C/min) and named NbTCP/HAp-0, NbTCP/HAp-I, and NbTCP/HAp-II, respectively. The NbTCP/HAp samples obtained were characterized by X-ray diffraction analysis (XRD, Rint2000, Rigaku, ■■■, ■■■) with  $\text{Cu K}_\alpha$  radiation (40 kV, 50 mA). The XRD profiles of  $2\theta$  angles between 20° and 60° with a step interval of 0.01° were collected at a scanning rate of 4°/min. Also, measurement of the lattice parameter was carried out using the 211, 112, and 300 planes of HAp, and data for the lattice parameter were collected with a scan rate of 0.025°/min. The observed interplanar spacing was corrected using elemental Si as a standard material.

Concentrations of Ca, P, and Nb ions in the precipitate were estimated by inductively coupled plasma analysis (ICP, HP4500, Hewlett-Packard, ■■■, ■■■) after the precipitate was dissolved in  $\text{HNO}_3$  solution. Microstructural evaluation of the precipitates was performed by scanning electron microscopy (SEM, JSM-5800LV, JEOL, ■■■, ■■■; acceleration voltage: 25 kV) and energy-dispersive X-ray spectroscopy (EDX) (LV5800, JEOL).

#### Osteogenic effects of NbTCP/HAp

NbTCP/HAp pellets were fabricated to investigate their effects on the osteogenic function of osteoblasts. In total, 100 mg of powdered NbTCP/HAp was put into a stainless steel mold and uniaxially pressed at 30 MPa for 1 min to form a pellet 0.5 mm in thickness and 12 mm in diameter. The pellets were sintered at 800°C for 2 h (temperature increase: 5°C/min).

NHOst were purchased from BioWhittaker (Walkersville, MD, USA) and maintained in ■■■ (αMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS, Kokusai Sinyakyo, Tokyo, Japan) in incubators at 37°C in a humid atmosphere with 5%  $\text{CO}_2$ . All assays were performed using αMEM containing 10% FCS supplemented with 10 mM  $\beta$ -glycerophosphate.

Cells were seeded on the pellets as described below. Each NbTCP/HAp pellet was immersed in 1 ml culture medium in a well of a 24-well cell culture plate (Corning, Corning, NY, USA) and incubated at 37°C for 24 h. After discarding the medium, 300  $\mu\text{l}$  of new culture medium was put into each well, followed by 1 ml of NHOst suspension ( $4 \times 10^4$  cell/ml), and incubation was carried out for 4 h. Finally, the cell-seeded NbTCP/HAp pellet was transferred to a new well of a 24-well plate with 1 ml of the test medium and incubated at 37°C in a humidified atmosphere with 5%  $\text{CO}_2$  for 7–14 days.

Extracts from various NbTCP/HAp samples were prepared to investigate their effects on dissolved ions. NbTCP/HAp powder (100 mg/ml) was added to the culture medium (αMEM) containing 10% FCS and immersed at 37°C for 24 h. After changing the medium, the suspensions were stirred by a shaker at 200 rpm for 72 h at 37°C. The suspension was centrifuged at 3600 rpm for 5 min, and the supernatant was collected to use as an extract for an osteogenesis test in vitro. The atomic concentrations of Nb in the extract were measured by ICP.

An NHOst suspension ( $4 \times 10^4$  cells/ml) was added to culture wells and incubated for 4 h. After the NHOst had adhered to the well, the suspension medium was discarded and 1 ml of the extract supplemented with 10 mM  $\beta$ -glycerophosphate was added. The NHOst were incubated at 37°C in a saturated humid atmosphere with 5%  $\text{CO}_2$  for 7–14 days.

We also examined the effect of Nb ions on the osteogenesis of NHOst. A solution of 0.2  $\mu\text{M}$   $\text{NbCl}_5/\alpha\text{MEM}$  and serial dilutions were prepared. In addition to the experiment using the extracts indicated above, NHOst were cultured in  $\text{NbCl}_5/\alpha\text{MEM}$  supplemented with  $\beta$ -glycerophosphate for 7–14 days.

Proliferation of NHOst cells in each experiment was estimated by a TetraColor One assay (Seikagaku, Tokyo, Japan), which incorporates an oxidation–reduction indicator based on detection of metabolic activity. After a 7-day incubation, the culture medium was discarded and 2% TetraColor One/αMEM solution was added to each well and was incubated for 2 h. The absorbance of the supernatant at 450 nm was measured using a  $\mu\text{Quant}$  spectrophotometer (Bio-tek, Winooski, VT, USA) to estimate the proliferation of the test cells. After estimating the proliferation, the cells were washed with phosphate-buffered saline [PBS(-)], followed by the addition of 1 ml of 0.1 M glycine buffer (pH 10.5) containing 10 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{ZnCl}_2$ , and 4 mM *p*-nitrophenylphosphate sodium salt. The absorbance of the added buffer at 405 nm after 5 min incubation at room temperature was detected to evaluate the ALP activity of the test cells. After measurement of ALP, the NHOst cultured in the extract were washed with PBS(-) three times and the calcium phosphate deposited by NHOst was estimated. The amount of deposited calcium phosphate dissolved in 0.1 N HCl solution was determined by a Wako Calcium C test kit (Wako), which is based on the *o*-cresolphthalein complex color development method. The NHOst in all assays were stained in 5% Giemsa solution and observed by light microscopy (Nikon, Eclipse TE300, ■■■, ■■■) to confirm their

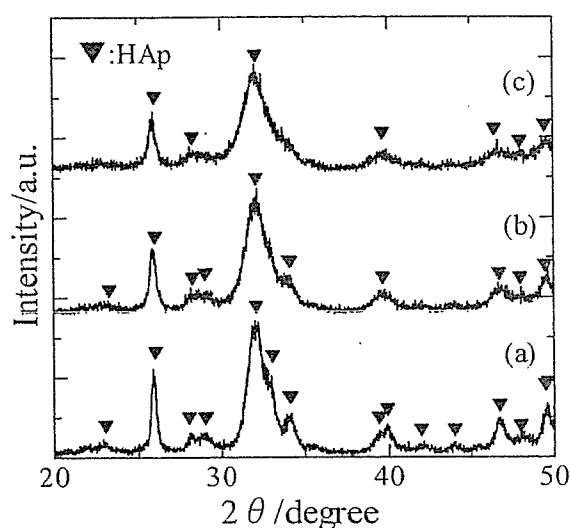
**Table 1.** Chemical composition and characteristics of the precipitates prepared in this study

Sample	Phase	Annealing temperature	Theoretical composition <sup>a</sup>		Measured composition <sup>a</sup>		Color of precipitate	Lattice parameter <sup>b</sup>	
			Ca/(P + Nb)	Nb/(P + Nb)	Ca/(P + Nb)	Nb/(P + Nb)		$\alpha$ -axis (nm)	$c$ -axis (nm)
NbHAp-0	HAp		1.60	0.000	1.60	–	White	–	–
NbHAp-I	HAp		1.60	0.017	1.56	0.013	Pale yellow	–	–
NbHAp-II	HAp		1.60	0.167	1.56	0.077	Buff yellow	–	–
NbTCPHAp-0	$\beta$ -TCP + HAp	800°C	1.60	0.000	1.60	–	White	0.939	0.687
NbTCP/HAp-I	$\beta$ -TCP + HAp	800°C	1.60	0.017	1.56	0.013	White	0.942	0.689
NbTCP/HAp-II	$\beta$ -TCP + HAp	800°C	1.60	0.167	1.56	0.074	White	0.943	0.690

HAp, hydroxyapatite; NbHAp, hydroxyapatite containing Nb ions; TCP, tricalcium phosphate

<sup>a</sup>Molar ratio

<sup>b</sup>Lattice parameter for HAp

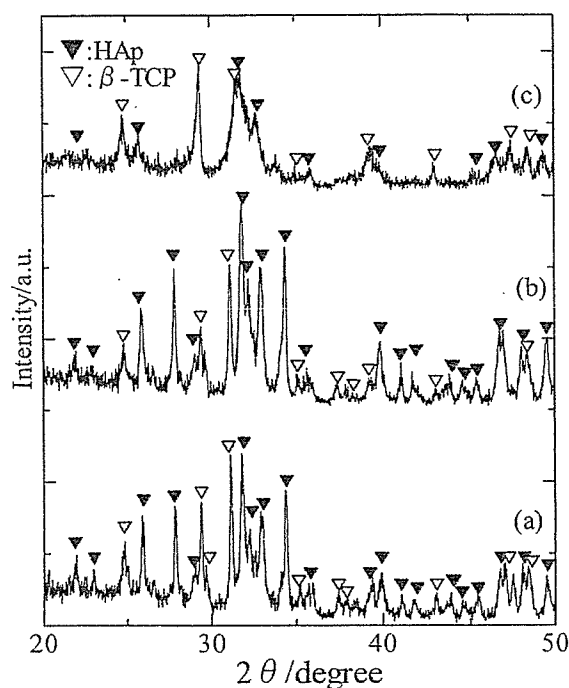


**Fig. 1.** X-ray diffraction (XRD) patterns of the precipitates with a Ca/(P + Nb) molar ratio of 1.50: a, Nb/(Nb + P) = 0; b, Nb/(Nb + P) = 0.0167; and c, Nb/(Nb + P) = 0.1667. Triangles represent XRD peaks due to the crystal structure of hydroxyapatite (HAp)

proliferation. All results were expressed as mean values  $\pm$  SD and were analyzed statistically with Student's *t* test.

## Results

XRD patterns of the precipitates prepared in this study are shown in Fig. 1. The XRD indicated that precipitates with Nb/(Nb + P) molar ratios from 0 to 0.167 had a monolithic apatite structure, irrespective of the Nb/(Nb + P) molar ratio of the starting solution, although the level of crystallite decreased as the Nb content increased. XRD patterns of the precipitates with various Nb/(Nb + P) molar ratios annealed at 800°C are shown in Fig. 2. The level of crystallites of the precipitates was high due to the annealing, and their diffraction peaks were composed of those of both HAp and



**Fig. 2.** XRD patterns of the annealed precipitates with a Ca/(P + Nb) molar ratio of 1.50: a, Nb/(Nb + P) = 0; b, Nb/(Nb + P) = 0.0167; and c, Nb/(Nb + P) = 0.1667. These precipitates were annealed at 800°C.  $\beta$ -TCP,  $\beta$ -tricalcium phosphate

$\beta$ -TCP. Interestingly, the crystallite level decreased when the Nb level increased.

The chemical compositions and characteristics of the precipitates prepared in this study are summarized in Table 1. Both the Ca/(Nb + P) and the Nb/(P + Nb) molar ratios in precipitates measured by ICP approximately agreed with their theoretical values, except for the Nb/(P + Nb) molar ratio of NbTCP/HAp-II: the measured Nb/(P + Nb) molar ratio of NbTCP/HAp-II was 0.074, which is lower than the theoretical value of 0.167. The lattice parameter of the HAp phase in NbTCP/HAp increased with increasing Nb content.

Fig. 3. Scanning electron microscopy–energy-dispersive X-ray spectroscopy spectra of NbTCP/HAp-II annealed at 800°C (a) and their mapping images from P-K $\alpha$ , Ca-K $\alpha$ , and Nb-M $\alpha$  lines (b)

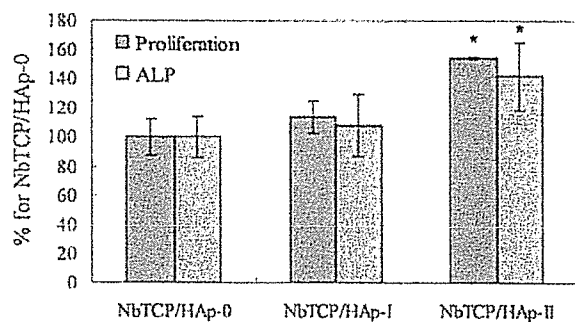
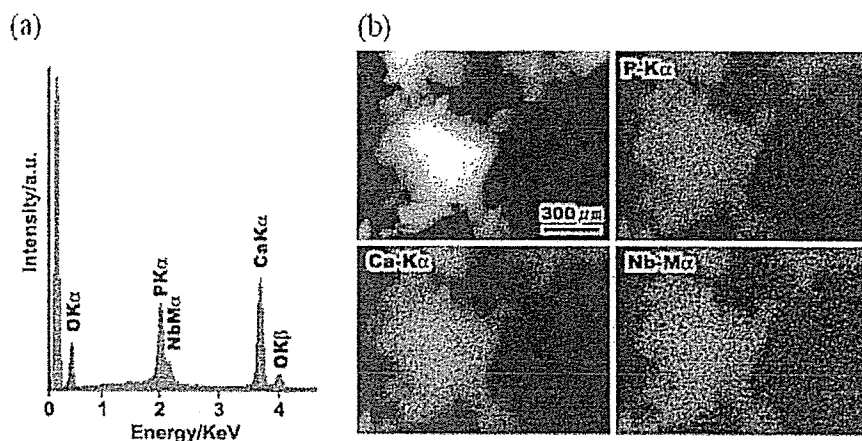


Fig. 4. Proliferation and alkaline phosphatase (ALP) activity of normal human osteoblasts (NHOst) cultured on various kinds of NbTCP/HAp pellets. \* $P < 0.01$  against NbTCP/HAp-0 (without Nb ions)

The lattice parameters of NbTCP/HAp-0 without Nb ions were 0.939 nm for the  $a$ -axis and 0.687 nm for the  $c$ -axis, while those of NbTCP/HAp-II were 0.943 nm for the  $a$ -axis and 0.690 nm for the  $c$ -axis. In addition, the color of the precipitates became dark yellow as the Nb/(P + Nb) molar ratio increased, while the annealed precipitates of NbTCP/HAp were white.

SEM observation of the precipitates before annealing revealed that all precipitates were present as aggregates composed of primary particles of less than 1  $\mu$ m in diameter, irrespective of the Nb/(P + Nb) molar ratio. Figure 3a shows SEM-EDX spectra of NbTCP/HAp-II. The EDX spectrum of Nb M $\alpha$  was separated from the P K $\alpha$  line and could be observed at 2.17 KeV, although its intensity was weak. The mapping images of the P-K $\alpha$ , Ca-K $\alpha$ , and Nb-M $\alpha$  lines are shown in Fig. 3b. As shown in Fig. 3b, Nb ions were present at the same site as the Ca and P ions, suggesting that the Nb ions were homogeneously distributed in the aggregates.

The proliferation and ALP activity of NHOst cultured on various kinds of NbTCP/HAp pellets is shown in Fig. 4. The proliferation of NHOst cultured on NbTCP/HAp-II pellets was approximately 60% higher than that on NbTCP/HAp-0 without Nb ions ( $P < 0.01$ ). As shown in Fig. 5, many

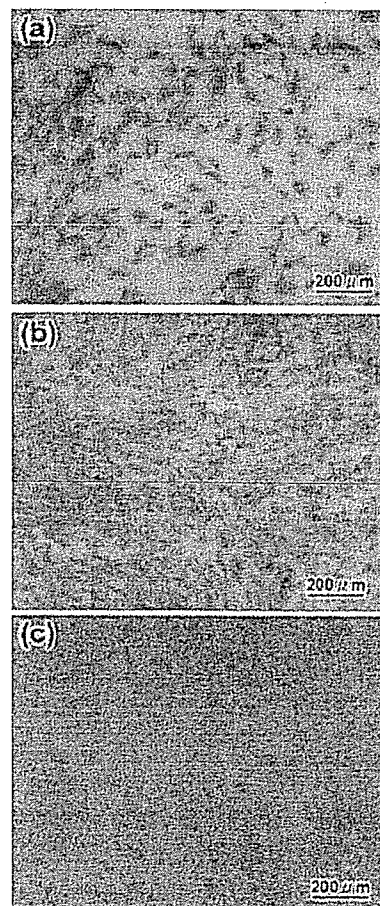


Fig. 5. Light microscopic images of NHOst cultured on various NbTCP/HAp samples for 7 days: a, NbTCP/HAp-0; b, NbTCP/HAp-I; and c, NbTCP/HAp-II. NHOst were stained by Giemsa solution