再生医療におけるリン酸カルシウムによる遺伝子導入は、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。また、Laurencin らは、リン酸カルシウム／DNA／PLAGA マイクロスフィアーを調製し、このマイクロスフィアー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。一方、リン酸カルシウムのうち HAp、α－TCP は、骨補填材料（人工骨）としてすで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良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更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。

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＜用意＞
・2.5M $CaCl_2$：フィルター滅菌後-20℃で保存
・2×BBS(BES buffered saline)：50mM BES [N, N- bis (2-hydroxyethyl)-2-aminoethanesulfonic acid]，280mM NaCl, 1.5mM Na_2HPO_4（pH 6.95）（2×HBSでもよい）
・滅菌水：超純水をフィルター滅菌
・DNA：1mg/ml溶液
・対数増殖期の細胞と培地

＜操作＞
①2.5M $CaCl_2$をフィルター滅菌したMilliQ水で10倍希釈する。
②20～30μgのDNAを調製した0.25M $CaCl_2$ 0.5mlにて溶解する。
③ボルッテクスをかけながら2×BBSを0.5ml滴下する。
④室温にて10～20分間静置する。
⑤対数増殖期の細胞に添加する。
⑥5%COで15~24時間培養する。

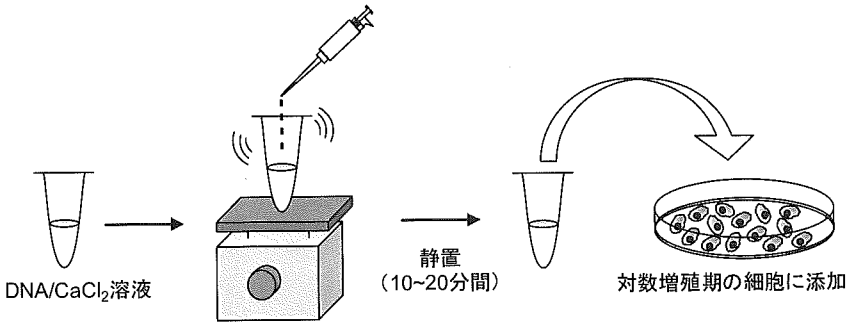

図１、リン酸カルシウム法による培養細胞への遺伝子導入[2)]

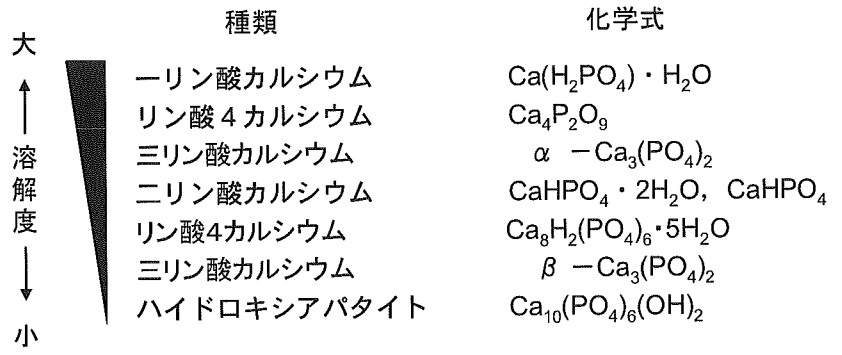

図2、リン酸カルシウムの種類と水中での溶解性
（出展：驚異の生体物質アパタイト、青木秀希著、医歯薬出版株式会社より改変）

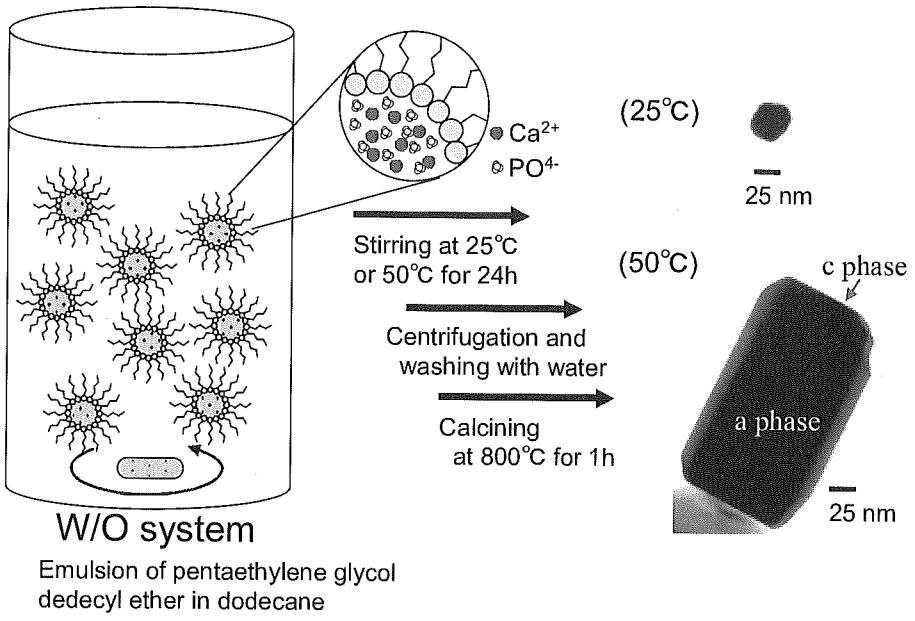

図３、ミクロエマルジョン法によるハイドロキシアパタイトの調製[7)]

Original Article

Preparation of PVA/DNA hydrogels via hydrogen bonds by ultra high pressure treatment and controlled release of DNA from hydrogels for gene delivery

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Abstract

We have developed polyvinyl alcohol (PVA) hydrogels incorporating DNA mediated hydrogen bonding interaction by ultra high pressure (UHP) technology for gene delivery system based on controlled release of DNA. Mixture solutions of DNA and PVA at various concentrations were treated by UHP processing at 980MPa for 10min. The good PVA/DNA hydrogels were obtained more than 5 w/v% of PVA concentration. The incorporation of DNA into hydrogels was confirmed by spectroscopic analysis and nucleic acid dye staining. The sustained release of DNA from the PVA/DNA hydrogels was investigated using hydrogels prepared by UHP treatment and freeze-thawing methods at different PVA concentrations. Various release profiles were attained by varying PVA concentration in hydrogels and preparation method. The total amount of DNA released from the hydrogels decreased with increasing the PVA content of the hydrogels. Furthermore, using PVA/DNA hydrogels obtained by UHP treatment, the cumulative release was reduced comparison with hydrogels prepared by freeze-thawing method. The intact fractions of DNA released from hydrogels were exhibited by agarose gel electrophoretic analysis, suggesting the possibility of transfection using PVA/DNA hydrogel prepared by UHP treatment.

Introduction

Non-viral gene delivery systems are of great interest in gene therapy due to their safety advantages compared with virus [1,2]. In many cases, several cationic synthetic materials such as cationic lipids, liposome [3], polyethyleneimine [4], polyamideamine dendrimer [5], poly-L-lysine(PLL), PLL derivatives [6], and other cationic peptides [7], were used as non-viral vectors. They were possible to form the complex with DNA by the electrostatic interaction of cationic groups of them and anionic groups of DNA,

resulting in the protection of DNA from nuclease degradation and the effective transfection into mammalian cells [8,9]. However, the cytotoxicity of cationic materials remains as one of important problems [10,11]. Moreover, cationic lipids and polymers have been described as displaying pro-inflammatory properties [12]. For more safe and efficient gene delivery system, it is necessary to develop novel, non or less cationic, gene delivery system, which not be based on electrostatic interaction with DNA. Previously, we reported that nano-particles consisting polyvinyl alcohol (PVA) and DNA via hydrogen bonding interaction were formulated using ultra high pressure (UHP) technology [13]. With high pressure process, it is well known that the inter-, intra molecular week hydrogen bonding interaction was empathized [14]. The PVA/DNA particles were transferred into mammalian cells.

Recently, controlled release of DNA was also investigated as one of possible trials to enhance the transfection efficiency using various biomaterials such as poly(lactide-co-glycolide)(PLGA)[15], hyaluronic acid[16], atelocollagen[17], and gelatin[18,19]. Mooney et al. reported that the sustained delivery of DNA from PLGA led to effective transfection of large number of cells in vitro and in vivo[15]. However, it is difficult to regulate the release of DNA owing to DNA molecules being loaded in PLGA without particular interaction forces, such as covalent, electrostatic and hydrogen bonding, with polymer molecules. Tabata et al. reported that the enhancement and prolongation of gene expression were achieved using the cationized gelatin hydrogel interacting with DNA electrostatically. The controlled release of DNA depended on hydrogel degradation, while the cationized gelatin hydrogel was crosslinked by glutaraldehyde, which has generally cytotoxic property, to obtain different cationization extents [18,19]. Therefore, in this study, we investigated the preparation of PVA/DNA hydrogels having hydrogen bonding interaction of DNA and

PVA molecules using UHP technology for controlled release of DNA. PVA is one of the most widely used for biomedical applications and possible to interact with DNA using UHP technology described above. We examined the release of DNA from PVA/DNA hydrogels prepared by UHP processing and freeze-thawing method, which is general method for the preparation of PVA hydrogel via hydrogen bonds [20]. The stability of DNA released from hydrogels was also studied.

Materials and Methods

Preparation of PVA/DNA hydrogels by ultra high pressure treatment

PVA having average molecular weight of 22000 and degree of saponification of 99.8 % was supplied from Kuraray Co., Ltd (Osaka, Japan). PVA solutions with various concentrations (6, 8, 10, 14 and 20 w/v%) were prepared by autoclaving for 30min at 121°C three times. They were mixed with distilled water at the ratio of 1:1 to obtain PVA solution of 3, 4, 5, 7, 10 w/v%. Salmon sperm DNA (Wako, Ltd, Osaka, Japan) was dissolved in Tris-EDTA buffer (TE, pH=7.8) at the concentration of 16.3mg/ml. The DNA solution was mixed with PVA solutions (10, 14, 20 w/v%) at the ratio of 1:1 in order to obtain PVA/DNA hydrogels. The 0.7ml of the mixture or PVA solutions was transferred in silicon tube (Ø9mm × 25mm) and both ends of them were capped by silicon plugs. They were treated under 980MPa at 37 °C for 10 min (UHP treatment) using high-pressure machine (Dr.Chef, Kobe Steal, Kobe, Japan). As a control, PVA/DNA hydrogels were also obtained by 4 cycles of freeze-thawing. Each cycle consisted of 2h at -80 °C and 1h at room temperature.

Confirmation of DNA included in the PVA/DNA hydrogels

The DNA content in PVA/DNA hydrogel was confirmed by two methods, staining with nucleic acid dye and UV-Visible spectroscopy. The PVA/DNA hydrogels were soaked into nucleic acid dye solution (Mupid blue, ADVANCE Co., Ltd, Tokyo, Japan) for 1 min. For depolarization, the gel was soaked by 70% ethanol for 1min and ion exchanged water for 1min. On the other hand, the PVA/DNA hydrogels were melted at 98 °C for 10 min. DNA concentration of them was measured by spectrophotometer (V-560, JASC Co., Tokyo, Japan).

DNA release from PVA/DNA hydrogels

The PVA/DNA hydrogels prepared by UHP and freeze-thawing methods were immersed in 5ml of PBS for 144h at 37°C. At 15min, 30min, 2, 3, 15, 27, 48, 111 and 144h, samples of the outer solution (20 μl) was withdrawn and then DNA concentration was measured spectrophotometerically at 260nm(Gene Quant pro S, Amersham pharmacia biotec, Tokyo, Japan).

Agarose gel electrophoresis

DNA released from PVA/DNA hydrogels was analyzed by agarose gel electrophoresis. Ten μl of the outer solution collected at several times was electrophoresed by 0.8% agarose gel for 30 min at 100V. The gel was immersed in ethidium bromide solution to stain DNA.

Plasmid DNA (pDNA) (pEGFP-N1, BD science, CA, USA) was used instead of salmon sperm DNA in order to prepare PVA/pDNA hydrogel. The pDNA solution (200

ng/ml) was mixed with PVA solution of 10, 14 and 20w/v%, and then treated by UHP processing. The PVA/pDNA hydrogels were inserted into the slots of 0.8% agarose gel, after 1h incubation, the electrophoretic analysis was carried out at 100V for 30min. Moreover, after the electrophoresis for 30min, the agarose gel including the PVA/DNA hydrogels was left for further 1h and then the electrophoretic analysis was performed again. The gel was immersed in ethidium bromide solution to stain DNA.

Results and discussion

Figure 1 shows the PVA and PVA/DNA hydrogels prepared by UHP treatment. In the case of 3 w/v% PVA solution, the clear aqueous PVA transformed into a white turbid and viscous solution by UHP treatment (Fig.1 (A)). By scanning electron microscopic (SEM) observation of the PVA solution, the aggregation of PVA particles having average diameter of 1μm were revealed (data not shown). With more than 4 w/v% of PVA concentration, the PVA hydrogels were obtained by UHP treatment. Although the surface of 4 w/v% PVA hydrogels was fragile and sol state (Fig. 1 (B)), the good hydrogels were obtained with more than 5 w/v% of PVA concentration (Fig.1 (C), (D)). They were melted by heating at 90°C for 10min, indicating that the UHP treatment for PVA solution caused the aqueous PVA to cross-link via hydrogen bonding of hydroxyl groups on the PVA molecules. The strength of them also increased with increasing PVA concentration, indicating that more tightly cross-linked polymer gel structure was formed with increasing PVA concentration.

When the mixture solutions of PVA and DNA were treated by UHP processing, hydrogels were formed at more than PVA concentration of 5 w/v% (Fig.1 (E), (F)). In order to confirm whether the hydrogels contain DNA, the hydrogels were melted by

heating at 90 °C for 10 min and the DNA concentrations were measured spectrophotometrically at 260 nm. Almost equal amount of DNA was contained in every hydrogels (Fig.2 (A)). Also, when the hydrogels were stained by nucleic acid dye, the blue stained hydrogels were observed in the only case of PVA/DNA hydrogels, but not PVA hydrogels (Fig.2 (B)). The PVA/DNA hydrogel of 5 w/v% was strongly stained comparison with the 10 w/v% of PVA/DNA hydrogels, suggesting the tight and multipoint interaction of PVA and DNA molecules via hydrogen bonds in the case of 10 w/v% PVA/DNA hydrogel.

In order to investigate the release of DNA from PVA/DNA hydrogels, as a control, PVA/DNA hydrogels were prepared by freeze-thawing methods, which is general method for PVA hydrogel preparation [20]. With freeze-thawing method, it took 12 hours to prepare PVA/DNA hydrogels, while PVA/DNA hydrogels were obtained for only 10 minutes using UHP processing, indicating that the UHP is one of powerful tools for material preparation. The release profiles of DNA from PVA/DNA hydrogels are shown in figure 3. Various release profiles were observed with hydrogels varying PVA content in hydrogels and preparation method. At 5w/v% of PVA concentration, DNA was released out very fast in early incubation stage in the case of freeze-thawing method. The initial burst release is contributed to the loose network structure that could not retard the rapid diffusion of DNA. With UHP treatment, the initial burst and the total amount of DNA released from PVA/DNA hydrogel markedly reduced comparison with freeze-thawing method. It indicates that the hydrogen bonding interactions of PVA and DNA molecules were formed in hydrogel, resulting in the formation of tight network structure. Additionally, different structures in hydrogels prepared by freeze-thawing and UHP methods were also observed by SEM observation (data not shown), suggesting the effect of the formulation of network structure on the release of DNA. At

10w/v% of PVA/DNA hydrogels, the release profiles of DNA from PVA/DNA hydrogels showed much reduced burst releases and sustained release patterns comparison with 5w/v% of that. This is good accordance with the result of being stained easily with 5 w/v% PVA/DNA hydrogels by nucleic acid dye. It is considered that the extent of cross-linking and the entanglements of PVA and DNA molecules increased, resulting in prolonged retention of DNA. Also, in the case of UHP method, the cumulative release of DNA was slightly degreased comparison with freeze-thawing method. These results indicate that the release pattern of DNA from PVA/DNA hydrogels is affected by not only the cross-linking degree in hydrogels, but also the interaction of PVA and DNA molecules.

It is important that DNA is released from hydrogels without structural change and degradation[2,21]. In order to confirm the stability of DNA released from PVA/DNA hydrogels, the equal amount of DNA in the outer solution immersing hydrogels was electrophoresed with agarose gel at 100V for 30min (Fig 4). The similar smear DNA band was observed in every case as well as naked salmon sperm DNA, indicating that the intact DNA was released from hydrogels. Also, the shift for low molecular weight was observed in DNA released from PVA/DNA hydrogels. Particularly, in the case of 10w/v% PVA/DNA hydrogels, the DNA fractions were shifted to the low molecular weight side. It is considered that the release of low molecular weight of DNA from hydrogels is easier than that of high molecular weight. As a result, the bands appeared to migrate for low molecular weight side when same amount of DNA was loaded on agarose gel electrophoresis. This is good agreement with the result that the higher molecular weight of DNA tended to form PVA/DNA nano-particles [13]. Also, the further shift was observed slightly using UHP treatment, indicating the enhancement of interactions between DNA and PVA molecules.

Plasmid DNA (pDNA) was used instead of salmon sperm DNA. PVA/pDNA hydrogels of 5, 7 and 10w/v% of PVA concentration were obtained by UHP treatment and then agarose gel electrophoretic analysis was carried out. In the case of naked pDNA, the fractions of the pDNA were observed in the gel. On the other hand, for PVA/pDNA hydrogels, the bands of pDNA ware observed in the gel and slot, signifying pDNA released from hydrogel and remained in hydrogel, respectively. Also, the amount of pDNA release decreased with increasing PVA content in hydrogel. These results indicate that PVA/pDNA hydrogels are prepared by UHP treatment and pDNA is released from that as well as salmon sperm DNA. After further 1h incubation, when gel electrophoresis was carried out again, the new bands of pDNA in the proximity of slot appeared in addition to primary bands, indicating sustained release of pDNA from hydrogels.

Conclusion

We have developed hydrogels consisting PVA and DNA via hydrogen bonds using UHP technology. The incorporation of DNA into hydrogels was confirmed by spectroscopic analysis and nucleic dye stain. The sustained release of DNA from PVA/DNA hydrogels was achieved and then its profile was highly dependent on the PVA concentration used. The stability of DNA released from hydrogels was also shown, and PVA/DNA hydrogels are considered as a potential candidate for gene delivery.

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Captions

Figure 1.

Photos of PVA hydrogels (A-D) and PVA/DNA hydrogels (E,F) at the concentration of (A) 3 w/v%, (B) 4w/v%, (C),(E) 5w/v% and (D),(F) 10w/v% obtained by ultra high pressure treatment.

Figure 2

Confirmation of containing of DNA in PVA/DNA hydrogels. (A) Amount of DNA in solution obtained by melting PVA/DNA hydrogels prepared using UHP processing. (B) Photos of hydrogels stained by nucleic acid dye.

Figure 3

DNA release profiles from hydrogels prepared by different method; (○,●) 5w/v% and (□,■) 10w/v% of PVA concentration. Opened and closed symbols indicate hydrogels prepared by freeze-thawing and UHP methods, respectively.

Figure 4

Agarose gel electoroporetic analysis of salmon sperm DNA released from PVA/DNA hydrogels prepared by freeze-thawing and UHP methods. An equal amount of DNA was loaded into each lane.

Figure 5

The formation of PVA/plasmid DNA hydrogels by UHP methods and the release of plasmid DNA. Agarose gel electrophoresis was carried out twice. (A) After PVA/

plasmid DNA hydrogels were inserted into slot of agarose gel and incubated for 1 hour, the electrophoresis was carried out. (B)After further 1 hour incubation, the electrophoresis was carried out again.

Figure 1

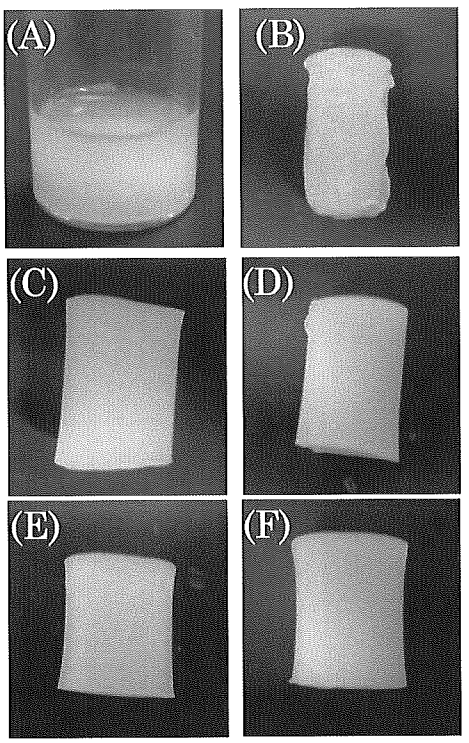
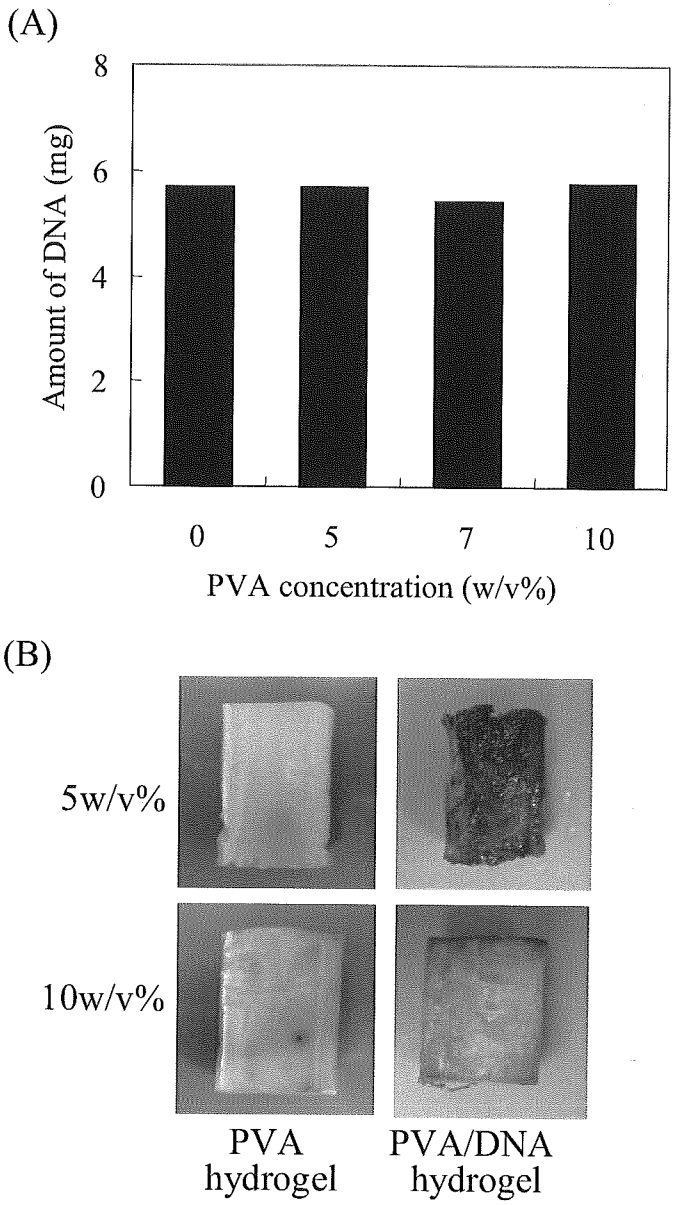


Figure 2


(A)
Amount of DNA (mg)
8
6
4
2
0
0
5
7
10
PVA concentration (w/v%)
(B)
5w/v%
10w/v%
PVA hydrogel
PVA/DNA hydrogel