子導入は，コラーゲン，ポリ(乳酸-グリコール酸)(PLAGA)などのスキャフォールドとの組み合わせで検討されている。Kasugaiらは，リン酸カルシウム/DNA共沈殿物を含有するアテロコラーゲンの凍結乾燥ペレットあるいはゲルを作製し，骨髄，ラット皮下での導入遺伝子の発現を報告している[17]。また，Laurencinらは，リン酸カルシウム/DNA/PLAGAマイクロスフェアを調製し，このマイクロスフェア表面に生着させた培養細胞への遺伝子導入に成功している[18]。一方，リン酸カルシウムのうちHAp，β-TCPは，骨補填材料（人工骨）として既に実用化されており，骨・軟骨の再生に向けた骨髄細胞，骨芽細胞，骨膜細胞などのスキャフォールドとして数多く検討されている。また，再生医療においては細胞を大量に使用するため細胞を効率よく増幅させるバイオリアクター技術を必要とし，HApは細胞担時体としても検討されている。

おわりに

本稿では，セラミックのうち遺伝子導入に汎用されているリン酸カルシウムに重点をおき，遺伝子導入におけるリン酸カルシウムの合成（結晶化）および物性の重要性を述べた。サイズ，結晶化度，安定性が遺伝子導入効率に強く影響し，微小な粒子で高い導入効率が示される。今後，ナノテクノロジー，有機材料とのハイブリッド化技術などのさらなる技術進展により物性制御がなされ，安全かつ高効率な遺伝子導入素材としての開発が期待される。

参考文献

1) Graham FL, van der Eb AJ : Virology 52, 456-467, 1973.
2) Chen C, Okayama H : Mol Cell Biol 7, 2745-2752, 1987.
3) 青木秀希，驚異の生体物質アパタイト，医歯薬出版，10-11，1999.
4) Yang YW, Yang JC : Biomaterials 18, 213-217, 1997.
5) Chowdhury EH, Sasagawa T, et al : Anal Biochem 314, 316-318, 2003.
6) Jordan M, Schallhorn A, et al : Nucleic Acids Res 24, 596-601, 1996.
7) Orrantia E, Chang PL : Exp Cell Res 190, 170-174, 1990.
8) Furuzono T, Walsh D, et al : J Mater Sci Lett 20, 111-114, 2001.
9) Roy I, Mitra S, et al : Int J Pharm 250, 25-33, 2003.
10) Bisht S, Bhakta G, et al : Int J Pharm 288, 157-168, 2005.
11) Bhakta G, Mitra S, et al : Biomaterials 26, 2157-2163, 2005.
12) Chowdhury EH, Megumi K, et al : Anal Biochem 328, 96-97, 2004.
13) Chowdhury EH, Kunou M, et al : Gene 341, 77-82, 2004.
14) Kakizawa Y, Furukawa S, et al : J Control Release 97, 345-356, 2004.
15) Sandhu AP, Lam AM, et al : Anal Biochem 341, 156-164, 2005.
16) Zaitsev S, Buchwalow I, et al : Acta Histochem 104, 85-92, 2002.
17) Kuroda S, Kondo H, et al : J Pharmacol Sci 97, 227-233, 2005.
18) Kofron MD, Laurencin CT : Biomaterials 25, 2637-2643, 2004.

参考図書

＊驚異の生体物質アパタイト，青木秀希，医歯薬出版，1999.

参考ホームページ

・東京医科歯科大学生体材料工学研究所分子制御分野岸田研究室
http://www.labonet.net/kishida/index.php
・東京医科歯科大学生体材料工学研究所
http://www.tmd.ac.jp/i-mde/www/index.html
・国立循環器病センター研究所生体工学部
http://www.ncvc.go.jp/res/seitai/seitaij.html

木村　剛

1997年　京都工芸繊維大学繊維学部高分子学科卒業
京都工芸繊維大学大学院工芸科学研究科高分子学専攻
1999年　京都工芸繊維大学大学院工芸科学研究科機能科学専攻
2003年　工学博士（京都工芸繊維大学）
国立循環器病センター研究所生体工学部流動研究員
2004年　東京医科歯科大学生体材料工学研究所分子制御分野助手

J Artif Organs (2007) ■:■■-■■
DOI 10.1007/s10047-006-0367-7

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 ultrahigh 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 ultrahigh 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 10 min. 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 15 h 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.

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. Lurai · T. Maritan
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 ultrahigh pressure (UHP) at 10000 atmospheres (980 MPa) and 40°C for 10 min.[13] It is well known that intra- and intermolecular hydrogen bond-

ing 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 22000 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, ■■ 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 pressurization (Fig. 1B–D). The PVA hydrogel of 4% w/v was fragile (Fig. 1B), but increasing the PVA concentration enhanced

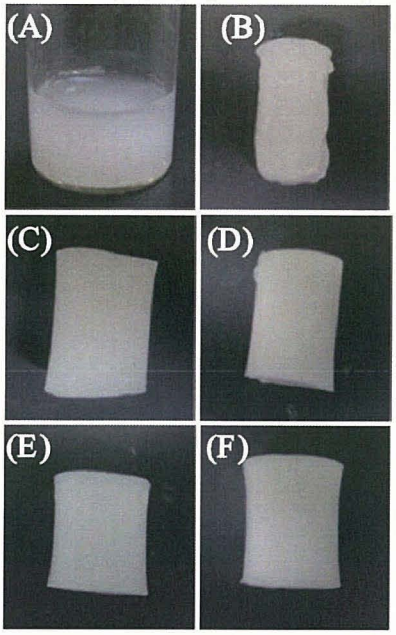


Fig. 1. Photographs of poly (vinyl alcohol) (PVA) hydrogels (**A–D**) and PVA/DNA (**E,F**) hydrogels at concentrations of **A** 3% w/v, **B** 4% w/v, **C,E** 5% w/v, and **D,F** 10% w/v obtained by ultrahigh-pressure treatment

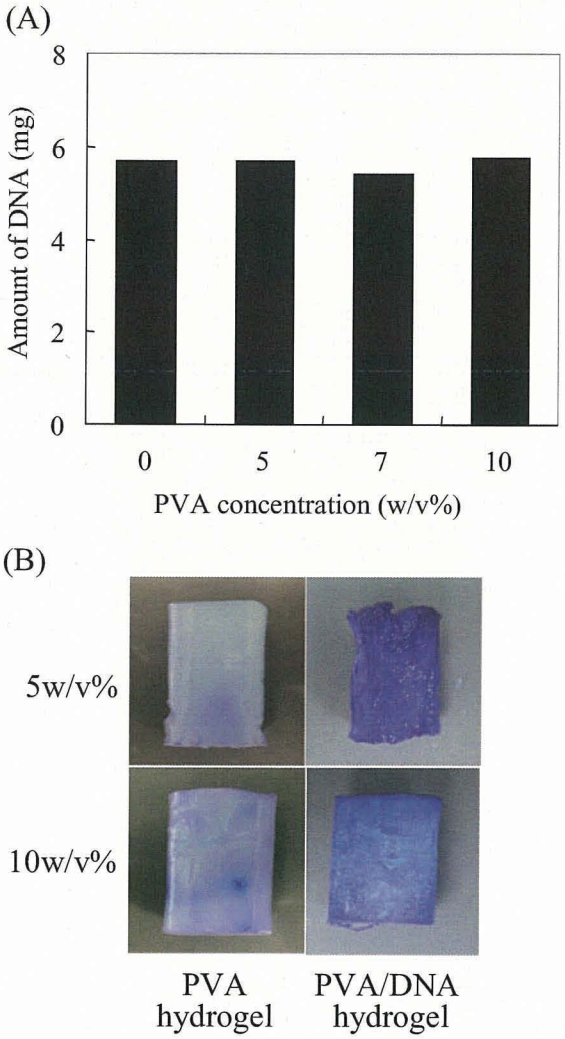


Fig. 2A,B. Presence of DNA in PVA/DNA hydrogels. **A** Amount of DNA in solution obtained by melting PVA/DNA hydrogels prepared using ultrahigh-pressure processing. **B** Photographs of PVA hydrogels and PVA/DNA hydrogels stained with nucleic acid dye

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 (3.3 M), 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 10 min and then the DNA concentration of the solutions obtained was measured spectrophotometrically at 260 nm. 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 10000 atm at 37°C for 10 min 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 15 h followed by a sustained release phase. However, the amount of DNA released was dependent on PVA content and on

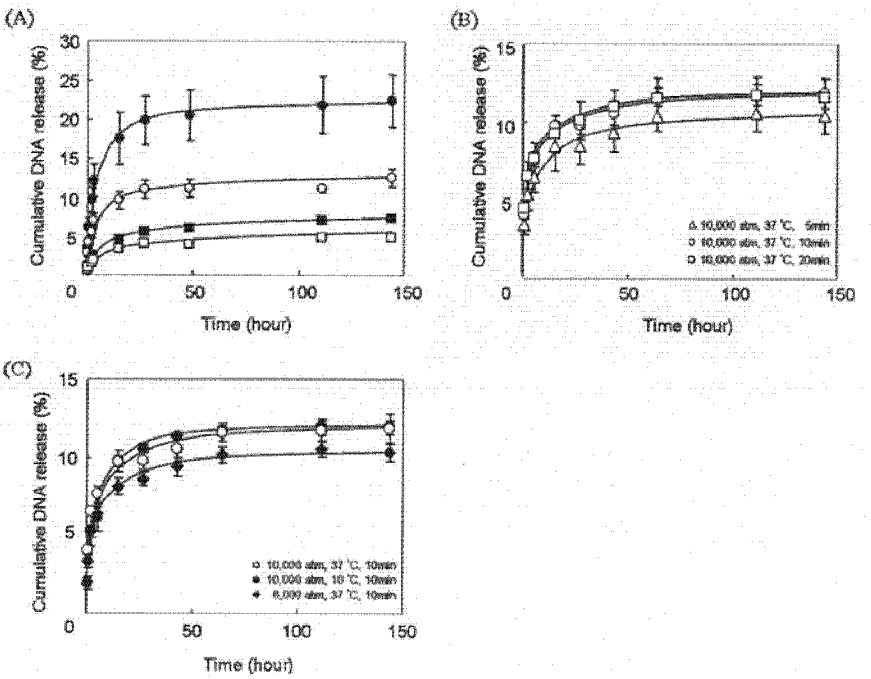


Fig. 3A–C. DNA release test from PVA/DNA hydrogels produced by pressurization under various conditions or by the freeze–thaw method. **A** Release profiles of DNA from hydrogels at PVA concentrations of 5% w/v (○, ●) and 10% w/v (□, ■) PVA concentration. *Open* and *solid* symbols indicate DNA from hydrogels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

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 from the PVA/DNA hydrogels was stable. Two bands of

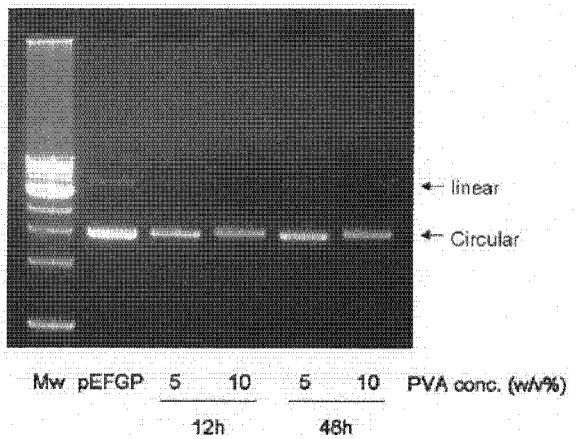


Fig. 4. Agarose gel electrophoresis of plasmid DNA (pDNA) released from PVA/pDNA hydrogels with PVA concentrations of 5% and 10% w/v produced by pressurization at 10000 atm and 37°C for 10 min after immersion in phosphate-buffered saline for 12 and 48 h. pEFGB, ■■

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.

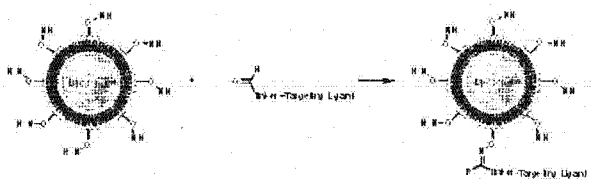
Acknowledgments This work was supported by grants from the Ministry of Health, Labor and Welfare, of Japan and the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Kuraray, Co., Ltd., for supplying the poly (vinyl alcohol).

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, OupickýD, 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 ultrahigh-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

systemic administration. This is achieved with a hydrophilic polymer of which polyethylene glycol (PEG) has been found to be best. Targeting cell-surface receptors is an attractive concept to achieve specific binding and internalisation of the liposome. To this end, cell-binding ligands are displayed from the surface of the liposome. The common denominator for these ligands is that their cell receptor targets are over-expressed in tumours.

We are developing a post-modification methodology for coupling cell or tissue targeting moieties onto liposomes via chemioselective oxime bond formation. Thus, liposomes comprising a highly nucleophilic aminoxy-functionalised lipid are formulated. The aminoxy lipid readily reacts with aldehyde functionalised targeting ligands in aqueous conditions forming a stable oxime bond conjugated targeted liposome.



A peptide displaying the RGD sequence was selected as the targeting ligands to validate our post-coupling methodology. A cyclic RGD peptide conjugate has been successfully synthesised using a combination of solution- and solid-phase synthesis. Additionally, control RGE peptide and PEG linker conjugates have been prepared. All the ligands terminate in an aldehyde functional group separated from the targeting moiety by a PEG linker.

The couplings between the RGD conjugate and aminoxy liposomes in water at pH ~4 were shown to be efficient, simple and reproducible. In vitro HUVEC cell uptake studies show a definite targeted uptake of the RGD-targeted liposomes relative to appropriate control liposomes.

193. Gene Transfection Using Inorganic Particle/PVA/DNA Complexes Prepared by Ultra High Pressure Technology

Tsuyoshi Kimura,[1] Kwangoo Nam,[1] Sihngo Mutsuo,[2] Hidekazu Yoshizawa,[2] Masahiro Okada,[3] Tsutomu Furuzono,[3] Toshiya Fujisato,[4] Akio Kishida.[1]

[1]Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo, Japan; [2]Department of Environmental Chemistry and Materials, Okayama University, Okayama, Japan; [3]Department of Biomedical Engineering, National Cardiovasucular Center Research Institute, Osaka, Japan; [4]Department of Regenerative Medicine and Tissue Engineering, National Cardiovasucular Center Research Institute, Osaka, Japan.

Various non-viral gene systems, such as naked DNA, lipoplexes, micelles and polyplexes, have been developed for effective and safe gene delivery into target cells. Although cationic compounds were employed as gene carriers due to the ability of complex formation with DNA electrostatically and effective gene transfer into cells, the intrinsic cytotoxicity of them is essential problem in non-viral gene delivery system. Therefore, we have tried the development of DNA complex with non-ionic, water soluble polymers via hydrogen bond using ultra high pressure (UHP) technology because the inter-, intra-molecular week hydrogen bonding interaction was empathized with high pressure process. Previously, polyvinyl alcohol (PVA) was utilized as the model hydrogen bonding polymers, and the PVA/DNA complexes were obtained by UHP treatment. Although the PVA/DNA complexes were up-taken by cells, a little enhancement of gene expression was observed using them. In this study, we hypothesize that inorganic particles, such as calcium phosphate (CP), calcium carbonate and hydroxy apatite (HAp), promote the endosomal escape of transferred DNA because the inorganic particles are dissolved under low pH condition in endosome vesicles and then the rupture of endosome is induced by osmotic shock. We performed the development of inorganic particle/PVA/DNA complexes using UHP technology. Plasmid DNAs encoding enhanced green fluorescent protein (EGFP) gene or luciferase gene under CMV promoter were used. Nano-HAps having the average diameter of 50nm were synthesized by modified micro-emulsion method. Nano-HAp was dispersed ultrasonically in PVA solution and then mixed with DNA solution. CP/DNA complexes were prepared by general method and mixed with PVA solution. Their mixtures were treated under 10000 atmospheric pressures at 40 degree for 10min. By SEM observation, the irregular surface of inorganic particles/PVA/DNA complexes was observed, indicating the encapsulation of inorganic particles in PVA/DNA particle. The nano-HAp/PVA/DNA complexes showed a higher transfection activity than DNA complexes with nano-HAp or PVA. With CP/PVA/DNA complexes, also, the transfection activity increased several fold than the PVA/DNA complexes. These results indicate the utility of the inorganic particle/PVA/DNA complexes prepared by the UHP treatment for DNA delivery.

194. Development of Novel DNA Formulations Based on Polymers and Cyclodextrin for Gene Delivery to the Muscle

Caroline Roques,[1,2] André Salmon,[1] Marc Y. Fiszman,[1] Elias Fattal,[2] Yves Fromes.[1]

[1]Gene Therapy Lab, Institut de Myologie - INSERM u582, Paris, France; [2]CNRS UMR 8612, University of Paris - Faculty of Pharmacy, Chathenay-Malabry, France.

So far gene transfer to the muscle has mainly been based on viral vectors, given their efficiency to transfer DNA in vivo. However, virus-derived vectors have numbers of limitations, such as insert size, tissue specificity or immunogenicity, therefore restricting the possibilities of repeated administrations. After intramuscular injection, major hurdles remain tissue diffusion and intracellular entry.

To improve these parameters with reference to naked DNA, our approach consisted in designing synthetic vectors. The first step intended to condense and protect plasmid DNA (pCMV-βGal, Invitrogen) through its association with various polymers differing by their charge, i.e. Polyethyleneimine (PEI, Sigma), Tetronic 304 (BASF) and PE6400 (BASF). For each polymer/DNA formulation, the morphological properties of the vectors were assessed by cryo-Transmission Electron Microscopy, their size by Dynamic Light Scattering and their zeta potential by Laser Doppler Velocimetry. Characterization revealed a great diversity of objects in terms of size, shape and zeta potential.

In vivo toxicity and efficiency of the systems were also evaluated after intramuscular injections into tibialis anterior and quadriceps muscles of wild type Syrian hamsters. X-Gal revelation and Haematein/Eosin staining were then performed on serial sections of each muscle. These experiments highlighted the extremely high cytotoxicity of PEI/DNA complexes towards skeletal muscle. On the contrary, no significant lesions were detected after injection of PE6400/DNA or Tetronic/DNA formulations. Both systems did significantly improve transfection with reference to naked DNA.

In order to promote cellular entry of the DNA, a second step in our study consisted in combining the previous polymeric vectors with randomly methylated beta-cyclodextrin (Rameb) since this compound has demonstrated its ability to destabilize the cell membrane through cholesterol complexation. In vivo toxicity of Rameb after intramuscular injection has been assessed as well as efficiency when associated to polymer/DNA formulations. Addition of Rameb to the polymeric vectors did not allow a significant increase

Available online at www.sciencedirect.com
ScienceDirect
Biomaterials 28 (2007) 1–8
Biomaterials
www.elsevier.com/locate/biomaterials

Preparation and characterization of cross-linked 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 5 June 2006; accepted 1 August 2006
Available online 7 September 2006

Abstract

2-methacryloyloxyethyl phosphorylcholine (MPC)-immobilized collagen gel was developed. Using 1-ethyl-3-(3-dimethyl aminopropyl)-1-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), we cross-linked a collagen film in 2-morpholinoethane sulfonic acid (MES) buffer (EN gel). EN gel was prepared under both pH 4.5 and pH 9.0 in order to observe changes in cross-linking ability. To cross-link MPC to collagen gel, poly(MPC-*co*-methacrylic acid) (PMA) having a carboxyl group side chain was chosen. E/N gel was added to the MES buffer having pre-NHS activated PMA to make MPC-immobilized collagen gel (MiC gel). MiC gel was prepared under both acidic and alkaline conditions to observe the changes in the cross-linking ability of PMA. X-ray photoelectron spectroscopy showed that the PMA was cross-linked with collagen under both acidic and alkaline conditions. Differential scanning calorimetry (DSC) results showed that the shrinkage temperature increased for the MiC gels and that the increase would be greater for the MiC gel prepared under alkaline conditions. The data showed that swelling would be less when the MiC gel was prepared under alkaline conditions. The biodegradation caused by collagenase was suppressed for the MiC gel prepared under alkaline conditions due to stable inter- and intrahelical networks.

Keywords: Collagen; Phospholipid; Cross-linking; Surface modification

1. Introduction

Collagen is an extracellular-matrix protein that plays an important role in the formation of tissues and organs and is involved in various functional expressions of cells [1]. Collagen is non-toxic, non-antigenic, favors cell adhesion, proliferation, and differentiation to mimic the natural cell environment. However, favoring cell adhesion can be both advantageous and disadvantageous, for its strong affinity to cells and blood is uncontrollable, which may soon lead to blood coagulation and mineralization when applied for use as artificial blood vessels. Furthermore, the collagen that is prepared in a matrix form such as a gel for tissue reconstruction is mechanically insufficient [2]. Without modification, the collagen gel cannot be applied for bioprosthesis [3].

To overcome the disadvantages of collagen while maintaining its biological performance, a prosthesis-tissue complex, or bioartificial polymeric material, was developed by blending or mixing biomolecules and synthetic materials. The chief purpose for developing such a bioartificial polymer material is to overcome the poor biological performance of synthetic polymers and to enhance the mechanical characteristics of biomolecules [4].

To control cell adhesiveness and to increase mechanical strength simultaneously, collagen must be modified by cross-linking or mixing with synthetic polymers. Polymers such as poly(vinyl alcohol), poly(acrylic acid), poly(vinyl pyrrolidone), and polyethylene are used as bioartificial polymer materials because of their favorable chemical reactivity with collagen, absence of toxicity, and good mechanical performance [4–8].

However, it is very important to consider biological response in the adoption of a cross-linker or synthetic polymer because of the possibilities of severe problems

*Corresponding author. Tel./fax: +81 03 5280 8028.
E-mail address: kishida.fm@tmd.ac.jp (A. Kishida).

0142-9612/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2006.08.002

such as toxicity, inflammatory response, or alteration of protein structure. Furthermore, some synthetic polymers that are known to be 'biocompatible' degrade in biological fluids, making the collagen structure unstable. Adoption of natural cross-linkers such as glutaraldehyde [9], genipin [10], or transglutaminase [11], and natural polymers like hyaluronic acid [12], heparin [13], or chondroitin-6-sulfate [14,15] is used as direct cross-linker or immobilizer to overcome the problems presented by the use of synthetic polymers, but cannot fully solve the problems.

To overcome these problems, we developed a biosynthetic hybrid material by cross-linking collagen with a 2-methacryloyloxyethyl phosphorylcholine (MPC) based copolymer using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as cross-linkers by activating the MPC polymer with EDC and NHS to cross-link the microfibrils and polymer chain using amide bond [3,16–18].

MPC is a blood compatible product developed in the early 1990s [19]. Design of the MPC polymer took into account the surface structure of the biomembrane. Recently, phospholipid-accumulated surfaces have been prepared by various methods, and it has been reported that the phosphorylcholine group plays an important role showing excellent blood compatibility and anti-protein adsorptivity [20–23]. The MPC units can then be introduced to conventional polymers by various methods of modification. They effectively reduce protein adsorption and denaturation and inhibit cell adhesion even when the polymer is exposed to whole blood in the absence of any anticoagulants [24]. By adopting the MPC polymer with the collagen gel, it is possible to expect a biocompatible collagen–polymer hybrid gel that is stable, has its molecular weight controlled, has no cross-linker leaking, and is mechanically tough.

In the present study, cross-linking ability between poly(MPC-*co*-methacrylic acid) (PMA) and collagen using EDC and NHS was investigated by altering several parameters, and the physical properties of PMA-immobilized matrices were characterized. In this article, the terms interchain cross-linking and immobilization are used synonymously.

2. Experimental method

2.1. Preparation of collagen–phospholipid polymer hybrid gel

2.1.1. Synthesis of PMA

PMA was synthesized by a method that has already been published [19]. In short, desired amount of MPC and MA was dissolved in ethanol in an ampoule. Then 2,2′-azoisobutyronitrile (AIBN) was added to the ethanol solution. The argon gas was bubbled into the ethanol solution to eliminate the oxygen. The ampoule was sealed and heated to 60 °C for 16 h. The solution was precipitated into diethyl ether, freeze-dried, and kept in vacuum until use. The mole ratio of PMA was controlled to MPC:MA = 3:7, and the number average molecular weight $\overline{M}_n$ of the PMA was approximately 300,000. The chemical structure of PMA is shown in Fig. 1.

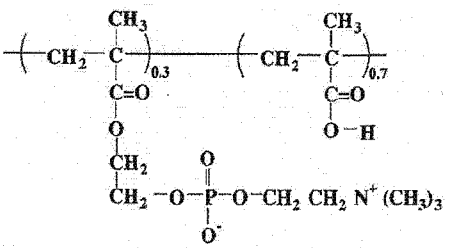


Fig. 1. Chemical structure of PMA.

Table 1
Terminology of collagen gels used in this study

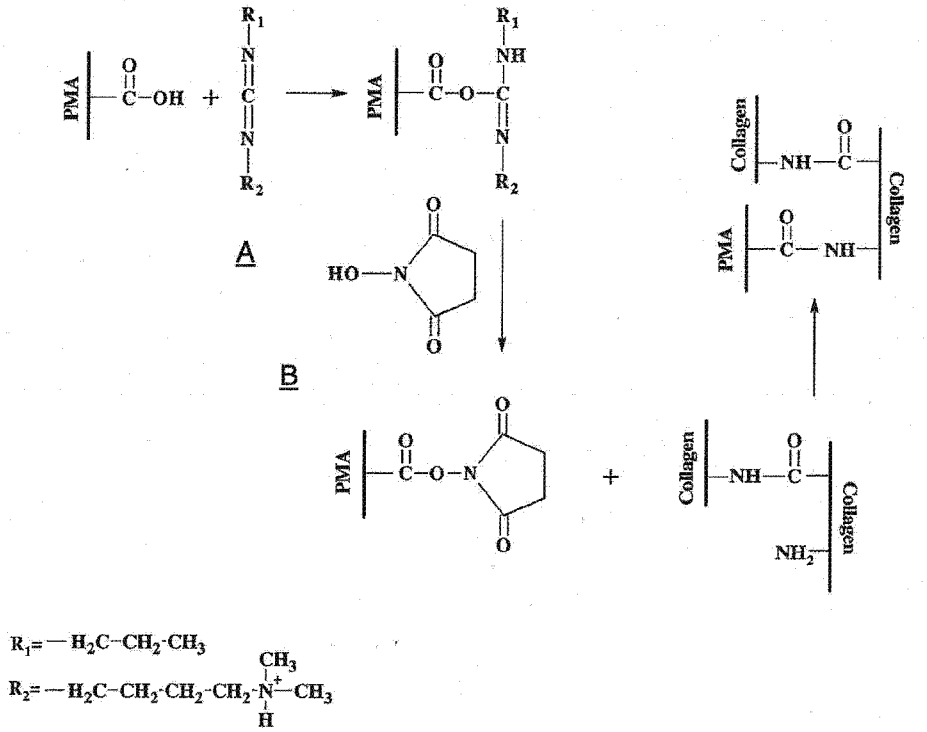
Terminology	Composition
Uc-gel	Uncross-linked collagen gel (immersed in alkaline pH conditions)
EN-1	EDC/NHS-cross-linked collagen gel under acidic pH conditions
EN-2	EDC/NHS-cross-linked collagen gel under alkaline pH conditions
MiC-11 gel	PMA immobilized to EN-1 gel under acid pH conditions
MiC-12 gel	PMA immobilized to EN-1 gel under alkaline pH conditions
MiC-21 gel	PMA immobilized to EN-2 gel under acid pH conditions
MiC-22 gel	PMA immobilized to EN-2 gel under alkaline pH conditions

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

Cross-linked collagen gel was prepared by using 0.5 wt% collagen type I solution (pH 3, KOKEN, Tokyo, Japan). Conventional film fabrication method was used for the film fabrication. The collagen solution was dropped onto the polyethylene film and dried in room temperature. The collagen film (thickness = 36 ± 2 μm) was immersed into a 0.05 M 2-morpholinoethane sulfonic acid (MES) buffer (pH 4.5) (Sigma, St. Louis, USA) containing 1-ethyl-3-(3-dimethyl aminopropyl)-1-carbodiimide hydrochloride (EDC) (Kanto Chemicals, Tokyo, Japan) and NHS (Kanto Chemicals, Tokyo, Japan). Each chemical was added at the mole ratio of EDC:NHS:collagen-carboxylic acid groups = 5:5:1 [11,13]. The cross-linking procedure was allowed to continue for 4 h at 4 °C to produce a cross-linked gel (EN-1 gel). After 4 h, the reaction was stopped by removing the gel from the solution. The gel was then washed with 4 M of Na_2HPO_4 aqueous solution for 2 h to hydrolyze any remaining *O*-acylisourea groups and then with distilled water for 3 days to remove any salts from the gel. Same preparation process was repeated under alkaline conditions (pH 9.0; adjusted with NaOH) to prepare an EN-2 gel.

2.1.3. Preparation of MPC-immobilized collagen gel (MiC gel)

Preparation of the MiC gel was done by using the EN-1 and EN-2 gels. PMA was added with EDC and NHS to the MES buffer (pH 4.5 and pH 9.0) and was pre-activated for 10 min before immersion of the EN-1 or EN-2 gel. The immobilization of PMA to the collagen was allowed to continue for 4 h at 4 °C. The gel was then washed with 4 M of Na_2HPO_4 aqueous solution for 2 h and then with distilled water for 1 day to remove any salts from the gel to prepare a salt-free MiC gel: MiC-11 gel (PMA immobilized under acidic conditions using the EN-1 gel), MiC-12 gel (PMA immobilized under alkaline conditions using the EN-1 gel), MiC-21 gel (PMA immobilized under acidic conditions using the EN-2 gel), and MiC-22 gel (PMA immobilized under alkaline conditions using the EN-2 gel). The terminology of the samples is listed in Table 1. PMA cross-linking with the collagen is shown in Fig. 2. Collagen film was immersed



R_1= —H_2C–CH_2–CH_3

R_2= —H_2C–CH_2–CH_2–CH_2–$N^+H(CH_3)$–CH_3

A: 1-ethyl-3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (EDC)
B: *N*-hydroxysuccinimide (NHS)

Fig. 2. Schematic picture of immobilization of MPC polymer with collagen.

into the MES buffer pH 9.0 for 1 day to obtain a non cross-linked collagen gel (Uc-gel) and was used as a reference.

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 scanning electron microscopy (SEM, SM-200, Topcon, Tokyo, Japan). The samples, which had been cut into small pieces, were lyophilized overnight. The chemical composition of the surfaces of the gel was determined by the take-off angle of the photoelectrons fixed at 90°. The morphologies of the gels were observed with SEM after gold coating with an ion coater (IB-3, Eiko Co., Ibaraki, Japan). The razor blade-cut surfaces of the respective gels were observed.

2.2.2. Shrinkage temperature

The shrinkage temperatures of the gels were determined using differential scanning calorimetry (DSC, DSC6000, Seiko, Chiba, Japan) in the range 0–150 °C at a scanning rate 5 °C/min. The samples were incubated with small amounts of phosphate buffer solution for 1 h at room temperature before being measured [9]. Instead of an empty container, a container of PBS was used for reference.

2.2.3. Mechanical properties

The stress–strain curves of the respective collagen gels were determined by uniaxial measurements using a universal testing machine (Orientec STA-1150, Tokyo, Japan). The sizes of the samples used for measurement were 4 cm × 1 cm. Each sample was strained at the rate of 10 mm/min. The obtained data were fitted to the stress–strain curves of the samples and the elongational modulus at 1% and 8% was calculated.

2.2.4. Swelling test

A swelling test of each sample was executed by cutting the lyophilized gels into small pieces and putting them into pH-controlled aqueous solutions at 37 °C. The pH of the aqueous solution was controlled to 2.1 or 7.4. The gels were gently shaken for 24 h and then removed for weighing. The swelling ratio was calculated in order to define the exact amount of swelling caused by water absorption. The equation used for the swelling ratio was

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

where W_h is hydrated weight and W_d is dried weight of the gel.

2.2.5. Enzymatic degradation test

Degradation tests of the gel samples were executed using collagenase from Clostridiopeptidase histoluticum (EC 3.4.24.3, *Sigma*, St. Louis, USA) with collagenase activity of 300 units/mg. In this experiment, 30±2 mg of collagen gels were immersed into 2 mL of 0.1 M Tris-HCl buffer (pH 7.4) with 5×10^{-3} M of calcium chloride (Kanto Chemical, Tokyo, Japan) and 8×10^{-4} M of sodium azide (Kanto Chemicals, Tokyo, Japan) and was shaken for 1 h at 37 °C. Then, 2 mL of collagenase Tris-HCl buffer solution with a concentration of 1.32 mg/mL was added to the solution containing the gel to determine the total concentration of collagenase at 100 units/mL. The container was returned to the shaking water bath. The remaining weights of the samples were measured for 72 h.

2.2.6. Statistical analysis

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

3. Results and discussion

3.1. Basic characteristics of collagen gels

The reaction between EDC and the carboxyl groups are shown elsewhere; the mechanism is well known [25,26]. According to Nakajima and Ikada [26], proton and ionized carboxyl groups are required for the reaction with EDC. The excess amount of EDC against the carboxyl groups should be used up, and no reaction occurred when the molar ratio of EDC to the carboxyl groups was below 0.5. Using EDC for cross-linking might cause hydrolysis, which makes the carboxyl groups return to the original carboxyl groups.

The use of NHS is to prohibit the hydrolysis of the carboxyl groups. NHS would lead to formation of NHS-ester, which prevents the side reaction of the *O*-acylisourea groups [25,26]. This is because the reactive species relative to the neucleophilic attack of the free amine group of collagen are the NHS-activated carboxyl groups rather than the *O*-acylisourea groups.

Fig. 3 shows the XPS result of Uc-gel, EN-2 gel, MiC-21 gel, and MiC-12. All gels showed XPS signals attributed to carbon in CH_3– or –CH_2–, –COC–, C(=O)–, and nitrogen in –CONH– was observed at 285, 286.6, 288.5, and 400.8 eV, respectively. The phosphorus peak and one nitrogen peak in $-N^+(CH_3)_3$ were observed at 134 and 403.2 eV, respectively, indicating that PMA was a properly cross-linked collagen [21,24].

SEM images of the outer surfaces and razor blade-cut surfaces (vertical cross-section) of the respective collagen gels are shown in Fig. 4. The razor blade-cut surfaces of the Uc-gel and the EN-1 and EN-2 gels are porous. For MiC, the non-porous layer is shown to be deposited on the porous layer. Non-porosity can be seen for the pure PMA film prepared using same method (image not shown). This implies that PMA covers the collagen gel instead of being blended, making it a heterogeneous phase. However, the outer surface of the gel is entirely one phase showing no defects, indicating that PMA is immobilized on the collagen surface and is distributed homogeneously. This is because the high molecular weight of the PMA causes the polymer to be located primarily on the surface of the collagen gel. When the PMA and collagen are premixed and gelled, the razor blade-cut surface shows that the porous and non-porous structures coexist (picture not shown). In the case of MiC-11 and MiC-12, the non-porous outer layer is very thin and the pore size is bigger, indicating that a sizeable amount of swelling had occurred.

3.2. Network structure of collagen gels

Shrinkage temperature T_s is considered as the rupture of the inter-chain bonds bringing the fusion of the oriented peptide chains [27], which is responsible for the shrinkage, and the cross-linking will result in the stabilization of the triple helix structure and an increase in the shrinkage temperature [28]. Table 2 lists T_s of the respective collagen gels. The result indicates that T_s would increase when the gels are cross-linked. Because PMA is immobilized, T_s of the gels would shift to a higher temperature, eventually reaching approximately 85 °C, which is about a 30 °C increase from that of uncross-linked collagen gel. The EN-1 gel and the MiC-11 and MiC-12 gels showed that T_s is lower than the EN-2 gel and the MiC-21 and MiC-22 gels. This implies that formation of inter- and intrahelical cross-links, which prevent the fusion of the peptide chains, is very important for stabilization of the network. The immobilization of PMA made the extra cross-link, that is, the bond between the PMA chain and collagen microfibril by the amide bond, eventually increasing T_s further. Comparing T_s of MiC-11 and MiC-12, we can see that the numeric value is almost the same. The same phenomenon can be seen for MiC-21 and MiC-22, implying that the immobilization of PMA would be affected by the pH of the MES buffer. Under pH 4.5, the carboxyl groups of PMA would be protonated, leading to the formation of COO–NHS, because the pK_a of PMA is known to be 2.7 [29].

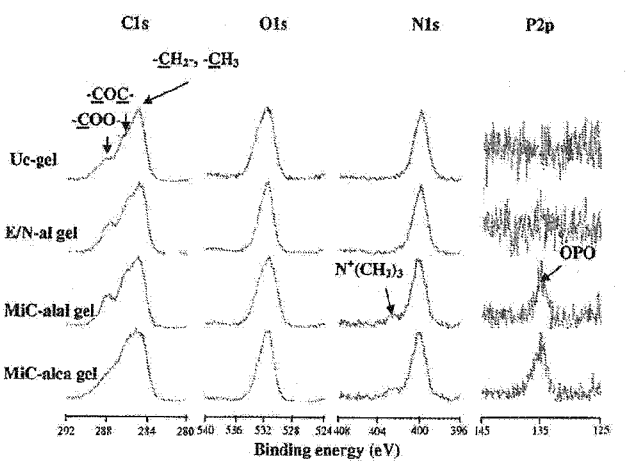


Fig. 3. XPS chart of Uc-gel, E/N-2l gel, MiC-22 gel, and MiC-21 gel. The takeoff angle of photoelectron was 90°.

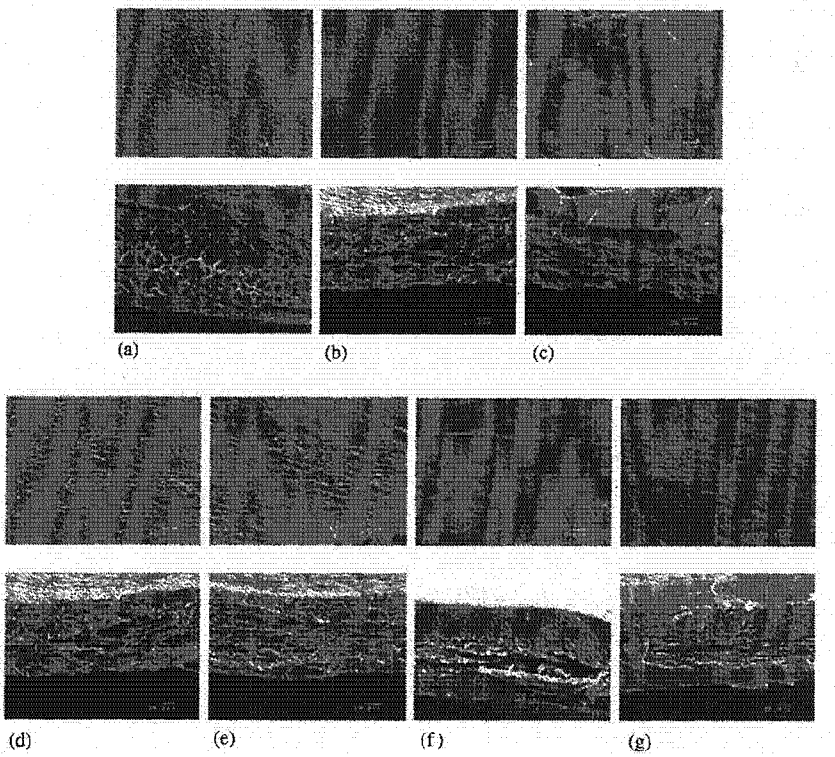


Fig. 4. Outer surface morphology (upper) and razor blade cut morphology (below) of respective gels: (a) Uc-gel, (b) E/N-1 gel, (c) E/N-2 gel, (d) MiC-11 gel, (e) MiC-12 gel, (f) MiC-22 gel, and (g) MiC-21 gel.

Table 2
Shrinkage temperatures of collagen and collagen gels

Sample	T_s (°C)
Uncross-linked	56.4±8.1
EN-1	67.4±0.9
EN-2	76.5±2.9
MiC-11	74.1±3.9
MiC-12	75.1±2.0
MiC-21	84.8±2.0
MiC-22	84.1±3.9

Fig. 5 shows the strain–stress curve of the Uc-gel, EN-2 gel, MiC-22, and MiC-21 gels. It can be seen that all collagen gels are J-shaped. This shape indicates that, after the cross-linking and immobilization processes, the collagen maintains its soft tissue viscoelastic behavior, which is soft and tough [30]. Table 3 shows the results of the elongational strain modulus of the respective gels at 1% and 8% of strain. Cross-linking with EDC/NHS increased the elongational strain modulus approximately five times and immobilization of PMA increased the elongational strain modulus about 12.5 times that of the uncross-linked collagen gel. The cross-linking process and immobilization of PMA made the collagen gel much tougher. This strongly suggests that the PMA must be immobilized onto the surface of the collagen gel in order to maintain its biomolecular property and stronger mechanical property

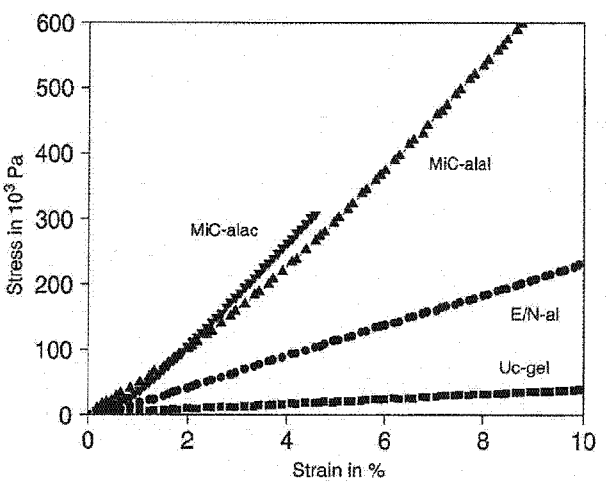


Fig. 5. Stress–strain curve of respective collagen gels.

simultaneously. The EN-1 gel, MiC-21, and MiC-11 gels were too fragile to measure the strain modulus.

Fig. 6 shows the swelling of the respective gels under pH 2.4 and pH 7.4. For uncross-linked gels, the gel dissolved under pH 2.4, while it swelled approximately 1400% under pH 7.4. When collagen gels absorb water, the triple helix structure is known to turn into a random coil conformation because the collagen peptide chains increase the accessibility to hydration. In the neutral and alkaline

Table 3
Mechanical strength of the collagen gels

Sample	Strain modulus at 1% (MPa)	Strain modulus at 8% (MPa)
Uncross-linked	0.4±0.1	0.6±0.1
EN-2 gel	2.1±0.1	2.9±0.2
MiC-21	5.6±1.1	8.7±1.6
MiC-21	5.1±0.6	8.0±1.0

Mechanical strength of EN-1, MiC-11 and MiC-12 was not measured due to fringe nature of the samples.

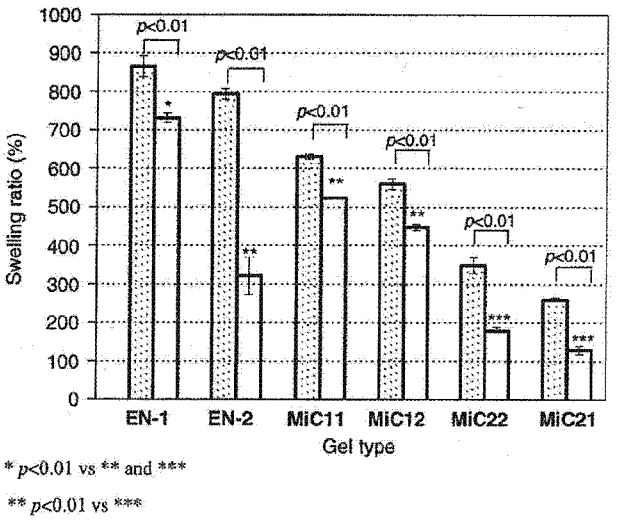


Fig. 6. Swelling ratio of respective collagen gels under pH 2.1 (hatched bar) and under pH 7.4 (empty bar) aqueous solutions. Each value represents the mean±SD ($n = 5$).

conditions, collagen film would be stabilized by forming an entanglement of fibrils formed by hydrophobic and electrostatic bonds [31–33]. Since the pK_a of collagen type I is known to be approximately 5.5 [34,35], a stable gel without any cross-linker can be formed under neutral and alkaline conditions.

The EN-1 and EN-2 gels showed a high swelling ratio under pH 2.1, but had different swelling ratios under neutral pH conditions. The EN-1 gels showed a swelling ratio of about 870% under pH 2.4 and 730% under pH 7.4, while the EN-2 gels showed 800% under pH 2.4 and 320% under pH 7.4. The swelling ratio was relatively higher for the EN-1 gel than the EN-2 gel because the network density was much higher for the EN-2 gel. The EN-2 gel, for which cross-linking was executed under alkaline conditions, is thought to possess a denser cross-linking network. As mentioned earlier, EDC and NHS are known to bring inter- and intrahelical cross-links, holding the α-helices together tightly. [36,37].

Immobilization of PMA on the collagen gels brought different swelling ratios according to the conditions of preparation. For the MiC-11 and MiC-12 gels, the swelling ratio was lower than that for the EN-1 gel, implying that a network between collagen and PMA is formed by the interchain cross-links. However, their swelling ratio under pH 2.4 was lower than that for the EN-2 gel, but was higher under pH 7.4. PMA could not penetrate into the collagen gel during the immobilization process, leaving much of the amine groups unreacted. In contrast, MiC-21 and MiC-22 showed that the swelling ratio under pH 2.4 and pH 7.4 would be lowest among all collagen gels. As mentioned earlier, the formation of a denser network brought a lower swelling ratio. The low swelling ratio of the MiC-11 and MiC-12 gels under pH 2.4 and pH 7.4 implies that the intra- and interhelical cross-links play important roles in the stabilization of the collagen gels.

3.3. Degradation of collagen gels by collagenase

Fig. 7 shows the degradation of collagen gels caused by the activation of collagenase in Tris-HCl buffer. The collagenase absorbed into the collagen gel would cleave the helical segment, hydrolyzing the collagen gels. Collagenase is known to be adsorbed onto the collagen fibers once it penetrates into the fiber [36–39]. Therefore, it is thought that the swelling ratio is related to this biodegradation process.

Our study shows that the collagen gel that is not cross-linked would degrade within 2 or 3 h. Cross-linking the collagen with EDC and NHS would strongly maintain the helical structure, extending the time of complete degradation from 6 to 24 h according to the cross-linking conditions. Low swelling collagen gels lead to slow degradation. For the MiC-22 and MiC-21 gels, almost 80% of the original collagen gel remained after 24 h. The E/N gels have only intra- and interhelical cross-links while the MiC gels possess interchain cross-links. For the E/N

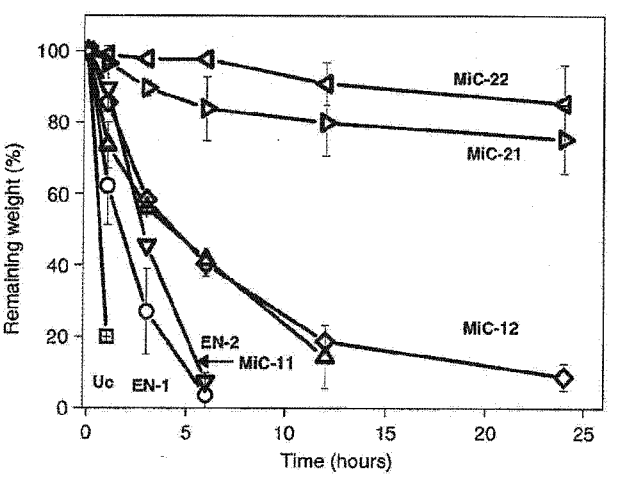


Fig. 7. Degradation of collagen gels by collagenase in Tris-HCl buffer (pH 7.4) at 37 °C. Each value represents the mean±SD ($n = 4$).

gels, the intra- and interhelical cross-links maintained the helical structure after cleavage by collagenase [39]. However, the absorption of water eventually made the E/N gels dissociate within 24 h, with slightly faster degradation for the EN-1 gel. In contrast, the MiC gels possess interchain cross-links that link the microfibrils and the PMA chains, making the degree of swelling much lower [38]. The cleavage by collagenase would be prevented by the PMA-collagen network, which links microfibrils together, shielding the helices.

4. Conclusion

We were able to successfully immobilize MPC to collagen and prepare a stable gel. By using collagen film prepared from 0.5 wt% collagen solution, MiC gels were prepared under MES buffer. EDC/NHS and PMA polymer could form a cross-link with the collagen film. The physical behaviors of the gels changed according to the preparation conditions such as the pH of the MES buffer. Inter- and intrahelical cross-links were formed by EDC/NHS. Higher cross-link efficiency can be obtained under an alkaline condition because the pK_a of collagen is approximately 5.5. A pre-NHS activated PMA polymer chain could be located on the collagen gel and cross-linked with the amine collagen group, forming an interchain cross-link. Since the pK_a of PMA carboxyl groups is 2.7, the immobilization of PMA was successful at any pH. The coexistence of intra- and interhelical cross-links and intermolecular cross-links make the network much denser, which leads to difficulty in either penetration or hydrolyzation by the collagenase. Mechanical and enzyme stability enable this gel to be applied as a biosynthetic hybrid biomaterial.

We will report on the biological properties of the collagen–phospholipid polymer hybrid gel in the near future.

Acknowledgment

This study was financially supported by a grant from the Research on Health Sciences Focusing on Drug Innovation (KH61060) of the Japan Health Sciences Foundation and Health and Labour Sciences Research Grants (HLSRG).

We would like to thank Professor Kazuhiko Ishihara of The University of Tokyo for his helpful advice on phospholipid polymer.

References

[1] Fujioka K, Maeda M, Hojo T, Sano A. Protein release from collagen matrices. Adv Drug Deliv Rev 1998;31:247–66.
[2] Kato YP, Silver FH. Formation of continuous collagen fibers: evaluation of biocompatibility and mechanical properties. Biomaterials 1990;11:169–75.
[3] Olde Damink LHH, Dijkstra PJ, van Luyn MJA, van Wachem PB, Nieuwenhuis P, Feijen J. Crosslinking of dermal sheep collagen using a water-soluble carbodiimide. Biomaterials 1996;17:765–73.
[4] Cascone MG, Barbani N, Cristalli C, Giusti P, Ciardelli G, Lazzeri L. Bioartificial polymer materials based on polysaccharides. J Biomater Sci Polym Edn 2001;12:267–81.
[5] Barbani N, Lazzeri L, Cristalli C, Cascone MG, Polacco G, Pizzirani G. Bioartificial materials based on blends of collagen and poly(acrylic acid). J Appl Polym Sci 1999;72:971–6.
[6] Cascone MG, Martinti S, Barbani N, Laus M. Effect of chitosan and dextran on the properties of poly(vinyl alcohol) hydrogels. J Mater Sci Mater Med 1999;10:431–5.
[7] Sionkowska A. Interaction of collagen and poly(vinyl pyrrolidone) in blends. Eur Polym J 2003;39:2135–40.
[8] Dascălu MC, Vasile C, Silvestre C, Pascu M. On the compatibility of low density polyethylene/hydrolyzed collagen blends. II: new compatibilizers. Eur Polym J 2005;41:1391–402.
[9] Olde Damink LHH, Dijkstra PJ, van Luyn MJA, van Wachem PB, Nieuwenhuis P, Feijen J. Glutaraldehyde as a crosslinking agent for collagen-based biomaterials. J Mater Sci Mater Med 1995;6:460–72.
[10] Sung HW, Chang WH, Ma CY, Lee MH. Crosslinking of biological tissue using genipin and/or carbodiimide. J Biomed Mater Res 2003;64A:427–38.
[11] Orban JM, Wilson LB, Kofroth JA, El-Kurdi MS, Maul TM, Vorp DA. Crosslinking of collagen gels by transglutaminase. J Biomed Mater Res 2004;68:756–62.
[12] Segura T, Chung PH, Shea LD. DNA delivery from hyaluronic acid–collagen hydrogels via a substrate-mediated approach. Biomaterials 2005;26:1575–84.
[13] Wissink MJB, Beernink R, Pieper JS, Poot AA, Engbers GHM, Beugeling T, et al. Immobilization of heparin to EDC/NHS-cross-linked collagen, characterization and in vitro evaluation. Biomaterials 2001;22:151–63.
[14] Perper JS, Hafmans T, Veerkamp JH, van Kuppevelt TH. Development of tailoir-made collagen-glycosaminoglycan matrices: EDC/NHS crosslinking, and ultrastructural aspects. Biomaterials 2000;21:581–93.
[15] Jaworski J, Klapperich CM. Fibroblast remodeling activity at two- and three-dimensional collagen–glycosaminoglycan interfaces. Biomaterials 2006;23:4212–20.
[16] van Luyn MJA, van Wachem PB, Olde Damink LHH, Dijkstra PJ, Feijen J. Relations between in vitro cytotoxicity and crosslinked dermal sheep collagens. J Biomed Mater Res 1992;26:1091–110.
[17] van Wachem PB, van Luyn MJA, Olde Damink LHH, Dijkstra PJ, Nieuwenhuis P. Biocompatibility and tissue regenerating capacity of crosslinked dermal sheep collagen. J Biomed Mater Res 1994;17:353–63.
[18] van Wachem PB, Zeeman R, Dijkstra PJ, Feijen J, Hendriks M, Cahalan PT, et al. Characterization and biocompatibility of epoxy-crosslinked dermal sheep collagens. J Biomed Mater Res 1999;47:270–7.
[19] Ishihara K, Ueda T, Nakabayashi N. Preparation of phospholipid polymers and their properties as polymer hydrogel membranes. Polym J 1990;22:355–60.
[20] Iwasaki Y, Mikami A, Kurita K, Yui N, Ishihara K, Nakabayashi N. Reduction of surface-induced platelet activation on phospholipid polymer. J Biomed Mater Res 1997;36:508–15.
[21] Ishihara K, Nomura H, Mihara T, Kurita K, Iwasaki Y, Nakabayashi N. Why do phospholipid polymers reduce protein adsorption? J Biomed Mater Res 1998;39:323–30.
[22] Kitano H, Imai M, Mori T, Gemmei-Ide M, Yokoyama Y, Ishihara K. Structure of water in the vicinity of phospholipid analogue copolymers as studied by vibrational spectroscopy. Langmuir 2003;19:10260–6.
[23] Ueda H, Watanabe J, Konno T, Takai M, Saito A, Ishihara K. Asymmetrically functional surface properties on biocompatible phospholipid polymer membrane for bioartificial kidney. J Biomed Mater Res A 2006;77:19–27.

[24] Watanabe J, Ishihara K. Phosphorylcholine and poly(D,L-lactic acid) containing copolymers as substrates for cell adhesion. Artif Organs 2003;27:242–8.
[25] Olde Damink LHH, Dijkstra PJ, van Luyn MJA, van Wachem PB, Nieuwenhuis P, Feijen J. In vitro degradation of dermal sheep collagen crosslinked using a water-soluble carbodiimide. Biomaterials 1996;17:679–84.
[26] Nakajima N, Ikada Y. Mechanism of amide formation by carbodiimide for bioconjugation in aqueous media. Bioconjugate Chem 1995;6:123–30.
[27] Flory PJ, Garrett RR. Phase transitions in collagen and gelatin systems. J Am Chem Soc 1958;80:4836–45.
[28] Khor E, Li HC, Wee A. Animal tissue-polypyrrole hybrid biomaterials: shrinkage temperature evaluation. Biomaterials 1996;17:1877–9.
[29] Nam K, Watanabe J, Ishihara K. Modeling of swelling and dissociation mechanism, and release behavior of spontaneously forming hydrogel composed of phospholipid polymers for oral delivery carrier. Int J Pharma 2004;275:259–69.
[30] Gentleman E, Lay AN, Dickerson DA, Nauman EA, Livesay GA, Dee KA. Mechanical characterization of collagen fibers and scaffolds for tissue engineering. Biomaterials 2003;24:3805–13.
[31] Ripamonti A, Roveri N, Briga D. Effects of pH and ionic strength on the structure of collagen fibrils. Biopolymers 1980;19:965–75.
[32] Wallace D. The relative contribution of electrostatic interactions to stabilization of collagen fibrils. Biopolymers 1990;29:1015–26.
[33] Rosenblatt J, Devereux B, Wallace D. Dynamic rheological studies of hydrophobic interactions in injectable collagen biomaterials. J Appl Polym Sci 1993;50:953–63.
[34] Luescher M, Ruegg M, Schindler P. Effect of hydration upon the thermal stability of tropocollagen and its dependence on the presence of neutral salts. Biopolymers 1974;13: 2489–503.
[35] Zhang J, Senger B, Vautier D, Picart C, Schaaf P, Voegel J-C, et al. Natural polyeletrolyte films based on layer-by-layer deposition of collagen and hyaluronic acid. Biomaterials 2005;26: 3353–61.
[36] Zeeman R, Dijkstra PJ, van Wachem PB, van Luyn MJ, Hendriks M, Cahalan PT, et al. Successive epoxy and carbodiimide crosslinking of dermal sheep collagen. Biomaterials 1999;20:921–31.
[37] Vizárová K, Bakos D, Reháková M, Petríkova M, Panáková E, Koller J. Modification of layered atelocollagen: enzymatic degradation and cytotoxicity evaluation. Biomaterials 1995;16: 1217–21.
[38] Park S-N, Park J-C, Kim HO, Song MJ, Suh H. Characterization of porous collagen/hyaluronic acid scaffold modified by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide cross-linking. Biomaterials 2002;23:1205–12.
[39] Ma L, Gao C, Mao Z, Zhou J, Shen J. Enhanced biological stability of collagen porous scaffolds by using amino acids as novel cross-linking bridges. Biomaterials 2004;25:2997–3004.

Transactions of the Materials Research Society of Japan 31[3] 735-738 (2006)

Influence of Cross-linking on Physicochemical and Biological Properties of Collagen-Phospholipid Polymer Hybrid Gel

Kwangwoo Nam, Tsuyoshi Kimura and Akio Kishida
Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University Kanda-Surugadai 2-3-10, Chiyoda-ku, Tokyo 101-0062, Japan.
FAX:81-3-5280-8029, e-mail: bloodnam.fm@tmd.ac.jp

To adopt collagen as a biomaterial, collagen should be modified due to disadvantages such as poor mechanical strength and high thrombogenicity. Preparation of collagen-polymer hybrid gel for application as an artificial vascular graft was executed by immobilizing 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer, poly(MPC-*co*-methacrylic acid) (PMA), using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide and *N*-hydroxysuccinimide as cross-linkers. In order to alter the density of interchain cross-links (intermolecular bonding) between collagen fibrils and the MPC polymer chains, collagen-polymer hybrid gel was prepared by changing the mole ratio of MPC moiety of PMA. The intra- and interhelical cross-links made the gel thermodynamically stable. The interchain cross-links made the gel mechanically and dimensionally stable by supporting the network structure of the hybrid gel, which is thought to be achieved by connecting collagen fibrils. Enzymatic stability was depending on the density of interchain cross-links, because the adsorption of collagenase was prohibited. Increase in the MPC moiety made the gel cell adhesion property decrease. This implies that the interaction between cells and surface of the hybrid gel is being regulated by the MPC groups, making the hybrid gel much efficient for artificial vascular graft use.
Key words: collagen, phospholipids polymer, cross-link, gel, cell adhesion

1. INTRODUCTION

In order to use collagen for a biomaterial product, the cross-linking of collagen and/or immobilizing synthetic polymer with collagen to is indispensable. Non-treated natural collagen cannot directly be applied to the biological system due to disadvantages such as poor mechanical strength, calcium deposition, and high thrombogenicity. However, the collagen is biocompatible and non-antigenic, synergic with bioactive component, easily modifiable, and available in abundance, which makes it suitable for medical application [1-3]. While keeping the advantageous property of collagen, disadvantageous property should be eliminated or be complemented.

Cross-link method using *N*-(3-dimethyl-aminopropyl)-*N'*-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) was chosen for this study [4,5]. Cross-linking collagen with EDC and NHS makes 'zero-length' amide cross-links between carboxylic acid groups from aspartic and glutamic acid residues, and ε-amino groups from (hydroxy-) lysine residues forming intra- and interhelical cross-link to prepare an EDC/NHS cross-link collagen gel [5]. And, 2-methacryloyloxyethyl phosphorylcholine (MPC) based copolymer, which is known for its excellent biocompatibility [6], was used to cross-link the microfibrils of collagen to produce a hybrid gel having biocompatibility and improved mechanical strength.

In this study, we investigated the network structure of the collagen-phospholipid polymer hybrid gel and the effect to the mechanical strength, thermal stability, dimensional stability, and enzymatic stability against collagenase. Furthermore, the biological property of the collagen gel was examined to evaluate the application as an artificial blood vessel.

2. EXPERIMENTAL

2.1 Preparation of EDC and NHS Cross-linked Collagen Gel

Preparation of EDC and NHS cross-linked collagen gel (E/N gel) was executed by using 0.5wt% collagen type I solution (pH 3, KOKEN, Tokyo, Japan). Collagen solution was fabricated into film. Then the collagen film was immersed into the 0.05M 2-morpholinoethane sulfonic acid (MES) buffer (pH 9) (Sigma, St Louis, USA) containing EDC (Kanto Chemicals, Tokyo, Japan) and NHS (Kanto Chemicals, Tokyo, Japan). The cross-linking procedure was executed for 4 hours at 4°C to make a cross-linked gel (E/N-al gel). After 4 hours, the reaction was stopped and the gel was then washed with 4M of Na_2HPO_4 aqueous solution for 2 hours to hydrolyze any remaining *O*-acrylisourea groups and then with distilled water for 3 day to remove salt from the gel. The molar ratio of each chemical was fixed to EDC:NHS:collagen-carboxylic acid groups=5:5:1.

2.2 Preparation of MPC-immobilized Collagen gel

Preparation of the MPC-immobilized Collagen gel (MiC gel) was executed by using E/N-al gel. poly(MPC-*co*-methacrylic acid) (mole ratio; MPC:methacrylic acid=3:7, PMA30) (Figure 1) was added with EDC and NHS in MES buffer (pH 10) and was pre-activated for 10 minutes before E/N-al gel was immersed. The molar ratio of each chemical was fixed to EDC:NHS:carboxylic acid groups of PMA =5:5:1. The immobilization of PMA to the collagen was continued for 4 hours at 4°C. Then the gel was washed with 4M of Na_2HPO_4 aqueous solution for 2 hours and then with distilled water for 1 day to remove salt from the gel to prepare a salt-free MiC30 gel. To increase the MPC moiety of the collagen-polymer hybrid gel, PMA90 (MPC:methacrylic acid=9:1) was prepared and immobilized to the collagen to make a MiC90 gel.

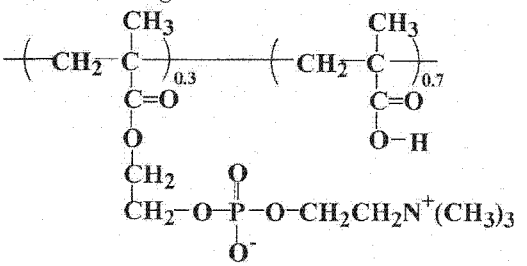


Figure 1.Chemical structure of PMA.

2.3 Surface Characterization

The surface analysis was executed using X-ray photoelectron spectroscopy (XPS; AXIS-HSi, Shimadzu/KRATOS, Kyoto, Japan) and Scanning electron microscopy (SEM; SM-200, Topcon, Tokyo, Japan) Samples which had been cut into small pieces were lyophilized for overnight. The chemical composition of the surfaces of the gel (upper part of the gel) was determined by releasing angle of the photoelectrons fixed at 90°. The morphologies of the gels were observed with a SEM. The razor-blade cut surfaces of respective gels were observed.

2.4 Network Characterization

The shrinkage temperature of the gels were determined using differential scanning calorimeter (DSC; DSC6000, Seiko, Chiba, Japan) in the range of 0°C to 150°C at the scanning rate of 5°C/minute.

Stress-strain curves of respective collagen gels were determined by uniaxial measurements using a tensile strength tester (STA-1150, Orientec, Tokyo, Japan). The sample for the measurement was prepared in the size of 4cm×1cm. The obtained data were changed to stress-strain curve of the samples and the elongation modulus was calculated.

The swelling test of respective samples was executed by cutting lyophilized gels into small pieces and putting into pH aqueous solution at 37°C. The pH of the aqueous solution was controlled to make 7.4. The gels were shaken gently for 24 hours and taken out to measure the changed weight of the sample. Swelling ratio was calculated in order to define the exact swelling phenomenon brought up by water absorption.

2.5 Enzymatic Degradation

The degradation test of the gel samples were executed using collagenase from Clostridiopeptidase histoluticum (EC 3.4.24.3, *Sigma*, St Louis, USA) with collagenase activity of 320 units/mg. In this experiment, collagen gels were immersed into Tris-HCl buffer solution with total concentration of collagenase 100units/mL. The weight of the gels after reaction with collagenase was measured from 1 to 72 hours.

2.6 Cell adhesion test

L-929 cells (mouse fibroblast) were used to evaluate the interaction between collagen gels and the cells. The fibroblasts were culture 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 a 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 surface. The collagen gels were sterilized by putting gels into ethanol:water 50:50 for 2 hours, than 70:30 for 2 hours, and 100:0 for an overnight before lyophilizing. The lyophilized gels were hydrolyzed with E-MEM for 5 minutes and the E-MEM was disposed just before cell seeding. After 24 hours and 48 hours, the number of adhering cells was determined using lactate dehydrogenase (LDH) assay at 560nm with UV/VIS spectrophotometer (V-560, Jasco, Tokyo, Japan).

3. RESULTS AND DICUSSION

3.1 Surface Characterization

All gels showed XPS signals attributed to carbon in CH_3- or $-CH_2$-, -COC-, C(=O)-, and nitrogen in CONH- was observed at 285, 286.6, 288.5, and 400.8eV, respectively. A phosphorus peak and one nitrogen peak in $-N^+(CH_3)_3$ was observed at 134eV and 403.2eV, respectively, indicating that PMA was properly cross-linked collagen [6]. Figure 2 shows the images of the outer surface (upper part of the gel) and razor blade-cut surface (cross-section) morphology of respective collagen gels observed with SEM. All outer surfaces that are immobilized with PMA show non-porous homogenous structure. When the razor-cut surface was observed, relatively porous (or hollow) layer that is composed of many thin plates, and non-porous (or dense) layer was seen. Hollow layer is thought be the uncross-linked collagen (a collagen gel that is prepared under pH 9.0 MES buffer without any cross-linker; Uc gel) or intra- and interhelically cross-linked collagen layer. The non-porous layer representing PMA is deposited on the collagen layer and the thickness increases as more PMA is adopted. However, we are not sure yet how the deposited layer would affect the physical property of the hydrogel. We are working on this and would be reported soon.

3.2 Network Characterization

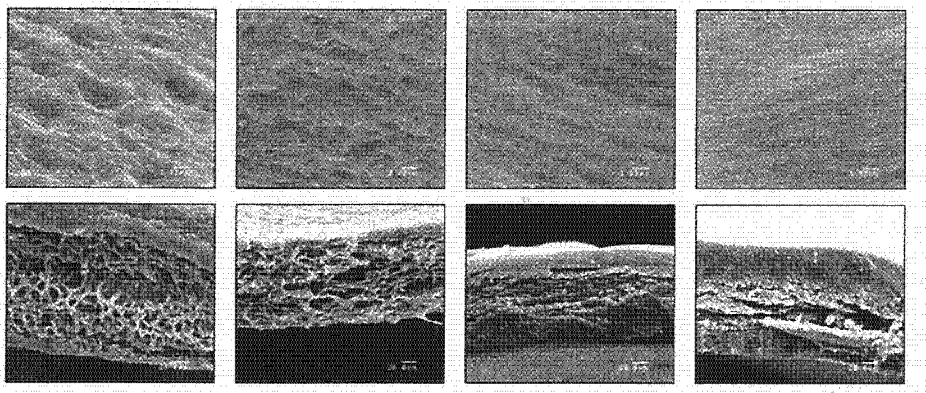


Figure 2. SEM images of collagen gels. (a) Uc gel, (b) E/N gel, (c) MiC90 gel, and (d) MiC30 gels. Upper images imply the outer surface and below images indicate the razor-cut surface of the gels.

Table I shows the change of the shrinkage temperature (T_s) of each collagen gels. The cross-linking brought the increase in the T_s. And the T_s would increase further as the PMA is immobilized, but would never cross 85°C. Since the denaturization temperature is the endothermic transition of the triple helix of the collagen molecules to the random coil, it is believed that intra- and interhelical cross-link controls the T_s [7]. When the higher amount of EDC and NHS was used, the T_s would increase up to 83°C (data not shown). This implies that the increase in the T_s is not due direct connection between collagen microfibrils and polymer chain but due to complexity of the network. So, the stability of the collagen gels against temperature is dependant not only on intra- and interhelical cross-links, but also on the density of the network.

Table I. Shrinkage temperature of respective collagen gels.

Sample	Shrinkage temperature (°C)
Uc gel	56±8
E/N gel	74±3
MiC90	76±3
MiC30	84±4

The elongational modulus increases as PMA is immobilized, indicating it is the interchain cross-link that reinforces the mechanical strength. The elongational modulus of MiC gels measured at 1% strain and 8% strain showed that approximately 10~13 times increase compared to Uc-gel while that of E/N gel showed approximately 5 times increase. This indicates that the network is much denser for MiC gels, which directly affected the mechanical strength.

All collagen gels showed 1.4~2 times increase in elongational modulus at 8% strain compared to that of at 1% strain, indicating soft tissue viscoelastic behavior can be maintained after immobilizing with PMA. So, biomaterial possessing biological property can be obtained.

Figure 3 shows the swelling of the respective gels under pH 7.4. For uncross-linked gel, the gel dissolved under pH 2.1, while swelled approximately 1400% under pH 7.4. When collagen gels absorb water, the triple helix structure is known to turns to random coil conformation, because collagen peptide chains increases accessibility to hydration. In the neutral and alkaline pH conditions, collagen film would be stabilized by forming entanglement of fibrils formed by hydrophobic and electrostatic bonds.

E/N-al gels shows swelling ratio of 320% under pH 7.4. As mentioned earlier, EDC and NHS is known to be bring inter- and intrahelical cross-links, holding the α-helices together tightly. However, its low cross-linking density due to high free amine group contents makes the gel to swell high.

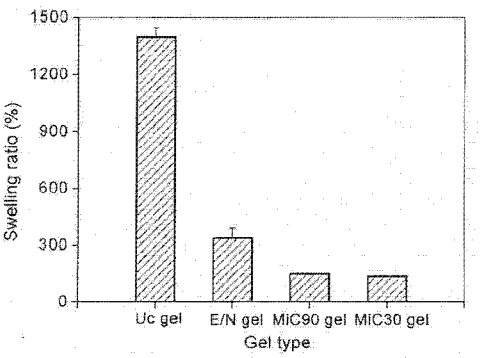


Figure 3. Swelling ratio of respective collagen gels in pH 7.4

MiC gels shows much suppressed swelling ratio. The decrease in the swelling ratio comparing to E/N gel indicates that the dense network has formed. Denser network between collagen and PMA is thought to be formed by interchain cross-links by connecting microfibrils together, increasing the toughness of the collagen gel. Furthermore, the high mechanical strength of the hybrid gel is suppressing the absorption of water, leading to low swelling ratio

3.3 Enzymatic Degradation

Figure 4 shows the degradation of collagen gels caused by the activation of collagenase in Tris-HCl buffer. Collagen gel would be degraded once it encounter with collagenase. Collagenase would cleave the helical segment, which makes the collagen gels to hydrolyze. Collagenase is known to absorb onto the collagen fibers once it penetrates into the fiber [7]. Our study shows that the collagen gel that is not cross-linked would be degraded within 2 or 3 hours. Cross-linking the collagen with EDC and NHS would maintain the helical structure firmly, extending the complete degradation time from 3 hours to 24 hours. And as mentioned previously, the E/N-al gel possesses higher intra- and intercross-link chains, making the gel to endure longer time against collagenase.

MiC gels showed higher stability against collagenase. The network of the collagen gel is thought to be denser than E/N gel, as described previously. For E/N gels, the absorption of collagenase eventually made it to be dissociated within 24 hours. On the other hand, MiC gels possess interchain cross-link, which links the microfibrils and the PMA chains, making the gel to swell much lower. And the cleavage by collagenase would be prevented by the PMA-collagen network which links fiber and polymer chain together, shielding the helices

Comparing the degradation rate between MiC gels, we can see that as MPC ratio increases, the degradation is much faster. This is because the network of the MiC90 is thought to be much sparse than MiC30, due to low mole ratio of methacrylic acid moiety. This makes the space between collagen and PMA larger, resulting in higher water absorbance.

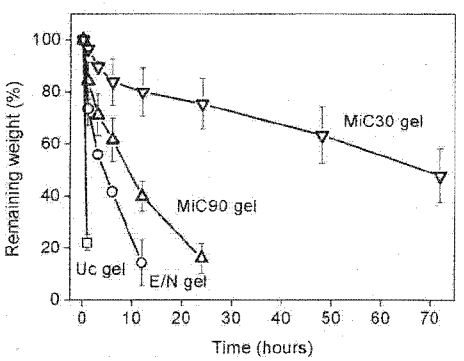


Figure 4. Degradation of collagen gels by collagenase in Tris-HCl buffer (pH 7.4) at 37°C.

3.4 Cell Adhesive property

When the number of adhered cells after 24 hours and 48 hours were compared among the collagen gels, and those with PMA immobilized on collagen gels were much lower than without PMA. This is clearly due to PMA polymer covering the surface of the collagen gel. The number of cell adhered on the surface decreased as the moiety of MPC unit increased. The difference between number of adhered cell on the surface after 24 and 48 hours was compared, the increase in the number of cells was observed for collagen gels. However, for MiC gels, increase was suppressed. Polymer immobilized on the collagen blocks the interaction between fibroblast and collagen, which is known to be the most decisive factor for cell adhesion [8].

4. CONCLUSION

The preparation of MiC gel was successfully achieved. Immobilization of MPC polymer made the gel tougher and stable. We could confirm that the stress-strain responded as generally observed for soft biological materials. Increase in the MPC unit brought the higher swelling, which lead to the faster degradation by collagenase. It is thought that the higher amount of adopted PMA have caused the formation of sparse cross-link network, which in turn make the surface of the MiC gel full of MPC head groups, reducing cell adhesion ability.

The increase in the MPC unit would bring higher biocompatilibty, while increase of MA unit would allow increment of mechanical strength. As the concentration of MPC increased, it is thought that the biocompatibility would increase but toughness decrease.

ACKNOWLEDGEMENT

This study was financially supported by Grant from the research on Health Science focusing on Drug Innovation (KH61060) of the Japan Health Sciences Foundation and Health and Labour Sciences Research Grants.

REFERENCES

[1] W. Friess, *Eur. J. Pharm. Biopharm.*, **45**, 112-136 (1998).
[2] K. Fujioka, M. Maeda, T. Hojo, and A. Sano, *Adv. Drug Del. Rev.*, **31**, 247-266 (1998).
[3] C.H. Lee, A. Singla, and Y. Lee, *Int. J. Pharma.*, **221**, 1-22 (2001).
[4] L.H.H. Olde Damink, P.J. Dijkstra, M.J.A. van Luyn, P.B. van Wachem, P. Nieuwenhuis, and J. Feijen, *Biomaterials*,**17**, 765-773 (1996).
[5] M.J.B. Wissink, R. Beernink, J.S. Pieper, A.A. Poot, G.H.M. Engbers, T. Beugeling, W.G. van Aken, and J. Feijen, *Biomaterials*, **22**, 151-163 (2001).
[6] K. Ishihara, T. Ueda, and N. Nakabayashi, *Polym. J.*, **22**, 355–360 (1990).
[7] R. Zeeman, P.J. Dijkstra, P.B. van Wachem, M.J. van Luyn, M. Hendriks, P.T. Cahalan, and J. Feijen, *Biomaterials*,**20**, 921-931 (1999).
[8] J. Watanabe and K. Ishihara, *Artif Organs*, **27**, 242-248 (2003).

(Received January 31, 2006;Accepted June 2, 2006)

ARTICLE IN PRESS

Available online at www.sciencedirect.com

ScienceDirect

Biomaterials

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

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.

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

Please cite this article as: Nam K, et al. Physical and biological properties of collagen-phospholipid polymer hybrid gels. Biomaterials (2007), doi:10.1016/j.biomaterials.2007.03.001