では、A型からZ型にらせん構造が変化する。また、RNA（poly(rG-C)$_6$）においても、B型からZ型に構造変化する。さらに、核酸の機能変化については、リボザイム（RNA）への圧力印加により、リボザイムが構造変化し、その結果、RNA切断活性が低下することが報告されている。以上のように、核酸の構造と機能には密接な関係があり、本研究では、DNAの圧力印加による構造変化と機能の関係について詳細に検討した。その結果、構造の変化に誘起されるDNAの凝縮が示された。

以上のことから本年度は、（1）水素結合性高分子／DNA複合体、（2）ナノ無機粒子／DNA複合体、（3）高圧凝縮DNAを用いたマウスへのin vivo遺伝子導入について検討した。

さらに、PVAハイドロゲルの調製については、その調製方法により異なる物性のハイドロゲルが得られることが知られている。具体的には、PVAは、濃厚水溶液を室温静置することでゲル化するが、不透明、低含水率、脆弱なゲルである。これまで、透明、高含水率、高強度のPVAゲルを調製するため、種々の化学的あるいは物理的架橋法が検討されてきた。グルタルアルデヒド、紫外線照射等による化学的架橋では、透明かつ高強度のゲルが得られるが、含水率は低い。一方、凍結－乾燥および反復凍結－融解法などの物理的架橋法によるPVAゲルは、高含水率、高強度であるが、不透明である。また、反復凍結－融解法では、ジメチルスルホキシド（DMSO）と水の混合溶媒を用いることにより、透明、高含水率、高強度のPVAゲルが得られる。ここでは、得られるPVAゲルの物性はDMSO／水の混合比により大きく変化し、PVAゲルの形成過程における相分離および結晶化の違いがPVAゲルの物性に強く影響することを示唆している。また、PVA水溶液への超高静水圧印加によるゲル形成では、得られたゲルが不透明であり、反復凍結－融解法に比して高含水率、同程度の強度であった。このことから、超高静水圧誘起PVAゲルの諸物性の制御を目的に、種々の混合比のDMSO／水混合溶媒を用いた異なる高静水圧印加条件でのPVAゲルの調製に関して検討を行った。

B．研究方法

（1）水素結合性高分子／DNA複合体を用いたマウスへのin vivo遺伝子導入

水素結合性高分子としてPVA（重合度1700）を用い、遺伝子としてはルシフェラーゼ遺伝子をコードするプラスミドDNA（pLuc）を用いた。PVA水溶液を調製し、終濃度0.001%となるようにDNA溶液（終濃度：25ng/μl）と混合した。超高圧印加処理（10000気圧、15分間、40℃）を施した。PVA/DNA複合体溶液200μlを生理的食塩水1.4～1.8mlと混合し、マウス（20～30g）の尾静脈より5～6秒間で投与した（ハイドロダイナミックス）。24時間後の肝臓でのルシフェラーゼ活性をルシフェラーゼ活性測定キットにて測定した。

（2）ナノ無機粒子／水素結合性高分子／DNA複合体を用いたマウスへのin vivo遺伝子導入

ナノ無機粒子としては、ハイドロキシアパタイト（HAp）と炭酸アパタイト（cHAp）を用い、水素結合性高分子としてPVA（重合度1700）を用い、遺伝子としてはルシフェラーゼ遺伝子をコードするプラスミドDNA（pLuc）を用いた。PVA水溶液（終濃度：0.001%）とナノ無機粒子（終濃度：0.001%）を混合し、超音波処理を5分間行った。その後、DNA溶液（終濃度：25ng/μl）と混合した。超高圧印加処理（10000気圧、15分間、40℃）を施した。ナノ無機粒子/PVA/DNA複合体溶液200μlを生理的食塩水1.4～1.8mlと混合し、マウス（20～30g）の尾静脈より5～6秒間で投与した（ハイドロダイナミックス）。12, 24, 48, 72時間後の肝臓でのルシフェラーゼ活性をルシフェラーゼ活性測定キットにて測定した。

（3）高圧凝縮DNAを用いたマウスへのin vivo遺伝子導入

遺伝子としてはルシフェラーゼ遺伝子をコードするプラスミドDNA（pLuc）を用いた。25ng/μlの

DNA溶液を調製し、超高圧印加処理（10000気圧、15分間、40℃）を施した。DNA溶液200μlを生理的食塩水1.4～1.8mlと混合し、マウス（20～30g）の尾静脈より5～6秒間で投与した（ハイドロダイナミックス）。12, 24, 48, 72時間後の肝臓でのルシフェラーゼ活性をルシフェラーゼ活性測定キットにて測定した。

また、高圧凝縮DNAの基礎的物性を検討するため、AFM観察および一本鎖DNA特異的切断酵素試験を行った。超高圧印加処理（10,000気圧、15分間、40℃）したプラスミドDNAをマイカ上に滴下し、所定時間静置した後に、エアーフラッシュし、AFM観察を行った。一本鎖DNA特異的切断酵素試験は、超高圧印加処理したプラスミドDNAに一本鎖DNA特異的切断酵素であるS1ヌクラアーゼを種々の単位で添加し、所定時間反応させた後、アガロースゲル電気泳動を行った。

（4）超高圧誘起PVAハイドロゲル

重合度1,700、鹸化度99.8% のPVA-HC((株)クラレ) を使用した。DMSOと水の体積比がDSMO/水=0/100, 20/80, 60/40, 80/20, 100/0の混合溶媒を調製した後、オートクレイブを用いて121℃、20分、3回加熱溶解し、10%(w/v) PVA/DMSO/水混合溶媒を調製した。PVAゲルは、高圧法と反復凍結-融解法により調製した。

超高圧装置(Dr.CHEF;(株)神戸製鋼所)を用いて種々の条件下(1~10,000気圧、10~40℃、10分)でPVA溶液への高圧処理を行った。

PVA溶液を-20℃で24時間凍結後、室温下30分の融解と-20℃、2時間の凍結を1サイクルとし、これを5回繰り返した。

24時間水中に浸漬後、凍結乾燥したPVAゲルを100mlの水中に24時間浸漬させた。時間毎にゲルの重量を測定した。膨潤度Qは次式より算出した。

$$Q=\frac{W_2-W_1}{W_1}$$

W1:凍結乾燥後のゲルの重量

W2:水に浸漬後のゲルの重量

クリープメーター(YAMADEN Co., Ltd)を用いて圧縮試験を行なった。1つのゲルにつき3ヶ所で圧縮試験を行ない、応力-歪曲線の近似曲線の傾きから初期弾性率を算出した。

C．研究結果

（1）水素結合性高分子／DNA複合体を用いたマウスへのin vivo遺伝子導入

ハイドロダイナミックス法は、肝臓特異的に遺伝子発現を示すことが知られており、遺伝子ベクターの遺伝子導入能を検討するのに適した方法である。図1には、ハイドロダイナミックス法にてin vivo遺伝子導入を行ったマウスの肝臓でのルシフェラーゼ活性（24時間後）を示す。未処理DNA単独の場合、約3×10^6/mg proteinのルシフェラーゼ活性を示した。一方のPVA/DNA複合体では、未処理DNA単独に比して約1/4にルシフェラーゼ活性が減少した。

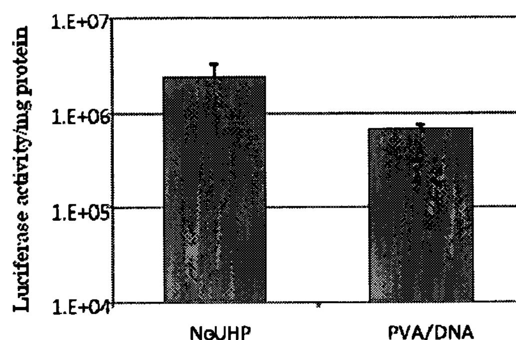

図1．ルシフェラーゼ活性（24時間後）

（2）ナノ無機粒子／水素結合性高分子／DNA複合体を用いたマウスへのin vivo遺伝子導入

図2には、炭酸アパタイト/水素結合性高分子／DNA複合体、ハイドロキシアパタイト/水素結合性高分子／DNA複合体を用いたハイドロダイナミックス法によるin vivo遺伝子導入後のマウスの肝臓でのルシフェラーゼ活性を示す。炭酸アパタイト/PVA/DNA複合体、ハイドロキシアパタイト/PVA/DNA複合体ともに未処理DNA単独に比して約10倍のルシフェラーゼ活性の増加が示された。これは、上述のPVA/DNA複合体では示されなかった結果であり、ナノ無機粒子の含有効果と言える。

一方、PVA濃度の高い場合は若干のルシフェラーゼ活性の減少が示された。

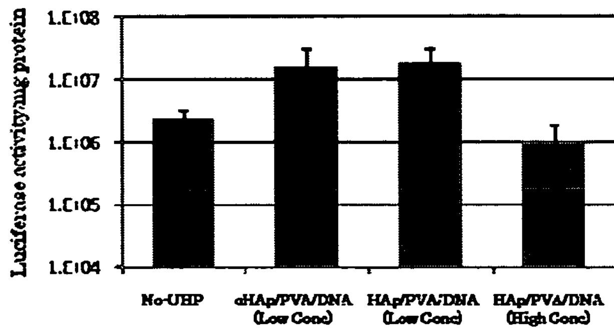

図２．ルシフェラーゼ活性（24時間後）

次に、遺伝子発現の経時変化について、未処理DNA単独とハイドロキシアパタイト/PVA/DNA複合体を用いて検討した（図３）。未処理のDNA単独の場合、投与後12時間後に遺伝子発現が最大となり、その後経時的に減少した。一方、ハイドロキシアパタイト/PVA/DNA複合体では、24時間後に最大の遺伝子発現を示し、その後は急激に減少した。

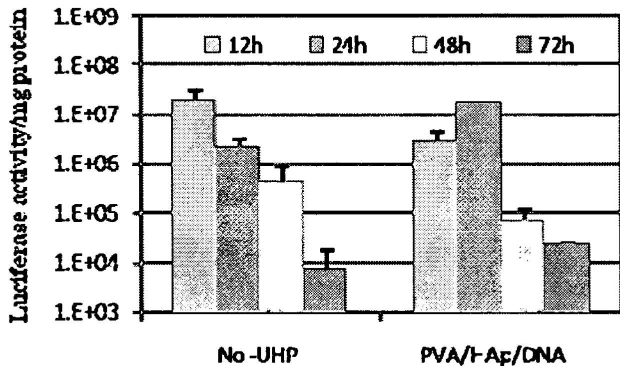

図３．ルシフェラーゼ活性（経時変化）

（３）高圧凝縮DNAを用いたマウスへのin vivo遺伝子導入

図４には、10000気圧、15分間、40℃の超高圧印加処理にて得られた高圧凝縮DNAを用いたマウスへのin vivo遺伝子導入におけるルシフェラーゼ活性の経時変化を示す。コントロールとしては、未処理DNA単独を用いた。未処理のDNA単独の場合、投与後12時間後に遺伝子発現が最大となり、その後経時的に減少した。一方、高圧圧縮DNAでは、12時間後では、未処理DNA単独に比して低いルシフェラーゼ活性であったが、24時間後には同程度の活性に増加し、さらに、48時間後には約10倍以上のルシフェラーゼを示した。

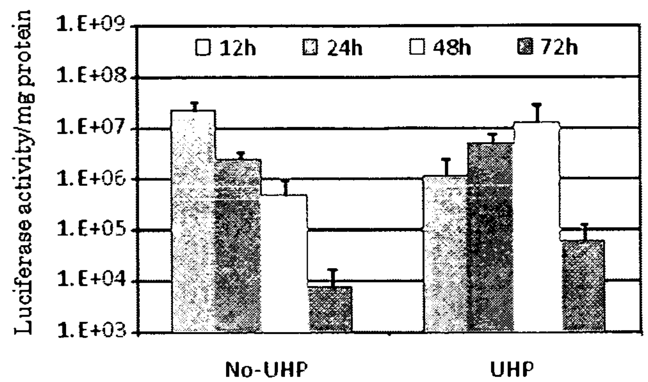

図４．ルシフェラーゼ活性（経時変化）

高圧凝縮DNAの基礎的物性について、AFM観察、一本鎖DNA特異的切断酵素試験を行った（図5,6）。AFM観察では、超高圧未処理のプラスミドDNAは環状およびスーパーコイル状のプラスミドDNAが観察された。一方の高圧凝縮DNAでは、スーパーコイル型プラスミドDNAとスーパーコイル型プラスミドDNAがさらに巻かれた凝縮構造をとるプラスミドDNAが観察された。スーパーコイル型プラスミドDNAにおいても部分的に球形が観察されていることから、高圧印加によりヘリシティーが増加し、徐々に部分的に巻かれていったと考えられる。一本鎖DNA特異的切断酵素試験においては、超高圧未処理のプラスミドDNA　ではS1ヌクレアーゼの添加量の増加に伴い、若干の切断が示された。一方の高圧凝縮DNAでは、S1ヌクレアーゼの添加量の増加に伴い、顕著な切断が認められ、100単位添加において切断されたDNAがスメアーなバンドが示され、さらに、100単位添加においては、オリジナルのバンドはほぼ消失し、スメアーなバンドだけとなった。

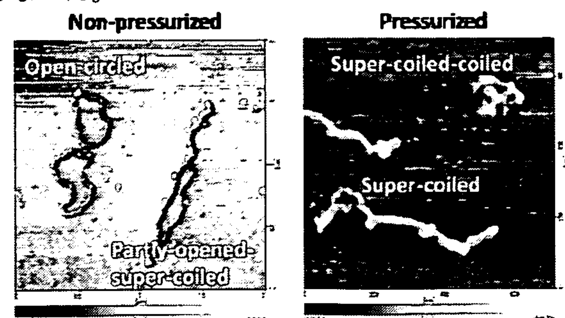

図５．高圧凝縮プラスミドDNAのAFM観察

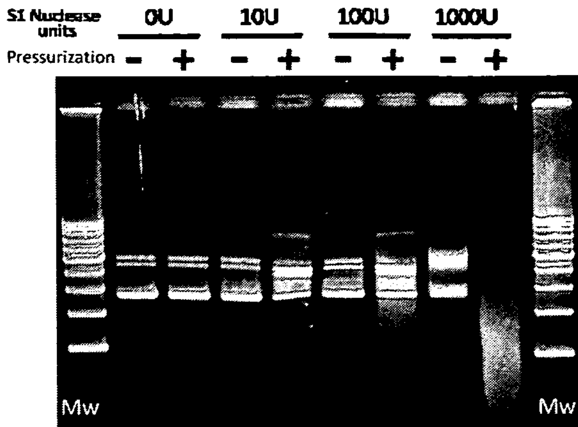

図６．一本鎖DNA特異的切断酵素試験

（４）超高圧誘起PVAハイドロゲル

種々のDMSOと水の混合比で調製したPVA/DMSO/水混合溶媒を種々の印加圧力および温度で高圧処理した。図7には、高圧処理後（10,000気圧, 10℃, 10分）の結果を示す。PVA水溶液では、温度に関係なく10,000気圧、10分間の圧力印加により不透明なゲルが得られた。これは超高圧下における脱水和によりPVA間で水素結合が形成されたためと考えられる。一方、PVA/DMSO/水混合溶液では、高圧、低温下においてゲル形成し易く、10,000気圧、10℃では透明性を有した成形性の良いゲルが得られた。中でも60/40の混合比にて最もゲルを形成し易かった。高分子溶液のゲル化は、相分離とゲル化の兼ね合いによりその物性が異なることが知られている。高温下ではまず相分離が誘起され、その後に濃厚相部分で架橋が生じるため不透明なゲルとなる。低温下ではまずゲル化が誘起され、その後に相分離が生じるため透明性を有したゲルとなる。高圧法ゲルは透明性を有していたことから、相分離よりもゲル化が誘起される傾向があることが示唆された。さらに、DMSOと水は1:2で水素結合を形成すると考えられている。PVAは水と水素結合を形成し溶解する。一方DMSOと水の混合溶媒では、DMSOと水の水素結合が生じるため溶解性が低下していく。混合比が60/40では、DMSOと水がほぼ完全に1:2で結合するため、PVAとの水素結合の形成が困難となりPVAに対して貧溶媒となる。このため60/40では相分離が誘起されやすしかし高圧法により得られたゲルは半透明であったことから、相分離とゲル化が同時に誘起されていると考えられる。さらにDMSOの割合が多くなると、PVAとDMSOの水素結合によりPVAは溶解する。よって80/20では高圧印加により脱溶媒和が生じるが、DMSOがPVA間の結合を阻害するため微結晶の成長が抑制され、透明性を有したゲルが得られたと考えられる。

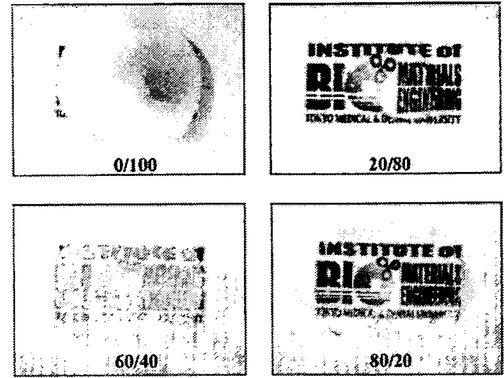

図７．超高圧誘起PVAハイドロゲル

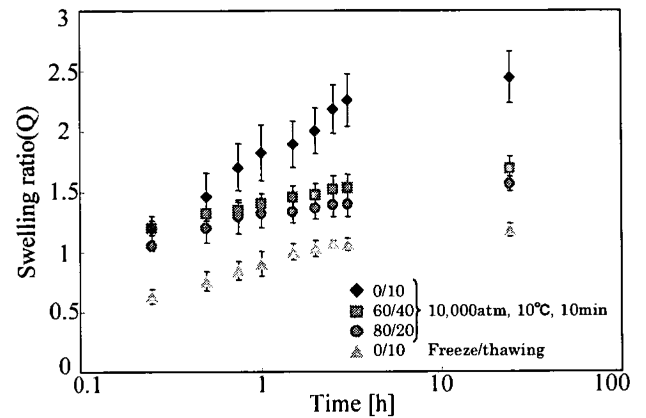

図８．超高圧誘起PVAハイドロゲルの膨潤度

く、PVA濃厚部分でゲル化が生じたと考えられる。

高圧法および一般的なPVAゲルの調製法として知られる凍結融解法により調製したPVAゲルの膨潤度測定した（図8）。PVA水溶液で調製したゲルは共に白濁したゲルとなるが、高圧法ゲルは反復凍結-融解法に比べ高い膨潤度を示した。これは高圧法ゲルが反復凍結-融解法に比べ内部空間が広い構造を有しており、さらに未架橋PVAの水和によるものと考えられる。60/40および80/20においても高圧法ゲルの方が高い膨潤度を示した。60/40では相分離が生じているため、水に浸漬した際に希

薄相中の未架橋PVAの溶出により空間が開いたため膨潤度が高くなったと考えられる。80/20では0/100同様、未架橋部分の水和によるものと考えられる。

高圧法と反復凍結-融解法により調製したPVAゲルの力学特性を比較するため圧縮試験を行なった。種々の条件により調製したゲルの応力-歪曲線より弾性率を算出した（図9）。各混合比による高圧法ゲルは反復凍結-融解法に比べて低い弾性率であったことから、柔軟性を有していることが示唆された。60/40で調製したゲルは80/20で調製したゲルに比べ高い弾性率を示した。60/40は濃厚相部分で架橋しているため強固になり、弾性率が高くなったと考えられる。一方、80/20は微結晶の架橋点が少なく、結晶のサイズも小さいため弱くなり弾性率が低くなったと考えられる。また、水に7日間浸漬後の高圧法ゲルの弾性率に差は見られなかったことから、DMSOを水に置換しても弾性率への影響は少ないことが示された。

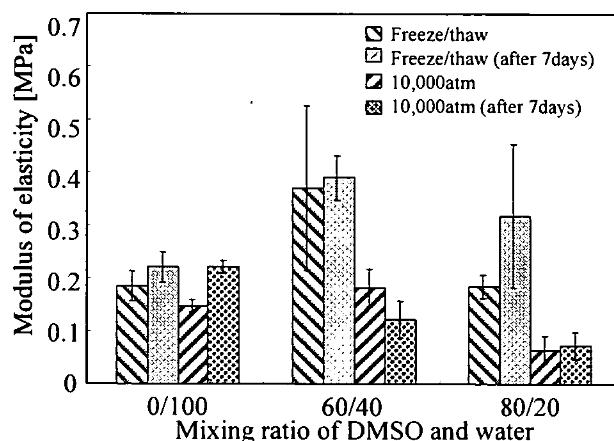

図9．超高圧誘起PVAハイドロゲルの弾性率

D．考察

超高圧印加処理にて得られた（1）PVA/DNA複合体、（2）ナノ無機粒子／PVA／DNA複合体、（3）高圧凝縮DNAを用いたハイドロダイナミックス法によるマウスへのin vivo遺伝子導入を行った。

（1）PVA/DNA複合体においては、これまでに培養細胞を用いた遺伝子導入にて効率良い細胞内導入が示されているものの、遺伝子発現は低いことが明らかとなっており、また、細胞内での遺伝子の被転写・翻訳モデルである無細胞系転写・翻訳においても、PVA/DNA複合体の低い転写・翻訳が示されている。これらの結果は、細胞内に導入されたPVA/DNA複合体の低い被転写・翻訳効率を示しており、図1に示されたin vivo遺伝子導入におけるPVA/DNA複合体の低い遺伝子発現の原因の一つと考えられる。

（2）ナノ無機粒子／PVA／DNA複合体においては、昨年度の培養細胞への遺伝子導入にてPVA/DNA複合体に比して高い遺伝子発現を示しており、今回のin vivo遺伝子導入でも高い遺伝子発現を示し、ナノ無機粒子の含有効果と言える。一方、培養細胞系においては、ハイドロキシアパタイトに比して炭酸アパタイトの細胞内導入促進が示されていたが、今回のin vivo遺伝子導入ではその違いが示されなかった。これについては、今回のハイドロダイナミックス法による遺伝子導入が細胞内への遺伝子送達をある程度強制的に行える手法であるため、培養細胞での遺伝子導入過程のエンドサイトーシスを経由していないためと考えられる。すなわち、ハイドロダイナミックス法においてはナノ無機粒子の含有がエンドサイトーシスからの遊離促進効果以外の何らかのメカニズムに作用しているものと考えられる。これについては今後詳細な検討を必要とする。

また、高濃度PVAのナノ無機粒子／PVA／DNA複合体では遺伝子発現の減少が示された。これについては、PVA濃度の低いナノ無機粒子/PVA/DNA複合体では粒子径が約600nmであり、PVA濃度が高いナノ無機粒子/PVA/DNA複合体では約1000nmであり、これらの粒子径の違いが影響したものと考えられる。

（3）高圧凝縮DNAにおいては、経時的な遺伝子発現の増加が示された。未処理DNA単独の場合は、遺伝子発現の経時的減少が示されており、細胞内での分解による減少と考えられる。一方の高圧凝縮DNAは、凝縮による核酸分解酵素の耐性が示されていることから遺伝子発現の安定化あるい

は、凝縮からの巻き戻りが考えられる。現在においては、初期のルシフェラーゼ活性が未処理DNA単独に比して低いことから、後者のDNAの巻き戻りによる遺伝子発現増加と考えている。

E．結論

本年度は、超高圧印加処理にて得られた（1）PVA/DNA複合体、（2）ナノ無機粒子／PVA／DNA複合体、（3）高圧凝縮DNAを用いたハイドロダイナミックス法によるマウスへのin vivo遺伝子導入を行った。（1）（2）については、昨年度の培養細胞系でのPVA/DNA複合体に比したナノ無機粒子／PVA／DNA複合体の遺伝子導入効率の増加が示されたと同様に、in vivo遺伝子導入においてもPVA/DNA複合体に比したナノ無機粒子／PVA／DNA複合体の遺伝子導入効率の増加が示された。これらの結果は、本研究ストラテジーであるナノ無機粒子効果（エンドソームでのナノ無機粒子の溶解によるエンドソームからの複合体の細胞内導入促進）を強く示している。また、（3）については、超高圧処理により誘起されるDNA構造変化（凝縮）が遺伝子発現の機能向上（遺伝子発現の持続）を示した。これは、全くの新しい知見であり、核酸構造科学の視点から意義深い知見と考えられる。また、遺伝子発現の持続は、現在の遺伝子治療臨床研究におけるNaked plasmid DNA法への応用においても有効でると考えられ、さらに、高圧印加のみの他の物質を利用しない点は、効率的な実用的開発が期待できる。加えて、現在の遺伝子キャリアーの主流である正電荷物質では、静電的相互作用による強度の凝縮が遺伝子発現を抑制していることが報告されており、これらの遺伝子キャリアー開発においても高圧凝縮DNAの応用が可能であると考えられる。具体的には、高圧凝縮DNAの利用では、同程度の凝縮度を得るにあたり正電荷物質の添加量を低減させることが出来ると考えられ、これは、正電荷物質に由来する細胞傷害性を低減でき、巻き戻りの効果（遺伝子発現の遅延）を付与できることから効率的に遺伝子導入することが可能と考えられる。

F．研究発表

1．論文発表

1). Tsuyoshi Kimura, Sayaka Iwai, Toshiya Moritan, Kwangwoo Nam, Shingo Mutsuo, Hidekazu Yoshizawa, Masahiro Okada, Tsutomu Furuzono, Toshiya Fujisato, Akio Kishida, Preparation of PVA/DNA hydrogels via hydrogen bonds by ultra high pressure treatment and controlled release of DNA from hydrogels for gene delivery, Journal of Artificial Organs, 10, 104-108, 2007

2). Kwangwoo Nam, Tsuyoshi Kimura, Akio Kishida, Physical and biological properties of collagen-phospholipid polymer hybrid gels, Biomaterials, 28, 3153-3162, 2007

3). Tsuyoshi Kimura, Seiichi Funamoto, Akio Kishida. Gene transfection on the tissue engineered bone decellularized by ultra high hydrostatic pressurization, Controlled Release Society Newsletter, 24(2), 10-11, 2007

4). Tsuyoshi Kimura, Kana Horiuchi, Kimio Kurita, Tsutomu Ono, Hidekazu Yoshizawa, Toshiya Fujisato, Akio Kishida, Characteristics of compacted plasmid DNA by high pressurization, Nucleic Acids Symposium Series, No51, 343-344, 2007

5). Shingo Mutsuo, Kazuya Yamamoto, Tsutomu Furuzono, Tsuyoshi Kimura, Tsutomu Ono, Akio Kishida, Pressure-induced Molecular Assembly of Hydrogen-bonded Polymers, Journal of Polymer Science Part B, Polymer Physics, 46, 743-750, 2008

6). Kwangwoo Nam, Tsuyoshi Kimura, Akio Kishida, Controlling Coupling Reaction of EDC and NHS for Preparation of Collagen Gels Using

Ethanol/Water Co-Solvents, Macromolecular Bioscience, 8, 32-37, 2008

7). Tsuyoshi Kimura, Kana Horiuchi, Kimio Kurita, Kwangwoo Nam, Tsutomu Ono, Hidekazu Yoshizawa, Tsutomu Furuzono, Toshiya Fujisato, Akio Kishida, Preparation and characterization of the condensed plasmid DNA by high hydrostatic pressurization, in preparation

２．学会発表

8). 木村剛、吉澤秀和、古薗勉、藤里俊哉、岸田晶夫、高圧凝縮ＤＮＡの基礎的検討と遺伝子デリバリーへの応用、遺伝子・デリバリー研究会　第７回シンポジウム要旨集、32、2007

9). 木村剛、三浦義之、栗田公夫、六雄伸悟、吉澤秀和、藤里俊哉、岸田晶夫、高圧印加による PEG/多糖水性二相形成の促進、Polymer Preprints, Japan, Vol.56, No.1, 1926 2007

10). 三浦義之、木村剛、栗田公夫、六雄伸悟、吉澤秀和、藤里俊哉、小林尚俊、岸田晶夫 超高圧誘起PVAハイドロゲルの調製と物性に関する検討、Polymer Preprints, Japan Vol.56, No.1, 1861, 2007

11). 木村剛、堀内可奈、栗田公夫、三浦義之、吉澤秀和、岸田晶夫、高圧凝縮 DNA の調製と機能解析、Polymer Preprints, Japan Vol.56, No.1, 1952, 2007

12). Tsuyoshi Kimura, Kwangoo Nam, Tsutomu Ono, Hidekazu Yoshizawa, Toshiya Fujisato, Akio Kishida, Assembling of hydrogen-bonding-polymers using high pressure technology, Proceedings of The International Symposium Organized by Institute of Chemical Research (ICR) Kyoto University, 107-108, 2007

13). 木村剛、吉澤秀和、古薗勉、藤里俊哉、岸田晶夫、pH 応答性ナノ無機粒子を含有する水素結合性ポリマー／DNA 複合体の細胞内送達、Drug Delivery System, 22-23, 363, 2007

14). 木村剛、三浦義之、栗田公夫、六雄伸悟、吉澤秀和、藤里俊哉、岸田晶夫、超高圧誘起ナノ高分子集合体のＤＤＳへの応用、Fiber Preprints, Japan, 62, No2,(symposia), 14, 62(2), 14, 2007

15). Tsuyoshi Kimura, Masahiro Okada, Tsutomu Furuzono, Hidekazu Yoshizawa, Toshiya Fujisato, Akio Kishida, Cellular delivery of DNA-polymer complex encupsulating inorganic nanoparticles prepared by ultra high pressurization, Tissue Engineering, 13, 1746-47, 2007

16). 木村剛、三浦義之、栗田公夫、小野努、吉澤秀和、藤里俊哉、岸田晶夫、高圧技術を用いたプラスミド DNA の凝縮操作、Polymer Preprints, Japan, 56(2), 5323-24, 2007

17). 三浦義之、木村剛、栗田公夫、小野努、吉澤秀和、藤里俊哉、岸田晶夫、高静水圧誘起ＰＶＡゲルの特性解析、Polymer Preprints, Japan, 56(2), 3832, 2007

18). 木村剛、南広祐、小野努、吉澤秀和、古薗勉、藤里俊哉、岸田晶夫、細胞への遺伝子送達における高圧技術応用、第５回生活支援工学系学会連合大会講演予稿集、102, 2007

19). 木村剛、南広祐、小野努、吉澤秀和、古薗勉、藤里俊哉、岸田晶夫、遺伝子送達における高技術の応用、第２９回日本バイオマテリアル学会大会予稿集、418, 2007

20). Tsuyoshi Kimura, Kana Horiuchi, Kimio Kurita, Tsutomu Ono, Hidekazu Yoshizawa, Toshiya

Fujisato, Akio Kishida, DNA condensation using hydrostatic pressurization for gene delivery, 1st Asiam Biomaterials Congress Abstract, 302, 2007

21). 木村剛、岸田晶夫、小野努、吉澤秀和、古薗勉、藤里俊哉、圧縮 DNA の遺伝子送達への応用、第 2 回バイオ・ナノテクフォーラム講演要旨集、A-06, 2008

G．知的財産権の出願・登録状況

1．特許取得

特願 2007-263704、岸田晶夫、木村剛、南広祐、藤里俊哉、「機能性 DNA の製造方法、形質転換体、及び疾患治療剤」、2007 年 10 月 9 日

2．実用新案登録

なし

3．その他

なし

別添５

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
1) Tsuyoshi Kimura, Sayaka Iwai, Toshiya Moritan, Kwangwoo Nam, Shingo Mutsuo, Hidekazu Yoshizawa, Masahiro Okada, Tsutomu Furuzono, Toshiya Fujisato, Akio Kishida	Preparation of PVA/DNA hydrogels via hydrogen bonds by ultra high pressure treatment and controlled release of DNA from hydrogels for gene delivery	Journal of Artificial Organs	10	104-108	2007
2) Kwangwoo Nam, Tsuyoshi Kimura, Akio Kishida	Physical and biological properties of collagen-phospholipid polymer hybrid gels	Biomaterials	28	3153-3162	2007
3) Tsuyoshi Kimura, Seiichi Funamoto, Akio Kishida	Gene transfection on the tissue engineered bone decellularized by ultra high hydrostatic pressurization.	Controlled Release Society Newsletter	24(2)	10-11	2007
4) Tsuyoshi Kimura., Kana Horiuchi, Kimio Kurita, Tsutomu Ono, Hidekazu Yoshizawa, Toshiya Fujisato, Akio Kishida	Characteristics of compacted plasmid DNA by high pressurization	Nucleic Acids Symposium Series	No51	343-344	2007
5) Shingo Mutsuo, Kazuya Yamamoto, Tsutomu Furuzono, Tsuyoshi Kimura, Tsutomu Ono, Akio Kishida	Pressure-induced Molecular Assembly of Hydrogen-bonded Polymers	J. Poly. Sci. Part B, Poly. Phyis.	46	743-750	2008
6) Kwangwoo Nam, Tsuyoshi Kimura, Akio Kishida	Controlling Coupling Reaction of EDC and NHS for Preparation of Collagen Gels Using Ethanol/Water Co-Solvents	Macromolecular Bioscience	8	32-37	2008
7) Tsuyoshi Kimura, Kana Horiuchi, Kimio Kurita, Kwangwoo Nam, Tsutomu Ono, Hidekazu Yoshizawa, Tsutomu Furuzono, Toshiya Fujisato, Akio Kishida	Preparation and characterization of the condensed plasmid DNA by high hydrostatic pressurization				in preparation
8) 木村剛、吉澤秀和、古薗勉、藤里俊哉、岸田晶夫	高圧凝縮ＤＮＡの基礎的検討と遺伝子デリバリーへの応用	遺伝子・デリバリー研究会　第７回シンポジウム要旨集		32	2007

9) 木村剛、三浦義之、栗田公夫、六雄伸悟、吉澤秀和、藤里俊哉、岸田晶夫	高圧印加による PEG/多糖水性二相形成の促進	Polymer Preprints, Japan	Vol.56, No.1,	1926	2007
10) 三浦義之、木村剛、栗田公夫、六雄伸悟、吉澤秀和、藤里俊哉、小林尚俊、岸田晶夫	超高圧誘起 PVA ハイドロゲルの調製と物性に関する検討	Polymer Preprints, Japan	Vol.56, No.1,	1861	2007
11) 木村剛、堀内可奈、栗田公夫、三浦義之、吉澤秀和、岸田晶夫	高圧凝縮 DNA の調製と機能解析	Polymer Preprints, Japan	Vol.56, No.1,	1952	2007
12) Tsuyoshi Kimura, Kwangwoo Nam, Tsutomu Ono, Hidekazu Yoshizawa, Toshiya Fujisato, Akio Kishida	Assembling of hydrogen-bonding-polymers using high pressure technology	Proceedings of The International Symposium Organized by Institute of Chemical Research (ICR) Kyoto University		107-108	2007
13) 木村剛、吉澤秀和、古薗勉、藤里俊哉、岸田晶夫	pH 応答性ナノ無機粒子を含有する水素結合性ポリマー／DNA 複合体の細胞内送達	Drug Delivery System	22-23	363	2007
14) 木村剛、三浦義之、栗田公夫、六雄伸悟、吉澤秀和、藤里俊哉、岸田晶夫	超高圧誘起ナノ高分子集合体のＤＤＳへの応用	Fiber Preprints, Japan 62, No2,(symposia) 14	62(2)	14	2007
15) Tsuyoshi Kimura, Masahiro Okada, Tsutomu Furuzono, Hidekazu Yoshizawa, Toshiya Fujisato, Akio Kishida	Cellular delivery of DNA-polymer complex encupsulating inorganic nanoparticles prepared by ultra high pressurization	Tissue Eng;	13	1746-47	2007
16) 木村剛、三浦義之、栗田公夫、小野努、吉澤秀和、藤里俊哉、岸田晶夫	高圧技術を用いたプラスミド DNA の凝縮操作	Polymer Preprints, Japan	56(2)	5323-24	2007
17) 三浦義之、木村剛、栗田公夫、小野努、吉澤秀和、藤里俊哉、岸田晶夫	高静水圧誘起ＰＶＡゲルの特性解析	Polymer Preprints, Japan	56(2)	3832	2007

18) 木村剛、南広祐、小野努、吉澤秀和、古薗勉、藤里俊哉、岸田晶夫	細胞への遺伝子送達における高圧技術応用	第５回生活支援工学系学会連合大会講演予稿集		102	2007
19) 木村剛、南広祐、小野努、吉澤秀和、古薗勉、藤里俊哉、岸田晶夫	遺伝子送達における高圧技術の応用	第２９回日本バイオマテリアル学会大会予稿集		418	2007
20) Tsuyoshi Kimura, Kana Horiuchi, Kimio Kurita, Tsutomu Ono, Hidekazu Yoshizawa, Tsutomu Furuzono, Kwangwoo Nam, Toshiya Fujisato, Akio Kishida	DNA condensation using DNA using high hydrostatic pressurization for gene delivery	1st Asiam Biomaterials Congress Abstract		302	2007
21)木村剛、岸田晶夫、小野努、吉澤秀和、古薗勉、藤里俊哉	圧縮 DNA の遺伝子送達への応用	第２回バイオ・ナノテクフォーラム講演要旨集		A-06	2008

J Artif Organs (2007) 10:104–108
DOI 10.1007/s10047-006-0367-7

© The Japanese Society for Artificial Organs 2007

ORIGINAL ARTICLE

Tsuyoshi Kimura, PhD · Sayaka Iwai
Toshiyuki Moritan, PhD · Kwangwoo Nam, PhD
Shingo Mutsuo · Hidekazu Yoshizawa, PhD
Masahiro Okada, PhD · Tsutomu Furuzono, PhD
Tosihya Fujisato, PhD · Akio Kishida, PhD

Preparation of poly(vinyl alcohol)/DNA hydrogels via hydrogen bonds formed on ultra-high pressurization and controlled release of DNA from the hydrogels for gene delivery

Abstract Poly(vinyl alcohol) (PVA) hydrogels interacting with DNA mediated by hydrogen bonds (PVA/DNA hydrogel) were developed using ultra-high pressure (UHP) technology. The goal was to create a new method of gene delivery by controlled release of DNA. Mixed solutions of DNA and PVA at various concentrations were pressurized at 10000 atmospheres at 37°C for 10min. PVA/DNA hydrogels with good formability were produced at PVA concentrations of more than 5% w/v. The presence of DNA in the obtained hydrogels was confirmed by spectroscopic analysis and nucleic acid dye staining. DNA release from the hydrogels was investigated using PVA/DNA hydrogel samples of 5% and 10% w/v formed by UHP treatment or by conventional freeze–thaw methods. The DNA release curves from both types of samples showed a rapid phase in the initial 15h followed by a sustained release phase. However, there was a difference in the amount of DNA released. Less DNA was released by the pressurized hydrogels than by the freeze–thaw hydrogels. Also, the cumulative amount of DNA released decreased as the PVA content in the hydrogels increased. These results indicate that DNA release from the hydrogels can be modulated by changing the preparation method and the PVA content. Furthermore, it was demonstrated that DNA release could be controlled by varying the amount and duration of pressurizing used to form the hydrogels. Intact fractions of plasmid DNA released from the hydrogels were separated by agarose gel electrophoretic analysis. These results suggest that, using controlled release, DNA from PVA/DNA hydrogels formed by UHP treatment can be transfected into cells.

Key words Controlled release · Ultra-high pressure · DNA · Hydrogel · Poly(vinyl alcohol)

Received: March 31, 2006 / Accepted: November 18, 2006

T. Kimura · K. Nam · A. Kishida (✉)
Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan
Tel. and Fax +81-3-5280-8028
e-mail: kishida.fm@tmd.ac.jp

S. Iwai · T. Moritan
Department of Medical Engineering, Suzuka University of Medical Science, Suzuka, Japan

S. Mutsuo · H. Yoshizawa
Department of Environmental Chemistry and Materials, Okayama University, Okayama, Japan

M. Okada · T. Furuzono
Department of Biomedical Engineering, National Cardiovascular Center Research Institute, Osaka, Japan

T. Fujisato
Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, Osaka, Japan

Introduction

Safe and biocompatible synthetic materials have been developed as biomaterials.[1] In gene therapy, nonviral synthetic gene carriers have been the focus of attention due to their biological safety advantages over viruses.[2] In many cases, cationic synthetic materials, such as cationic lipids, liposomes,[3] polyethyleneimine,[4] polyamideamine dendrimer,[5] poly-L-lysine (PLL), PLL derivatives,[6] and other cationic peptides,[7] have been used as nonviral vectors. It is possible to form complexes between these materials and DNA using the electrostatic interaction between their cationic groups and the anionic groups of DNA, making the DNA robust against nuclease degradation and enabling effective transfection into mammalian cells.[8,9] However, the cytotoxicity of cationic materials was reported to be a significant problem.[10,11] For safer and more efficient gene delivery, it is necessary to develop a noncationic or less cationic gene carrier through nonelectrostatic interaction with DNA. Sakurai et al. reported that a triple helical complex of single-strand DNA and double-strand schizophyllan, which is a kind of polysaccharide (β-1,3 glucan), was formed through hydrogen bonding.[12] In addition, we previously reported that nanoparticles of poly(vinyl alcohol) (PVA) bonded to DNA via hydrogen bonds were obtained when mixed solutions of PVA (less than 0.01% w/v) and DNA were treated under ultra-high pressure (UHP) at

10000 atmospheres (980 MPa) and 40°C for 10 min.[13] It is well known that intra- and intermolecular hydrogen bonding increases in these conditions.[14] The PVA/DNA nanoparticles could be internalized into mammalian cells, suggesting that they have utility as a novel nonviral vector that uses nonelectronic interactions.

Recently, controlled release of DNA was also investigated as a possible method of enhancing transfection efficiency using various biomaterials such as poly (lactide-co-glycolide) (PLGA),[15] hyaluronic acid,[16] atelocollagen,[17] and gelatin.[18,19] Shea et al. reported that the sustained delivery of DNA from PLGA led to effective transfection of a large number of cells in vitro and in vivo.[15] However, it was difficult to regulate the release of DNA owing to the lack of interaction forces, such as covalent, electrostatic, and hydrogen bonding, with which DNA molecules are loaded into PLGA with polymer molecules. Tabata et al. reported enhancement and prolongation of gene expression using a cationized gelatin hydrogel interacting with DNA electrostatically.[18,19] The controlled release of DNA depended on hydrogel degradation, but the cationized gelatin hydrogel was crosslinked by glutaraldehyde, which has generally cytotoxic properties, to obtain different degrees of cationization.

In the present study, we report the preparation of a novel PVA hydrogel with DNA crosslinked physically by hydrogen bonds using UHP technology and its application to the controlled release of DNA. The goal is to develop an effective, low-cytotoxic and gene-releasable biomaterial. PVA/DNA hydrogels were obtained for various pressurization conditions, temperatures, and processing times. DNA release from the hydrogels was investigated in vitro. PVA is widely used for biomedical applications because of its biocompatibility and neutrally charged nature.[20] It is also known that PVA hydrogel is formed by physical crosslinking with hydrogen bonds when PVA solution is frozen and thawed several times, which is called the freeze–thaw method.[21]

Materials and methods

Materials

In our experiments, we used PVA samples with an average molecular weight of 74800 and a degree of saponification of 99.8%, as supplied by Kuraray (Osaka, Japan). We also used salmon sperm DNA purchased from Wako (Osaka, Japan), plasmid DNA encoding enhanced green fluorescence protein under a cytomegalovirus promoter (pEGFP-N1, BD Science, Palo Alto, CA, USA), and nucleic acid staining dye solution (Mupid Blue) obtained from Advance (Tokyo, Japan).

Preparation of PVA/DNA hydrogels by UHP

Aqueous PVA solutions of 6%, 8%, 10%, 14%, and 20% w/v were prepared by autoclaving three times for 30 min at 121°C. Salmon sperm DNA was dissolved in a Tris-EDTA buffer (TE, pH = 7.8) at a concentration of 16.3 mg/ml. The DNA solution was mixed with PVA solutions of 10%, 14%, and 20% w/v at a ratio of 1:1. The 0.7-ml samples were transferred in silicon tubes (9 × 25 mm) with both ends capped by silicon plugs. The tubes were pressurized under various UHP conditions, using different pressures, temperatures, and durations, in a high-pressure machine (Kobe Steel, Kobe, Japan).

Confirmation of the presence of DNA in the PVA/DNA hydrogels

The presence of DNA in the PVA/DNA hydrogels produced by UHP treatment was confirmed by nucleic acid dye staining and UV-visible spectroscopy. For the former method, the PVA/DNA hydrogels were immersed in nucleic acid dye solution for 1 min and then transferred to 70% ethanol. After 1 min, they were immersed in ion-exchanged water for 1 min. For the latter method, after the PVA/DNA hydrogels were melted at 90°C for 10 min, their DNA concentration was measured by a spectrophotometer (V-560, JASC, Tokyo, Japan).

DNA release from hydrogels

The PVA/DNA hydrogels prepared by UHP were immersed in 5 ml of phosphate-buffered saline (PBS) for 144 h at 37°C. At 0.25, 0.5, 2, 3, 15, 27, 48, 111, and 144 h, 20 µl of the samples in the outer part of the PBS solution was collected and the DNA concentration was measured spectrophotometrically at 260 nm (Gene Quant Pro S, Amersham, Tokyo, Japan).

Stability of plasmid DNA released from hydrogels

Plasmid DNA (pDNA) was used instead of salmon sperm DNA and the mixed solutions of pDNA (100 µg/ml) and PVA (5% or 10% w/v) were treated by UHP under the conditions described above. The obtained PVA/pDNA hydrogels were immersed in PBS for 12 and 48 h, and then the samples in the outer part of the solution were collected and analyzed by agarose gel electrophoresis at 100 V for 45 min.

Results and discussion

Aqueous solutions of PVA at concentrations ranging from 3% to 10% w/v were hydrostatically pressurized at 10000 atm at 37°C for 10 min. With a PVA solution of 3% w/v, the clear solution was transformed into a turbid and viscous solution by pressurization (Fig. 1A). An aggregation of PVA particles with an average diameter of 1 µm was observed in the PVA solution on scanning electron microscopy (SEM, data not shown). For PVA concentrations of more than 4% w/v, hydrogels were produced on pressuriza-

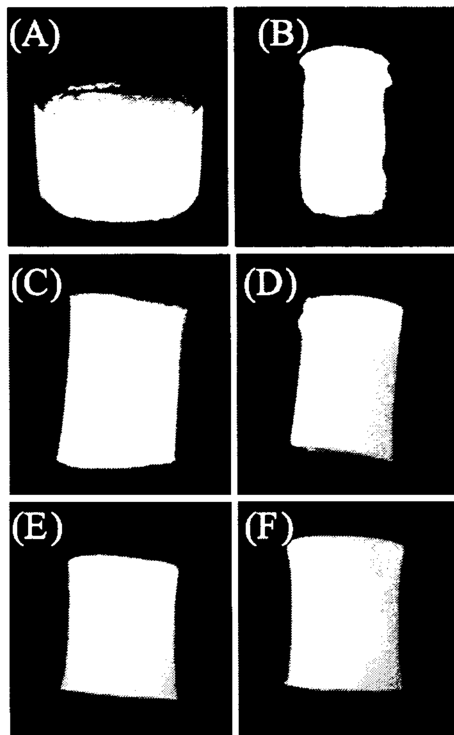

Fig. 1. Photographs of poly(vinyl alcohol) (PVA) hydrogels (**A–D**) and PVA/DNA (**E,F**) hydrogels at concentrations of **A** 3% w/v, **B** 4% w/v, **C,E** 5% w/v, and **D,F** 10% w/v obtained by ultra-high pressure treatment

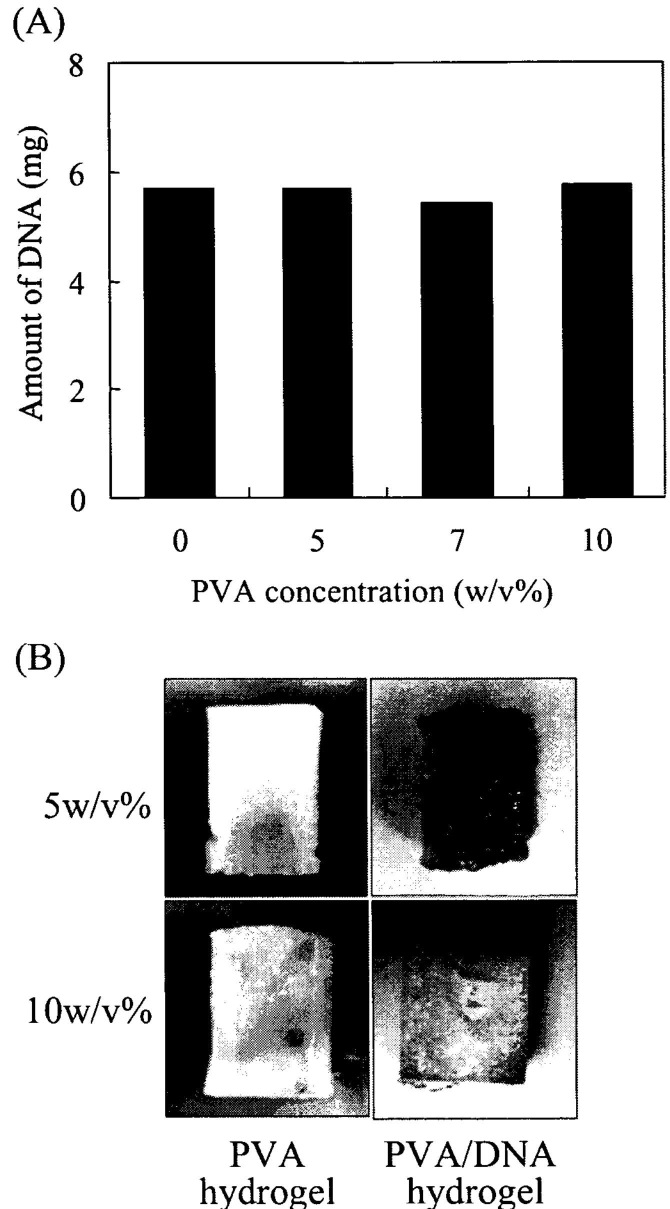

Fig. 2A,B. Presence of DNA in PVA/DNA hydrogels. **A** Amount of DNA in solution obtained by melting PVA/DNA hydrogels prepared using ultra-high pressure processing. **B** Photographs of PVA hydrogels and PVA/DNA hydrogels stained with nucleic acid dye

tion (Fig. 1B–D). The PVA hydrogel of 4% w/v was fragile (Fig. 1B), but increasing the PVA concentration enhanced hydrogel formability, and hard hydrogels were obtained at a PVA concentration of 10% w/v (Fig. 1D). These results indicate that pressurization induced physical cross-linking of PVA molecules and that the degree of cross-linking increased as the PVA concentration increased. To investigate whether the PVA molecules were physically cross-linked by hydrogen bonding, a PVA solution of 5% w/v with urea (3.3M), which was used as a hydrogen bond inhibitor, was treated under the above pressurizing conditions. The solution remained translucent (data not shown), indicating that the PVA hydrogel obtained by pressurization was mediated by hydrogen bonding.

The gelation of mixed solutions of DNA and PVA (5% and 10% w/v) was achieved by pressurization in the conditions described above (Fig. 1E,F). To confirm the presence of DNA in the hydrogels obtained, they were heat treated at 90°C for 10min and then the DNA concentration of the solutions obtained was measured spectrophotometrically at 260nm. Roughly equal amounts of DNA were contained in each hydrogel (Fig. 2A). Also, when the hydrogels were immersed in nucleic acid dye solution, which interacts electrostatically with the phosphate groups of DNA, the PVA hydrogel with DNA was stained, whereas the PVA hydrogel without DNA was not (Fig. 2B). These results indicate that a PVA hydrogel that sustains DNA (PVA/DNA hydrogel) was formed on pressurization. On the other hand, when urea was introduced, PVA/DNA hydrogel was not obtained on pressure treatment. This result suggests that hydrogen bonding between PVA and DNA took place in the pressurized PVA/DNA hydrogel.

DNA release from the PVA/DNA hydrogel formed by pressurization at 10000atm at 37°C for 10min was investigated. PVA/DNA hydrogels produced by the freeze–thaw method, a common method of forming PVA hydrogels,[21] were used as control samples. Figure 3A shows DNA release profiles from the PVA/DNA hydrogels at PVA concentrations of 5% and 10% w/v obtained by pressurization and the freeze–thaw method. Each release curve of DNA from a hydrogel consisted of a rapid phase in the initial 15h followed by a sustained release phase. However, the amount

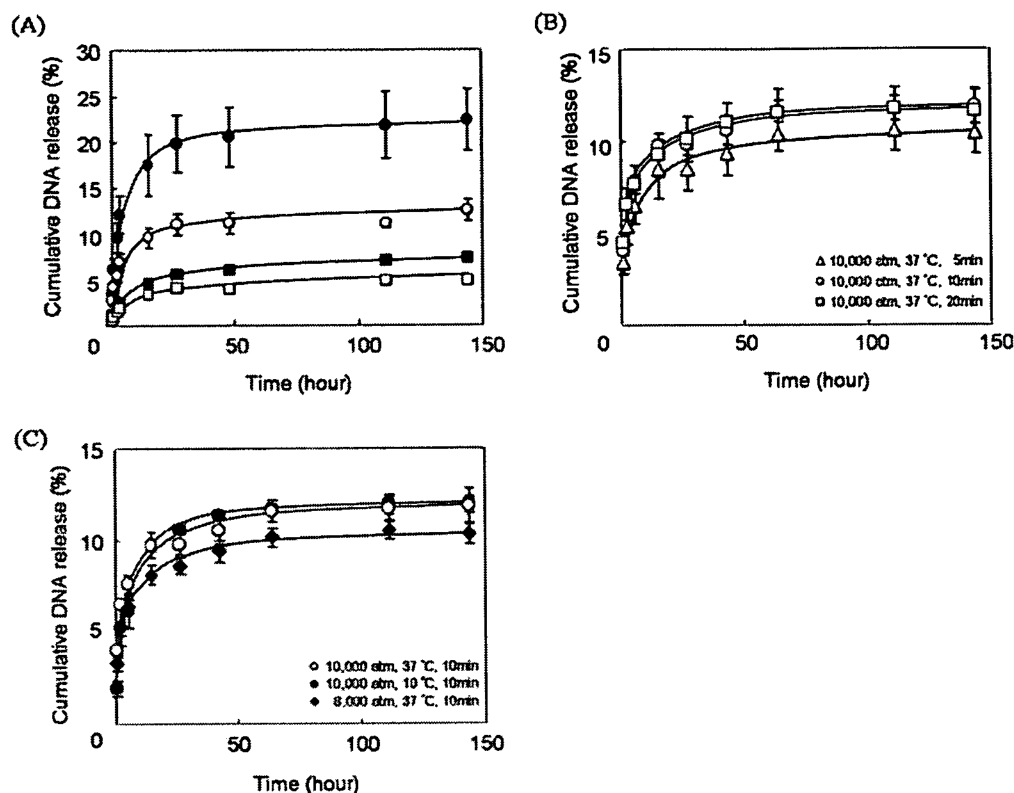

Fig. 3A–C. DNA release test from PVA/DNA hydrogels produced by pressurization under various conditions or by the freeze–thaw method. **A** Release profiles of DNA from hydrogels at PVA concentrations of 5% w/v (○, ●) and 10% w/v (□, ■) PVA concentration. *Open* and *solid* symbols indicate DNA from hydrogels obtained by pressurization (at 10000 atm and 37°C, 10 min) and the freeze–thaw method, respectively. **B** Release profiles of DNA from hydrogels of 5% w/v obtained by pressurization at 10000 atm and 37°C for 5 min (□), 10 min (○), and 20 min (□). **C** Release profiles of DNA from hydrogels of 5% w/v obtained by pressurization at 10000 atm and 37°C (○), 10000 atm and 10°C (●), and 8000 atm and 37°C (□) for 10 min

of DNA released was dependent on PVA content and on which procedure was used to prepare the hydrogels. The DNA release from the 10% w/v PVA/DNA hydrogels was lower than that from the 5% w/v PVA/DNA hydrogels, irrespective of the preparation methods. This is consistent with the fact that the 5% w/v samples were more easily stained by nucleic acid dye than the 10% w/v samples. We suppose that the increased crosslinking in the hydrogel caused by the increase in the PVA content contributed to the reduction of DNA released from the hydrogel. On the other hand, at the same PVA concentrations, DNA was more effectively released from the freeze–thaw hydrogels than from the pressurized hydrogels. Fibrous structures with large spaces (larger than 1 μm) were observed on SEM in the hydrogels made from 5% w/v PVA obtained by the freeze–thaw method, while many porous structures with diameters of 300 μm were observed in the pressurized hydrogels (data not shown). We believe that this difference in internal structure between sample types affected the interaction of PVA and DNA, resulting in the larger release of DNA from the freeze–thaw hydrogels.

To investigate the influence of the pressure conditions used to form hydrogels on DNA release, PVA/DNA hydrogels of 5% w/v were prepared by different levels of pressurization at different temperatures and for different durations. First, with pressure processing periods varying from 5 to 20 min at 10000 atm and 37°C, similar DNA release profiles were exhibited for the hydrogels obtained at pressurizing times of 10 and 20 min, but the amount of DNA released by hydrogel samples pressurized for 5 min (Fig. 3B) was less than that released by samples with longer pressurizing times. Second, the DNA release curves of the PVA/DNA hydrogel produced on pressurization at 10000 atm and 10°C for 10 min were the same as those for hydrogels produced on pressurization at 10000 atm and 37°C for 10 min. However, less DNA was released by hydrogels produced at pressures of 8000 atm and 37°C for 10 min than by hydrogels produced at 10000 atm and 37°C for 10 min (Fig. 3C). These results indicate that DNA release from pressurized hydrogels is dependent on the level and duration of pressure used in the hydrogel formation process. We previously reported that PVA gelation was promoted by increasing the pressure and by prolonging the pressurization time, by which close hydrogen bonds between PVA molecules are formed.[22] It seems that DNA was easily released from PVA/DNA hydrogels pressurized under conditions of more than 10000 atm for longer than 10 min because the hydrogen bonding interaction between PVA and DNA was more unstable than that between PVA molecules under more intense pressure conditions.

It is important for DNA to be released from hydrogels without structural change or degradation.[2,23] Plasmid DNA (pDNA), which is generally used as the DNA delivered by a nonviral vector, was used instead of salmon sperm DNA. PVA/pDNA hydrogels at PVA concentrations of 5% and 10% w/v were obtained by pressurization at 10000 atm at 37°C for 10 min and then immersed in 5 ml PBS. After 12 and 48 h of immersion, the outer part of the solution was collected and analyzed by agarose gel electrophoresis at 100 V for 30 min to investigate the stability of released pDNA from the hydrogels (Fig. 4). No degradation of DNA was observed, indicating that the plasmid DNA released

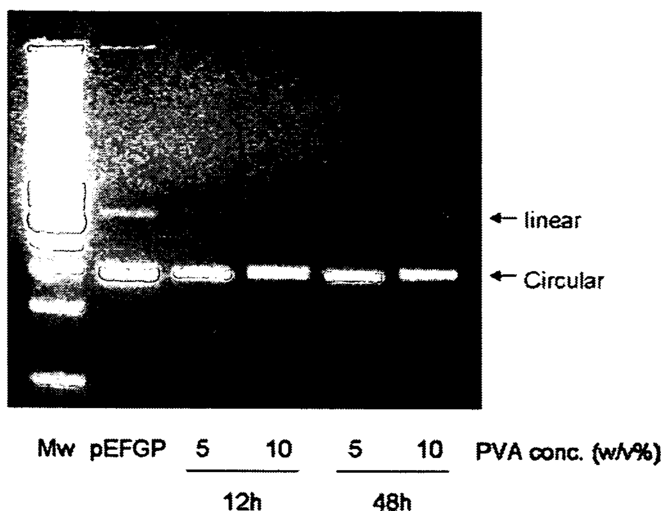

Fig. 4. Agarose gel electrophoresis of plasmid DNA (pDNA) released from PVA/pDNA hydrogels with PVA concentrations of 5% and 10% w/v produced by pressurization at 10000 atm and 37°C for 10 min after immersion in phosphate-buffered saline for 12 and 48 h

from the PVA/DNA hydrogels was stable. Two bands of linear and circular plasmid DNA were observed with 5% w/v PVA/DNA hydrogel, while circular plasmid DNA was released from the 10% w/v PVA/DNA hydrogel, indicating that the linear form of plasmid DNA tends to interact more strongly with PVA than the circular plasmid DNA.

Conclusions

Novel PVA/DNA hydrogels crosslinked physically by hydrogen bonds were developed using UHP technology. DNA released from the hydrogels was controlled by varying the PVA concentration and pressurization conditions, such as the level and duration of pressure used to form the hydrogels. The demonstrated stability of the DNA released from the hydrogels suggests that PVA/DNA hydrogels have potential as a candidate for gene delivery.

Acknowledgments This work was supported by grants from the Ministry of Health, Labor and Welfare, of Japan and the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Kuraray, Co., Ltd., for supplying the poly(vinyl alcohol).

References

1. Nowak T, Nishida K, Shimoda S, Konno Y, Ichinose K, Sakakibara M, Shichiri M, Nakabayashi N, Ishihara K. Biocompatiblility of MPC: in vivo evaluation for clinical application. J Artif Organs 2000;1:39–46
2. Glover DJ, Lipps HJ, Jans DA. Towards safe, non-viral therapeutic gene expression in humans. Nat Rev Genet 2005;6:299–310
3. Zhang S, Xu Y, Wan B, Qiao W, Liu D, Li Z. Cationic compounds used in lipoplexes and polyplexes for gene delivery. J Control Release 2004;100:165–180
4. Lungwitz U, Breunig M, Blunk T, Göpferich A. Polyethylenimine-based non-viral gene delivery systems. Eur J Pharm Biopharm 2005;60:247–266
5. Dufes C, Uchegbu IF, Scatzlein AG. Dendrimers in gene delivery. Adv Drug Deliv Rev 2005:57:2117–2202
6. Kimura T, Yamaoka T, Iwase R, Murakami A. Effect of physicochemical properties of polyplexes composed of chemically modified PL derivatives on transfection efficiency in vitro. Macromol Biosci 2002;2:437–446
7. Futaki S. Membrane-permeable arginine-rich peptides and the translocation mechanisms. Adv Drug Deliv Rev 2005;57:547–558
8. Reschel T, Koňák Č, Oupický D, Seymour LW, Ulbrich K. Physical properties and in vitro transfection efficiency of gene delivery vectors based on complexes of DNA with synthetic polycations. J Control Release 2002;81:201–217
9. Elouahabi A, Ruysschaert JM. Formation and intracellular trafficking of lipoplexes and polyplexes. Mol Ther 2005;11:336–347
10. Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterals 2006;24:1121–1131
11. Choksakulnimitr S, Matsuda S, Tokuda H, Takakura Y, Hashida M. In vitro cytotoxicity of macromolecules in different cell culture systems. J Control Release 1995;34:233–241
12. Sakurai K, Mizu M, Shinkai S. Polysaccharide–polynucleotide complexes. 2. Complementary polynucleotide mimic behavior of the natural polysaccharide schizophyllan in the macromolecular complex with single-stranded RNA and DNA. Biomacromolecules 2001;2:641–650
13. Kimura T, Okuno A, Miyazaki K, Furuzono T, Ohya Y, Ouchi T, Mutsuo S, Yoshizawa H, Kitamura Y, Fujisato T, Kishida A. Novel PVA-DNA nonoparticles prepared by ultra high pressure technology for gene delivery. Mater Sci Eng C 2004;24:797–801
14. Doi E, Shimizu A, Kitabatake N. Gel-sol transition of ovalbumin by high pressure. In: Hayashi R (ed) High pressure bioscience and food science. Kyoto: Sanei Press, 1993;171–177
15. Shea LD, Smiley E, Bonadio J, Mooney DJ. DNA delivery from polymer matrices for tissue engineering. Nat Biotech 1999;17: 551–554
16. Chun KW, Lee JB, Kim SH, Rark TG. Controlled release of plasmid DNA from photo-cross-linked pluronic hydrogels. Biomaterials 2005;26:3319–3326
17. Ochiya T, Takahama Y, Nagahara S, Sumita Y, Hisada A, Itoh H, Nagai Y, Terada M. New delivery system for plasmid DNA in vivo using atelocollagen as a carrier material: the Minipellet. Nat Med 1999;5:707–710
18. Fukunaka Y, Iwanaga K, Morimoto K, Kakemi M, Tabata Y. Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. Biomaterials 2005;26:3319–3326
19. Kushibiki T, Tomoshige R, Fukunaka Y, Kakemi M, Tabata Y. In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents. J Control Release 2003;90:207–216
20. Miyashita H, Shimmura S, Kobayashi H, Taguchi T, Asano-Kato K, Uchino Y, Kato M, Shimazaki J, Tanaka J, Tsubota K. Collagen-immobilized poly (vinyl alcohol) as an artificial cornea scaffold that supports a stratified corneal epithelium. J Biomed Mater Res Part B: Appl Biomater 2006;76B:56–63
21. Hyon SH, Cha WI, Ikada Y. Preparation of transparent poly (vinyl alcohol) hydrogel. Polymer Bull 1989;22:119–122
22. Yamamoto K, Furuzono T, Kishida A, Mustuo S, Yoshizawa H, Kitamura Y. Formation of a supramolecular assembly of poly (vinyl alcohol) by ultrahigh pressure. Meeting Report of the Poval Committee 2002;121:25–26
23. Walter E, Moelling K, Pavolvich HP. Microencapsulation of DNA using poly (D,L-lactide-co-glycolide): stability issues and release characteristics. J Control Release 1999;61:361–374

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

Biomaterials 28 (2007) 3153–3162

Biomaterials

www.elsevier.com/locate/biomaterials

Physical and biological properties of collagen-phospholipid polymer hybrid gels

Kwangwoo Nam, Tsuyoshi Kimura, Akio Kishida*

Division of Biofunctional Molecules, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan

Received 6 December 2006; accepted 8 March 2007
Available online 14 March 2007

Abstract

We successfully developed a novel method for immobilizing poly(2-methacryloyloxyethyl phosphorylcholine) [Poly(MPC)] polymer onto collagen using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as cross-linkers. In order to obtain the highest possible molar ratio of immobilized MPC moieties on the collagen gel, a collagen-phospholipid polymer hybrid gel was prepared by repeating the cross-linking process up to three times to create a dense network of collagen and PMA. Network formation by repeating the immobilization process was successful, resulting in decreased free amine group content and a low swelling ratio. The hybrid gel displayed very high stability against degradation by collagenase and possessed high hydrophilicity. Fibrinogen adsorption and cell adhesion were reduced and demonstrated less cell proliferation as compared to that by uncross-linked collagen gel. The collagen-phospholipid polymer hybrid gel did not exhibit toxicity, and the cell morphology remained intact (round); this implies that the interaction between the cell and the collagen-phospholipid polymer hybrid gel is safe and mild.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: Collagen; Phospholipid polymer; Immobilization; Protein adsorption; Cell adhesion

1. Introduction

In order to use collagen as a biomaterial product, cross-linking of collagen and/or immobilizing synthetic polymers onto collagen are indispensable measures. Non-treated natural collagen cannot be directly applied to a biological system due to drawbacks such as poor mechanical strength, calcium deposition, and high thrombogenicity. However, collagen is biocompatible, non-antigenic, synergic with bioactive components, easily modifiable, and abundantly available; these attributes render it suitable for medical application [1]. Hence, the undesirable properties of collagen should be eliminated while retaining its desirable properties.

When cross-linking the collagen gel, it should be ensured that the cross-linker is not toxic and does not affect biocompatibility. Preparing a cross-linked collagen gel does not necessarily require chemical cross-linking. Diverse methods such as chemical and physical cross-linking, UV irradiation, and blending have been used to cross-link collagen [2–6]. Among these, cross-linking using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) was chosen for this study [7–9]. Cross-linked collagen with EDC and NHS results in "zero-length" amide cross-links between the carboxylic acid groups from aspartic and glutamic acid residues, and the ε-amino groups from (hydroxy-)lysine residues [9]; these form intra- and interhelical cross-links to provide an EDC/NHS cross-linked collagen gel. A 2-methacryloyloxyethyl phosphorylcholine (MPC)-based copolymer, namely, poly(MPC-*co*-methacrylic acid) (PMA), which is also a well-known hemocompatible material [10], was used to cross-link the microfibrils of collagen to produce a collagen-polymer hybrid gel [11].

In our previous study, we discovered that the collagen-polymer hybrid gel could be prepared efficiently under alkaline pH conditions. Immobilization of PMA onto collagen would cover the entire collagen surface, increase the mechanical strength, reduce water absorption, and

*Corresponding author. Tel.: +81 3 5841 8028; fax: +81 3 5841 8005.
E-mail address: Kishida.fm@tmd.ac.jp (A. Kishida).

0142-9612/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2007.03.001

impart durability against collagenase degradation. However, a high percentage of MPC did not immobilize on the collagen gel surface. A longer coupling time or use of larger amount of EDC, NHS, or PMA did not result in an increase in the amount of immobilized PMA. This is believed to be attributable to spatial problems. For immobilization of heparin, low adoption of the polymer with the EDC/NHS coupling reaction was considered a problem. With regard to heparin, it is known that approximately 5.5% immobilization can be achieved [12]. However, activation of the carboxylic group can be triggered at any time point [7]. The coupling reaction continues when EDC/NHS is re-added to collagen gel. Based on this, we developed a novel technique to immobilize the polymer—the "repeat immobilization process."

The repeat immobilization process comprises simply repeating the process of immobilization on the surface of the pre-activated polymer, and a polymer-immobilized collagen gel is thus obtained. The rationale for this process is based on the following: (1) the carboxyl groups can be activated at any time point, and (2) a high percentage of unreacted amine groups are available. Re-activation may promote formation of additional amide bonds, which could not be achieved by a longer coupling time or the use of a higher amount of EDC, NHS, or PMA. In this study, we repeated the immobilization process until a collagen gel with the highest possible number of phospholipid head groups was obtained. Using this gel, we characterized the physical and biological properties of the collagen-polymer hybrid gel. To distinguish the collagen-polymer hybrid gel prepared by the repeat immobilization process from that prepared by time control, we named the former as "collagen-phospholipid polymer hybrid" (a CoPho gel).

2. Experimental methods

2.1. Preparation

2.1.1. Synthesis of PMA

PMA was synthesized according to a previously published method [11,13]. In brief, MPC and methacrylic acid (MA) were dissolved in an ethanol solution. Subsequently, a specific amount of 2,2-azobisisobutyronitrile (AIBN) was added to the ethanol solution. Polymerization was performed in a completely sealed round-bottom flask for 16 h at 60 °C. The solution was precipitated into diethyl ether, freeze-dried, and stored in vacuo until further use. The molar ratio of PMA was MPC:MA = 3:7, and the average molecular weight was 3×10^5.

2.1.2. Preparation of EDC and NHS cross-linked collagen gel (EN gel)

Cross-linked collagen gel was prepared by a previously reported method [11]. Instead of the 0.5 wt% collagen type I solution (pH 3; KOKEN, Tokyo, Japan), 2 wt% collagen type I solution was prepared and used for the film preparation. The collagen solution was dropped onto the polyethylene film and allowed to dry at room temperature. The collagen film (thickness = $56 \pm 3\,\mu m$) was immersed in a 0.05 M 2-morpholinoethane sulfonic (MES) acid buffer (pH 9.0) (Sigma, St Louis, USA) containing EDC (Kanto Chemicals, Tokyo, Japan) and NHS (Kanto Chemicals). The molar ratio of the constituents was EDC:NHS:collagen-carboxylic acid groups = 10:10:1. The cross-linking procedure was allowed to proceed at 4 °C for 4 h to produce a cross-linked gel (EN gel). After 24 h, the reaction was terminated by removing the gel from the solution. Subsequently, the gel was first washed with a 4 M aqueous Na_2HPO_4 solution for 2 h to hydrolyze any remaining O-acylisourea groups and subsequently with distilled water over a duration of 3 days to remove traces of salts from the gel.

2.1.3. Preparation of MPC-immobilized gel (MiC gel, MdC gel, and MtC gel; CoPho gel)

MPC-immobilized collagen gel (MiC gel) was prepared using uncross-linked collagen gel (immersed in an alkaline solution at pH 9.0 for 30 min) or EN gel [11]. PMA was added to the MES buffer (pH 9.0) along with EDC and NHS and was activated for 10 min before the uncross-linked collagen or EN gel was immersed. The molar ratios of each chemical was fixed; EDC:NHS:collagen-carboxylic acid groups = 10:10:1. The immobilization of PMA to collagen continued for 48 h at 4 °C. Subsequently, the gel was first washed with 4 M aqueous Na_2HPO_4 solution for 2 h and then with distilled water for 1 day to remove traces of salts and thus prepare a salt-free MiC gel. Fig. 1 illustrates the basic scheme for activation of PMA by EDC and NHS immobilization on collagen. To increase the number of MPC moieties on the collagen-polymer hybrid gel, a MPC-double immobilized collagen (MdC) gel was prepared by immobilizing PMA on the MiC gels by using the same procedure as earlier. To investigate the possibility of further immobilization, we prepared a MPC-triple immobilized collagen (MtC) gel using MdC as the base collagen for PMA immobilization. MiC-0, MdC-0, and MtC-0 were prepared from uncross-linked collagen gels; MiC-1, MdC-1, and

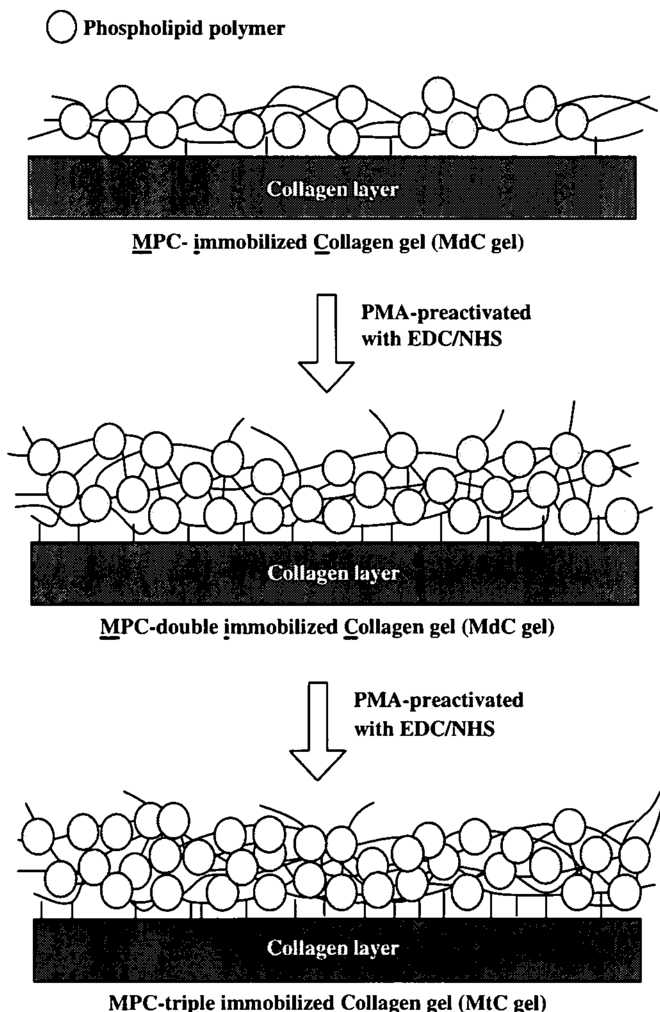

Fig. 1. Schematic diagram of the immobilization process of PMA on collagen.

MtC-1, from EN gels. The terminology used for the gel samples is listed in Table 1.

2.1.4. Preparation of glutaraldehyde cross-linked collagen gel (G-gel)

Cross-linking collagen gel to glutaraldehyde was performed by a previously reported method [14]. In brief, a 25% glutaraldehyde solution (Merck, Damstadt, Germany) was diluted to 0.5 wt% in phosphate buffer solution (PBS). The collagen film was immersed in the glutaraldehyde/PBS solution and was cross-linked for 3 h at room temperature. After cross-linking, the sample was first rinsed in running tap water for 30 min and then in 4 M NaCl for 2 h. In order to eliminate NaCl, the sample was rinsed with distilled water for 1 day to yield a glutaraldehyde cross-linked collagen gel (G-gel). The physical and biological properties of this gel were compared with those of the CoPho gels.

2.2. Characterization

2.2.1. Surface analysis

Surface analysis was executed using X-ray photoelectron spectroscopy (XPS; AXIS-HSi, Shimadzu/KRATOS, Kyoto, Japan) and static contact angle (SCA; ERMA-G1, Tokyo, Japan). The samples that had been cut into small pieces were lyophilized overnight. The chemical composition of the gel surface was determined by the releasing angle of the photoelectrons fixed at 90°. SCA measurement was performed by using a contact angle goniometer (ERMA-G1, Tokyo, Japan) and a Bil-mont syringe. The contact angle of the drop on the surface was measured at room temperature. The SCA experiment was repeated 7 times, and the average was calculated together with the standard deviation.

2.2.2. Determination of the reacted amine group content

The concentration of the primary amine group in tissue samples was determined using a colorimetric assay [15,16]. Three to four milligrams of each sample was prepared. These samples were placed in a 4 wt% aqueous $NaHCO_3$ solution (Kanto Chemicals, Tokyo, Japan) and 2,4,6 trinitrobenzene sulfonic (TNBS) acid. Subsequently, 0.5 wt% aqueous TNBS solution was added (Wako chemicals, Osaka, Japan). The reaction was allowed to proceed for 2 h at 40 °C; the samples were rinsed with saline solution in a vortex mixer to remove unreacted TNBS. Subsequent to freeze-drying the samples overnight, the dry mass was determined. The dry samples were immersed in 2 mL of 6 M aqueous HCl until fully dissolved. The resultant solution was subsequently diluted with distilled water (8 mL) and absorbance was measured at 345 nm (V-560, Jasco, Tokyo, Japan). The concentration of reacted amine groups was calculated using the following equation [16]:

$$[NH_2] = \frac{A \times V}{\varepsilon \times l \times m}, \quad (1)$$

where $[NH_2]$ denotes the reacted amine group content (mol/g collagen gel), ε the molar absorption coefficient of trinitrophenyllysine (1.46×10^4 mL/mmol cm), A the absorbance, V the volume of the solution (mL), l the path length (cm), and m the weight of the sample (mg). The reacted amine group contents of respective collagen gels were all compared with Uc gel.

2.2.3. Swelling test

The swelling test of the samples was executed by cutting the lyophilized gels into small pieces and placing them in a neutral pH aqueous solution at 37 °C. The pH of the aqueous solution was adjusted to 7.4. The gels were gently shaken for 24 h and were measured for assessing the change in weight of the sample. Swelling ratio was calculated in order to define the swelling phenomenon accomplished by water absorption. The experiment was repeated 5 times and the average was calculated along with standard deviation. The following equation was used to calculate the swelling ratio.

$$\text{Swelling ratio}, S(\%) = \frac{W_h - W_d}{W_d} \times 100,$$

where W_h denotes hydrated weight of the gel and W_d the dry weight of the gel.

2.2.4. Fibrinogen adsorption test

Bioresponse was evaluated in terms of protein adsorption by using bovine plasma fibrinogen. The concentration was adjusted to 1 mg/mL. First, the collagen gels were equilibrated by immersing them in PBS. Subsequently, the gels were transferred to the fribrinogen solution, and the solution was incubated for 3 h. After rinsing with PBS, the adsorbed fibrinogen was recovered by dipping the samples in 1 wt% *n*-sodium dodecyl sulfate (SDS) for 60 min [17]. The concentration of recovered fibrinogen was determined at 490 nm by using a Micro BCA kit (Bio-rad, Model 680, Tokyo, Japan).

2.2.5. Cell adhesion test

The interaction between the L929 cells (mouse fibroblast) and the collagen gels was evaluated. The fibroblasts were cultured in Eagle's Minimum Essential Medium (E-MEM, Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, NY, USA) at 37 °C in 5% CO_2 atmosphere. After treatment with 0.25% trypsin, the cell density was adjusted to 5×10^3 cells/mL and the cells were seeded on the gel surface [18,19]. The collagen gels were sterilized by placing the gels first in an ethanol:water (50:50) solution for 2 h, then in a 70:30 solution for 2 h, and overnight in a 100:0 solution before lyophilization. The lyophilized gels were hydrolyzed with E-MEM for 5 min, and the E-MEM was disposed immediately prior to cell seeding. After 24- and 48-h cycles, the number of adhering cells was measured using UV–vis spectrophotometer (V-560, Jasco, Tokyo, Japan) at 560 nm by lactate dehydrogenase (LDH) assay. All experiments were repeated 3 times and the average was calculated together with the standard deviation.

The morphology of the L929 cells after the 48-h incubation period was observed using scanning electron microscopy (SEM). The cells attached to the samples were rinsed with PBS and fixed with 2.5% glutaraldehyde. Subsequently, the samples were dehydrated for 15 min using an ethanol dilution series (10%, 30%, 50%, 70%, and 90%) before the final dehydration with 100% ethanol. After dehydration, the samples were first dried at room temperature and then in vacuum. In order to avoid deformation of the cells, all samples were fixed onto the glass cover prior to vacuum drying.

Table 1
Terminology of collagen gels used in this study

Terminology		Composition
Uc-gel		Uncross-linked gel (stabilized under pH 9.0)
EN gel		EDC/NHS-cross-linked collagen gel under pH 9.0
CoPho gel	MiC-0	PMA immobilized on Uc-gel under pH 9.0
	MdC-0	PMA immobilized on MiC-0 gel under pH 9.0
	MtC-0	PMA immobilized on MdC-0 gel under pH 9.0
	MiC-1	PMA immobilized on EN gel under pH 9.0
	MdC-1	PMA immobilized on MiC gel under al pH 9.0
	MtC-1	PMA immobilized on MdC gel under pH 9.0

2.2.6. Cell viability test

The cell viability test was executed using the 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, USA) assay. The cells were cultured on each test specimen (5000 cells/well) for 48 h and were washed twice with PBS. Subsequently, 200 μl of the MTT solution (0.5 mg/mL in medium, filter sterilized) was added to the culture wells. After incubation for 4 h at 37 °C in a 10% CO_2 atmosphere, the MTT reaction medium was removed and blue formazan was solubilized by the addition of 100 μL dimethylsulfoxide (DMSO). The optical density readings were subsequently performed at 570 nm by using the Micro BCA kit.

2.2.7. Statistical analysis

All the experiments were repeated at least 3 times and the values were expressed as mean ± standard deviation. In several figures, the error bars are not visible because they are included in the plot. Statistical analysis was performed using Student's *t*-test with the significant level set as $P<0.05$.

3. Results

Atomic concentration percentage of phosphorus is shown in Table 2. Phosphorus was not detected in the case of Uc gel and EN gel, while phosphorus was detected in the case of CoPho gels. The phosphorus concentration increased for MdC gels, but significant increase in the phosphorus atomic concentration was not shown for MtC gels.

SCA of the respective collagen gels was measured and is illustrated in Fig. 2. The SCA for Uc and EN gels was approximately 70°. SCA decreases as a result of repeating the immobilization process, and was 20° for MtC-0 and -1 gels, thus indicating that the CoPho gel was acquiring a hydrophilic nature.

Fig. 3 illustrates the results of the reacted amine group content of the respective collagen gels. It decreases from approximately 60–30% as the PMA is immobilized compared to Uc gel. The lowest reacted amine group content was observed for G-gel, which was approximately 15% of Uc gel.

Fig. 4 illustrates the swelling ratio of the collagen gels under acidic and neutral pH conditions. Uc gel dissolved in acidic pH conditions (pH 2.4) and swelled to approximately 350% in neutral pH conditions (pH 7.4). The swelling ratio decreased rapidly on execution of cross-linking. In terms of the swelling ratio, the two gels did not differ substantially under neutral pH conditions, contrary to the situation under acidic pH conditions. However, a decrease in the swelling ratio was observed; after re-immobilization it decreased from 130% for MiC-1 gel to 95% for MtC-1 gel.

Degradation by collagenase demonstrated that cross-linking decreases the degradation rate of collagen gels (Fig. 5). Uc gels, which completely degraded within 6 h, remained undegraded for 24 h when cross-linked with EDC/NHS alone. Immobilization stabilized the gels against degradation by collagenase.

Fig. 6 illustrates the results of fibrinogen adsorbed on the surface of the collagen gel. It can be clearly seen that fibrinogen adsorption decreased as cross-linking proceeded. The amount of adsorbed fibrinogen further decreases as the MPC polymer is immobilized.

Fig. 7 illustrates the results of the cell adhesion test. Here, we discovered that repeated immobilization of PMA suppressed cell adhesion. Comparison of cell adhesion on completion of 24- and 48-h cycles revealed that the number of adhered cells for in the case of the Uc gel after 48-h cycle had increased by approximately 2 times; the rate of

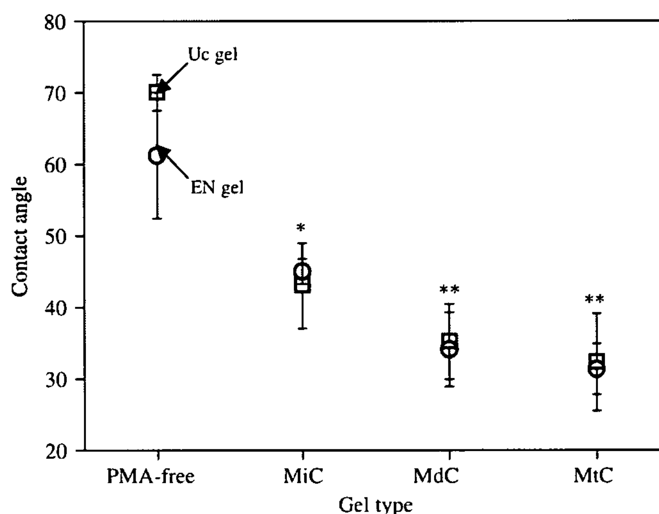

Fig. 2. The static contact angle of the collagen gels. Each value represents the mean ± SD ($n = 5$).

Table 2
Atomic phosphorus concentration of respective collagen gels

Gel type		Atomic phosphorus concentration (%)
Uc gel		0
EN gel		0
CoPho gel	MiC-0	0.21 ± 0.06
	MdC-0	0.36 ± 0.05
	MtC-0	0.37 ± 0.06
	MiC-1	0.18 ± 0.02
	MdC-1	0.37 ± 0.09
	MtC-1	0.36 ± 0.05