

An evaluation on PAH degradation and characteristics as media of PVA-derivative hydrogels prepared by using a CGA technique

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Abstract—We manufactured PVA-derived hydrogels with some crosslinkers by using a foam generation technique. Amino acids gels showed remarkably higher swelling ratios, probably because of the highly crosslinked network along with hydrogen bonds. Boric acid and starch would catalyze dehydration while structuring to result in much lower water content and accordingly high gel content, leading to less elastic, hard gels. Bulky materials such as ascorbic acid or starch produced, in general, large pores, and also nicotinamide, highly hydrophobic, was likely to enlarge its pore size, thus leading to reduced swelling. Hydrophilic (or hydrophobic), functional groups which are involved in the reaction or physical linkage, and bulkiness of crosslinkers were found to be more critical to the crosslinking structure and its density than molecular weights that seemed to be closely related to pore sizes. The average sizes of pores were 20 μm for methionine, 10-15 μm for citric acid, 50-70 μm for L-ascorbic acid, 30-40 μm for nicotinamide, and 70-80 μm for starch. Also, amino acid and glucose gels were more elastic than the others. The elasticity of a gel was reasonably correlated with its water content or swelling ratio. On the other hand, L-ascorbic acid among glucose, methionine, citric acid and vitamins, imparted not only the most favorable physical properties and the greatest cell density but also the highest PAH degradation on its derivative gels. The higher biomass ensured the higher degradation rate. The maximum cell density was 0.267 mg/g-hydrogel and degradation rates and efficiencies ranged 0.013-0.007 mM/mg/day and 92-48%, respectively.

Key words: PVA, Hydrogel, CGA Technique, Cell Adherence, Additive, Amino Acid, Organic Acid, Lipid, Saccharide, Microbial Immobilization

INTRODUCTION

Hydrogel, having a 3-dimensional network structure in a state of solid and liquid partly, is a physically and/or chemically cross-linked polymer whose peculiar feature is great restoration ability. Also, it has a strong water hold even under a reasonable pressure unlike a sponge or pulp because of its swelling capacity [1-3]. As a cross-linked polymer, hydrogel has been used for soil stabilizers [4], controlled release of drugs, separation of solutes, and particularly a part of the drug delivery system and immobilization of enzymes and microbes in biomedical applications [5]. Many researches on drug release *in vivo* by the relaxation of polymer chains in hydrogel have been carried out so far [6,7]. Ladam et al. used a hyaluronic acid-hydrogel for regeneration of brain, bone cells and blood vessel tissues [8]. Also, effects of swelling and contraction of hydrogels were investigated in terms of pH, temperature, and other surrounding changes [9-13]. Hydrogels are commercially applied to blood contact areas, i.e., blood vessels, blood conveyers, separation of blood serum proteins. In addition, they are used as artificial skin, contact lenses, and other implanting material, exploiting their exceptional biocompatibility and biodecomposability [5].

Porous hydrogels have been prepared by some conventional methods like salt leachate, templating, and freezing/thawing [14]. And recently, CGA (colloidal gas aphon) foaming method was intro-

duced. CGA foaming was developed by Sebba [15] first who used surfactant solutions with vigorous agitation in a foam generator. CGA-hydrogels, whose pores range from 50-300 μm can be formed with agitation of 4,000-8,000 rpm, followed by immediate freeze-drying [16]. The created pores and their structure are a necessity for microbial immobilization.

PAH (Polycyclic aromatic hydrocarbon) in nature is one of the most persistent chemicals. Its high hydrophobicity and strong residuality in water and soil environment make them toxic, refractory compounds which are apt to accumulate in the biosphere, including human beings, for a long time [17]. Degradation of PAHs has focused on the distribution, toxicity and biological decomposition for decades. In particular, biodecomposition has been acknowledged as a promising alternative to chemical methods that would be costly and cause secondary pollution because many isolated bacteria and most of the fungi found in nature could metabolite PAH with ease converting to detoxicated compounds including carbon dioxide [18-20]. In the US, the biological remediation occupies about 20% of all remediation cases such as organic solvent, surfactant flushing and so forth.

This work has two parts: one is to manufacture PVA hydrogels by using the CGA technique based on Sebba's. The other is to investigate biodegradation capability of a bacterial strain encapsured in the hydrogel. Several different hydrogels were prepared using additives like amino acids, sugars, etc. and were tested in terms of water content, swelling, and gel content. Cell (*Pseudomonas aeruginosa*) immobilized gels were submerged in PAH solutions to test the biodegradability. Also, microbial quantification along with deg-

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Table 1. Physical properties of additives used in this study

	Additive	Molecular formula	Molecular weight
Amino acid	DL-Methionine	$\text{CH}_3\text{SCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$	149.21
	Glycine	$\text{H}_2\text{NCH}_2\text{COOH}$	75.07
Organic acid	Citric acid anhydrous	$\text{C}_6\text{H}_8\text{O}_7$	192.12
	Boric acid	H_3BO_3	61.831
Vitamin	L-Ascorbic acid	$\text{C}_6\text{H}_8\text{O}_6$	176.13
	Nicotinamide	$\text{C}_6\text{H}_6\text{NO}_2$	123.11
Sugar	Starch	-	-
	Glucose	$\text{C}_6\text{H}_{12}\text{O}_6$	180.16

radation kinetics was done and some of the inside surfaces of the gels were observed with the SEM to verify the pores and existence of microbes.

EXPERIMENTAL

1. Manufacturing Hydrogels

Poly(vinyl alcohol) (PVA; 99+% hydrolyzed, Avg. Mw. 89,000-98,000) and glycine were purchased from Sigma-Aldrich. The other additives such as DL-methionine (D- and L-form mixture), citric acid anhydrous, nicotinamide, starch, glucose were used as received (Duksan Chemical, Korea). Boric acid and L-ascorbic acid were from Dong-Yang Silicon (Korea). Table 1 shows all the additives and their characteristics.

Twenty grams of PVA were stirred at 60-70 °C for 6 h to make 20 wt% PVA solution. Appropriate amounts of an additive (5, 10, 15, 20, and 30 wt%) were mixed well with the PVA solution to form a mixture. The mixture was agitated at 6,000 rpm for 10 min by using the CGA generator (SH-100, GLOBAL LAB) (Fig. 1). The foam mixture was frozen at -42 °C for 24 h and then was freeze-dried (FD-1000, RIKAKIAI) at -50.7 °C and 15.8 Pa for 24 h.

2. Gel Analysis

2-1. Water Content (WC)

We weighed (W_s) a gel cube (1 cm³) and weighed (W_d) it again

after ca 24 h drying in the 120 °C vacuum oven.

$$\text{WC}(\%) = \frac{W_s - W_d}{W_s} \times 100 \quad (1)$$

2-2. Swelling Ratio (SR)

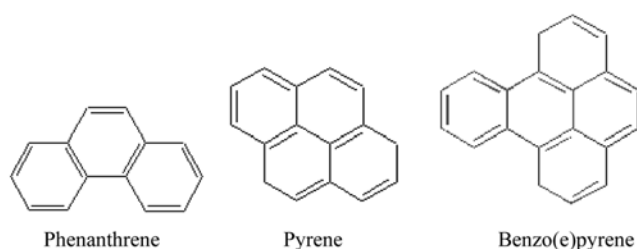
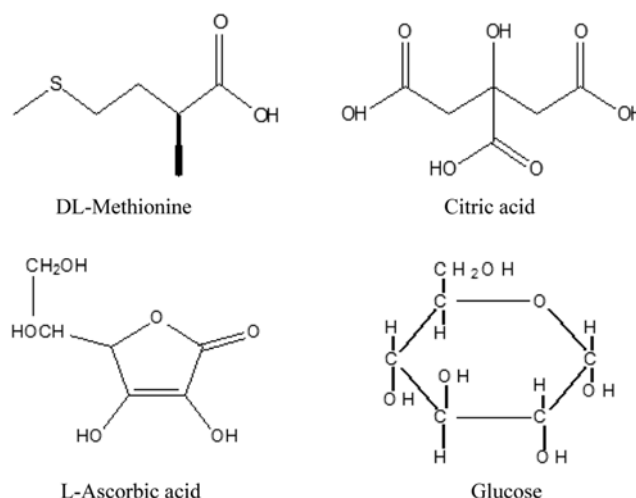
A gel cube was freeze-dried (W_d) and the gel was immersed in distilled water for 24 h and then the surface moisture was removed by using a filter paper before weighing (W_s).

$$\text{SR}(\%) = \frac{W_s - W_d}{W_d} \times 100 \quad (2)$$

2-3. Gel Content (GC)

A gel cube (W_d) was extracted in ethanol for 24 h and dried at 120 °C for 1 h (W_s).

$$\text{GC}(\%) = \frac{W_d}{W_s} \times 100 \quad (3)$$

**Fig. 1. Target PAHs used in this work.****Fig. 2. Structures of additives used in this work.****Table 2. Sensory test for hydrogels used in here**

Elastic	A little elastic	A little brittle	Brittle
Glycine (5%, 10%, 15%, 20%, 30%), DL-methionine (20%)	DL-Methionine (5%, 10%, 15%, 30%)	L-Ascorbic acid (30%)	Citric acid (15%, 20%, 30%)
	Glucose acid (10%, 15%, 20%)	Nicotinamide (5%, 10%)	Boric acid (5%, 10%, 15%, 20%, 30%)
	L-Ascorbic acid (5%, 10%, 15%, 20%)	Glucose acid (5%, 30%)	Nicotinamide (15%, 20%, 30%)
	Citric acid (5%, 10%)	Boric acid (15%, 20%)	Boric acid (30%)
	Boric acid (5%, 10%)		

2-4. Sensory Test

Mechanical and material characteristics sensed by touch are tabulated in Table 2.

2-5. SEM Observation

A cutting specimen of a gel was prepared for electron microscopic observation (JSM-5200, JEOL, Japan).

3. Strain and Cultivation

Pseudomonas aeruginosa (KCCM 40396) from KMCC (Korea) was used for microbial degradation. The cells were cultivated in a yeast extract growth medium at 37 °C and 120 rpm for 24 h. The average cell concentration was 1.3 g/L ($\mu_{max}=0.031\text{ h}^{-1}$).

4. Cell Adhesion and Immobilization

4-1. Cell Adhesion

A gel piece (0.4 g) was immersed in a solution containing the cells for 48 h. Then, the gel was washed with distilled water thoroughly before further experiment.

4-2. Cell Immobilization

We completely mixed 100 ml of 20 w% PVA solution and 10 ml of additive solution. Then added 50 ml of the cell culture to the mixture and then froze it at -50 °C for 24 h. The freeze-dried sample for another 24 h was washed with water.

4-3. Observation of Microbe-embedded Gel

Hydrogel was sliced into thin specimens. A carbon-taped speci-

men without any coat was observed and photographed at 20 KV with magnifications of 100 and 2,000.

4-4. Microbial Quantification

Nitrogen content of a completely disrupted cell lysate was measured with the BCA (Bicinchoninic acid) kit (Sigma-Aldrich) at 562 nm. The nitrogen content was converted to the dry weight of *Pseudomonas* whose average nitrogen content per cell was determined in the lab.

5. Degradation of PAH

Ten mM of each PAH (phenanthrene, pyrene, and benzo(e)pyrene) was prepared. Ten ml of the solution was positioned in a 20 ml-bottle and a microbe-carrying gel was put in the solution. At every 24 h the residual PAH concentration was measured with the fluorospectrometer (Hitachi FL-4500) under the conditions of the excitation/emission wavelengths, 250.0/360.0 nm; width of the slit, 5.0/1.0 nm; 400 V; room temperature.

RESULTS AND DISCUSSION

1. General PVA-Hydrogels

PVA hydrogels were prepared in 20 wt% and their physical prop-

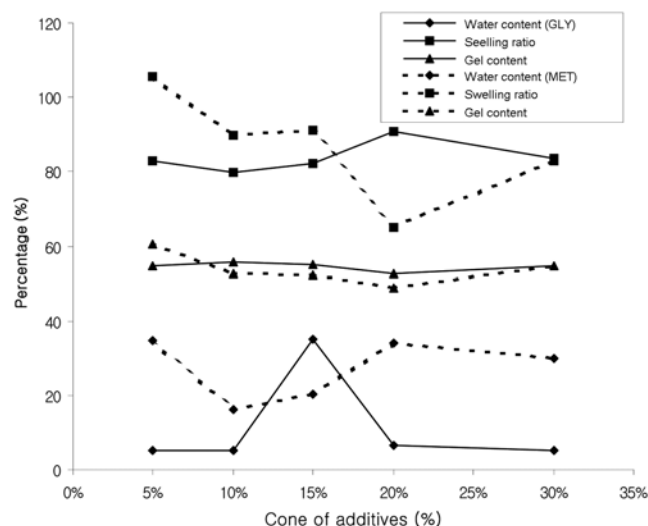


Fig. 3. Comparison of PVA/amino acid hydrogels: additives are glycine and DL-methionine.

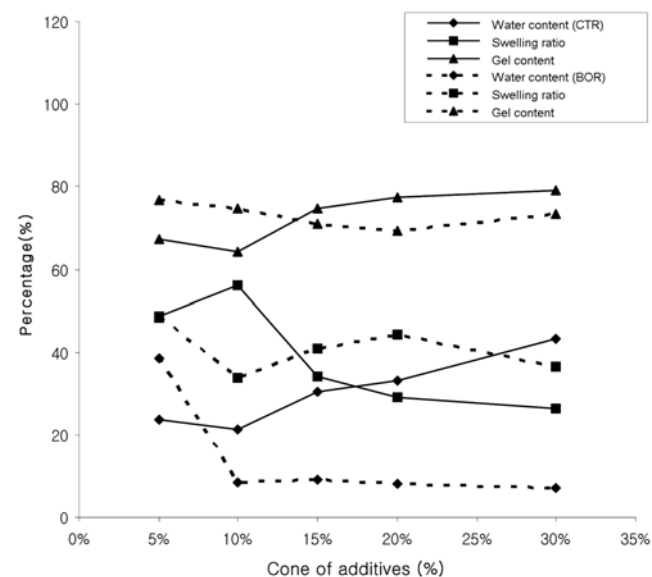


Fig. 5. Comparison of PVA/organic acid hydrogels: additives are citric acid and boric acid.

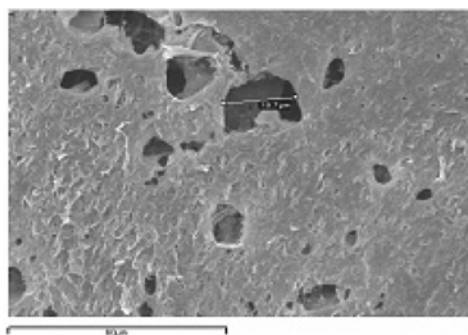
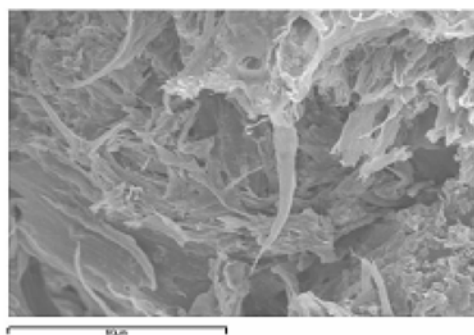


Fig. 4. SEM photographs for PVA/15%-glycine (left) and PVA/5%-DL-methionine (right) ($\times 1,000$).

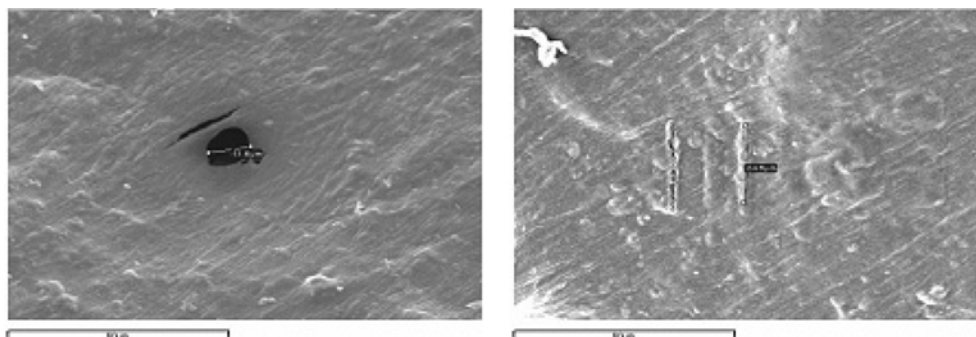


Fig. 6. SEM photographs for PVA/30%-citric acid (left) and PVA/5%-boric acid (right) hydrogels ($\times 1,000$).

erties such as water content, swelling ratio and inner linking structure were measured. The average water content and swelling ratio were 35.0%, and 92.6%, respectively. The hydrogels had pores (continuous and/or isolated) which were of about 70 to 80 μm and were mostly homogeneous (gels with lower PVA amount had produced much fewer pores).

2. PVA-Additive Hydrogels

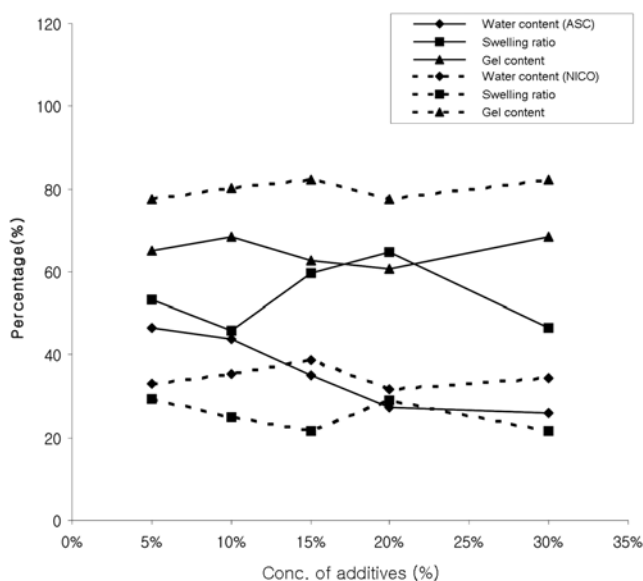


Fig. 7. Comparison of PVA/vitamin hydrogels: additives are L-ascorbic acid and nicotinamide.

The effect of additives on PVA hydrogels was investigated in terms of water content, swelling ratio, and gel content (Figs. 3-10). Amino acids, acids, vitamins and saccharides were used as additives. We expected some structural modifications with the addition of the additives, probably depending on the molecular characteristics such as molecular weight, moiety of hydrophilic patches, etc. Glycine led to a more compact network structure than methionine because of its smaller molecular weight, while methionine showed higher water content (30-40%) because it can easily absorb water due to the more loosened network. However, extra amount of methionine - presumably not used in forming the network structure - did not affect water content but further led to less elasticity of the gel or lower swelling ratio, increasing brittleness. We found that there was a maximum of swelling ratio, for example, for glycine, which was 92%. With fewer additive molecules the 'crosslinked network' should be loosened so as not to accommodate plenty of water molecules tightly; meanwhile, too many 'not-structure-involved molecules' could interfere with the inflow of water, depending on the hydrophilicity of the extra additive molecules. Amino acids among the additives to be tested showed the highest swelling capacity (90% and more), which was attributed to their multiple ways of chemical and/or physical crosslinking, coming from two or more active functional terminals such as NH-terminal, carboxylic terminal - hydrogen bonding or other chemical linkage - thereby resulting in a more compact but flexible network for water flow-in.

Boric acid exerted particularly low water content (about 10% or so) and few pores but high gel content (71-76.7%) because it excluded much of the water at the beginning of the 'crosslinking.' For citric acid the pore size was 10-15 μm (Figs. 5 & 6). The acid gels

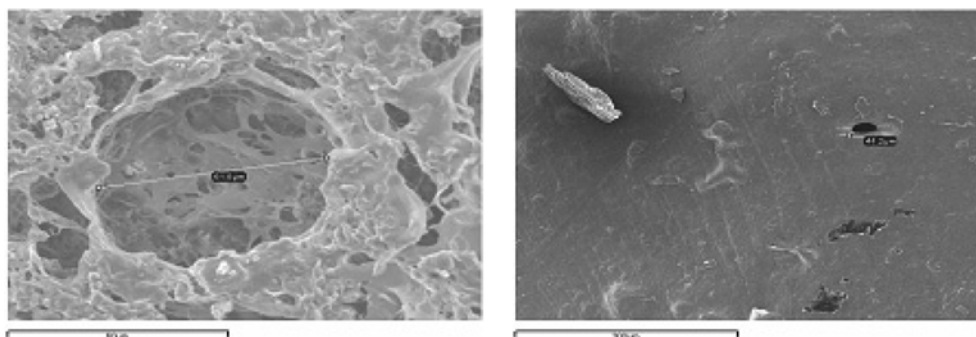


Fig. 8. SEM photographs for PVA/5%-L-ascorbic acid (left) and PVA/15%-nicotinamide acid (right) hydrogels ($\times 1,000$).

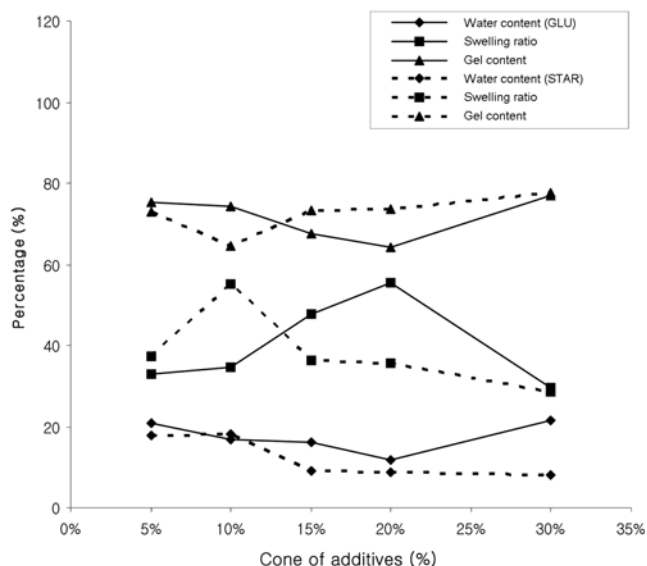


Fig. 9. Comparison of PVA/sugar hydrogels: additives are glucose and starch.

were hard as expected.

PVA-L-ascorbic acid gel (Fig. 7) showed relatively high water content in general (max 43.4% at 5% of the acid) and above averaged gel content. The cutting face of the hydrogel (Fig. 8) revealed about 50-70 μm of pores that were uniformly distributed, leading to a good microbe-fixing media. Nicotinamide as well as L-ascorbic acid was a little bulky in molecular structure (probably rings), so that it should have high water content but low swelling. Note that nicotinamide gel was of 30-40 μm in pores, which was not that uniform, hardened and of extraordinarily high gel content due to early loss of water as in boric acid.

Saccharides - glucose and starch - were tested as additives (Figs. 9 & 10). The great solubility of glucose created a fragile gel due to the excessive water holdup. In contrast, starch could form a sound gel, effectively entangling with PVA polymer, which showed reasonable elasticity and mechanical strength. Starch provided 70-80 μm of pore size with low water content and swelling.

The influential factors - measurable physical properties - were mutually correlated and analyzed in Fig. 11. In summary, water content was against the swelling ratio in general, and the two factors did not show a high relevancy to gel content. The water content



Fig. 11. Correlations between key factors affecting gel characteristics.

tended to increase with increased concentration of additives, which were hydrophilic, whereas it decreased for hydrophobic ones. It was likely that the pore size increased with higher molecular weight of the additives, more specifically with higher bulkiness or hydrophobicity of the molecules. For instance, ascorbic acid, which was bulkier and more hydrophobic but lower in molecular weight than citric acid provided much larger pores. The elasticity of the gels was well correlated to the their swelling ratio.

3. Microscopic Analysis of PVA Hydrogels

Outer gel surfaces were observed by SEM in Fig. 12. PVA-L-Ascorbic acid and PVA-DL-methionine hydrogels when the microbial cells were fixed were chosen for microscoping. Many pores were found and the attached (fixed) microbes in biofilm were seen in the magnified versions. Some pores were covered with the biofilm.

4. PAHs Degraded by Microbial Fixed PVA-Additive Hydrogels

4-1. Phenanthrene

Phenanthrene was degraded by PVA-L-ascorbic acid hydrogel with the microbial cells (Fig. 13). During 72-96 h after the cells were fixed on the gel, the cells kept growing until reaching a stationary level with a proportional decrease in phenanthrene remaining. In other words, the microbial growth in the gel and reduction of phenanthrene occurred simultaneously, suggesting the acting enzymes,

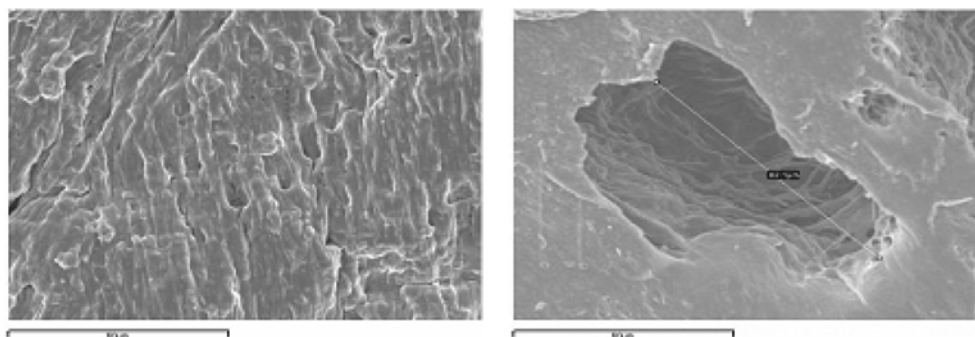


Fig. 10. SEM photographs for PVA/30%-glucose (left) and PVA/10%-starch hydrogels ($\times 1,000$).

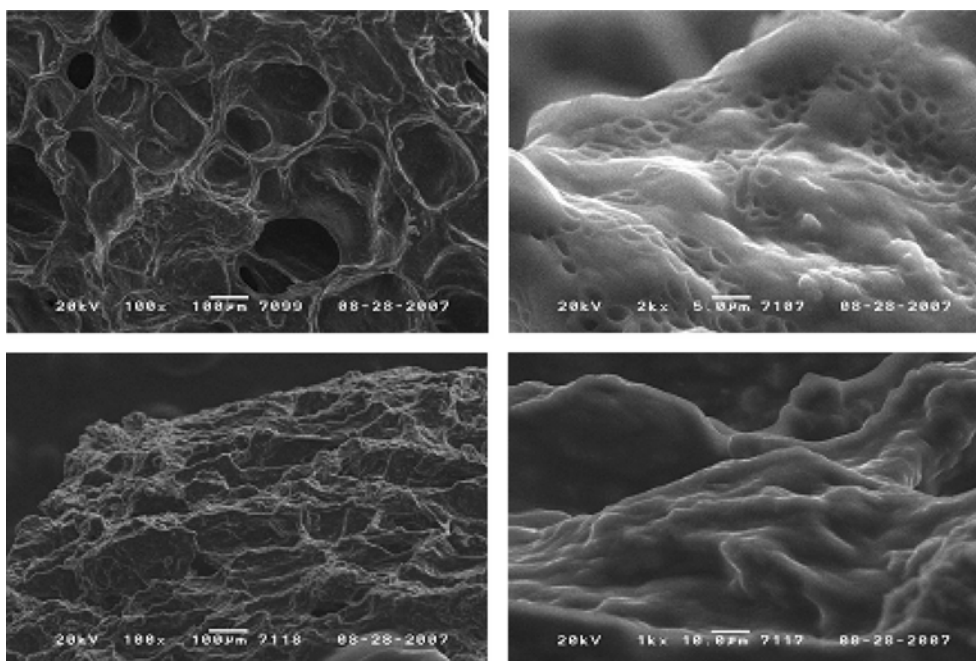


Fig. 12. SEM photographs of a PVA-glycine hydrogel (top; left, $\times 100$; right, $\times 2,000$) and a PVA-L-ascorbic acid hydrogel (bottom; left, $\times 100$; right, $\times 2,000$).

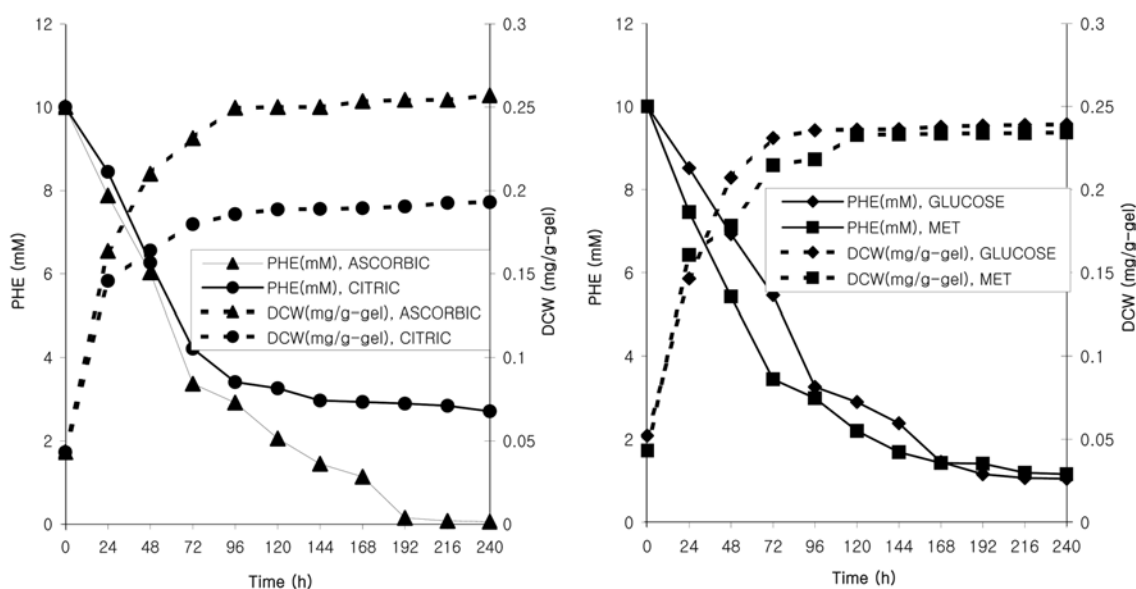


Fig. 13. Time-course curves of phenanthrene degradation and the corresponding microbial growth in the PVA hydrogels incorporating the four representative additives.

responsible for the degradation, were produced at a similar pace with the active growth phase. The highest removal percentage and cell density were max. 92% and 0.267 mg/g-gel, respectively. As found in the figure, for citric acid, the percentage went down to 88% at 0.24 mg/g-gel of cell population. PVA-DL-methionine gels also maintained as much microbial population and removal efficiency as L-ascorbic acid, while the two figures in PVA-glucose gels dropped substantially (0.20 mg/g-gel, 73%). The fragility found in the PVA-glucose gels was thought to induce the lower cell population entrapped in the gel matrix, and then the lower degradation of phenan-

threne, as well.

4-2. Pyrene

Fig. 14 shows the degradation performance for pyrene in the four PVA gels. L-ascorbic acid was the highest performing additive (0.26 mg/g-gel and 85%) and methionine, citric acid in order. Glucose was the lowest in cell mass and in removal efficiency (0.22 mg/g-gel and 62%). On the other hand, pyrene was found to be harder to decompose than phenanthrene (about 15% lower in removal efficiency while the cell masses at the comparable level). When citric acid and methionine were compared, methionine was likely to per-

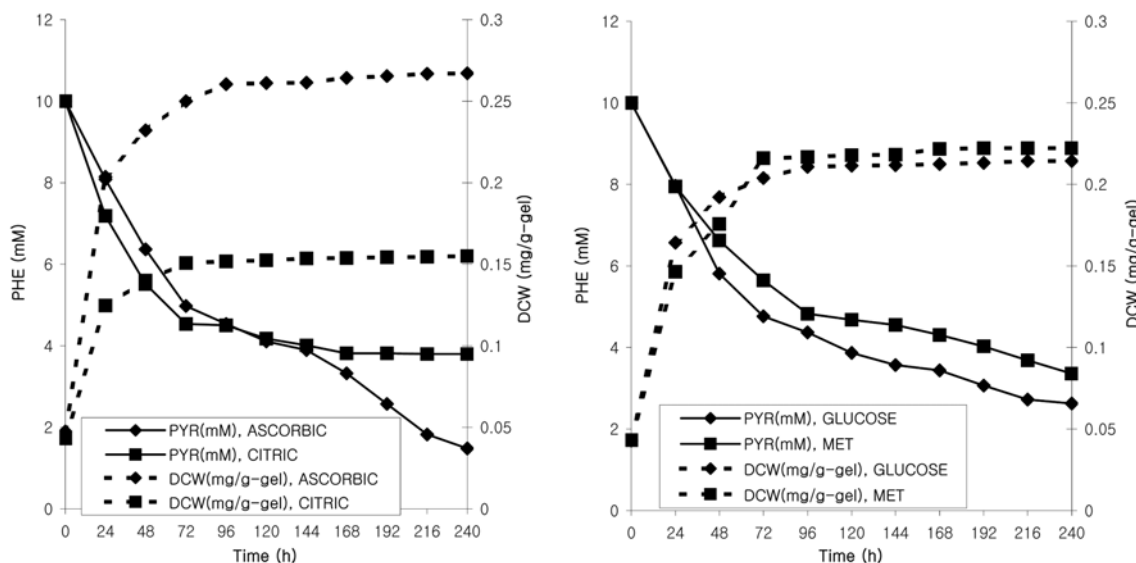


Fig. 14. Time-course curves of pyrene degradation and the corresponding microbial growth in the PVA hydrogels incorporating the four representative additives.

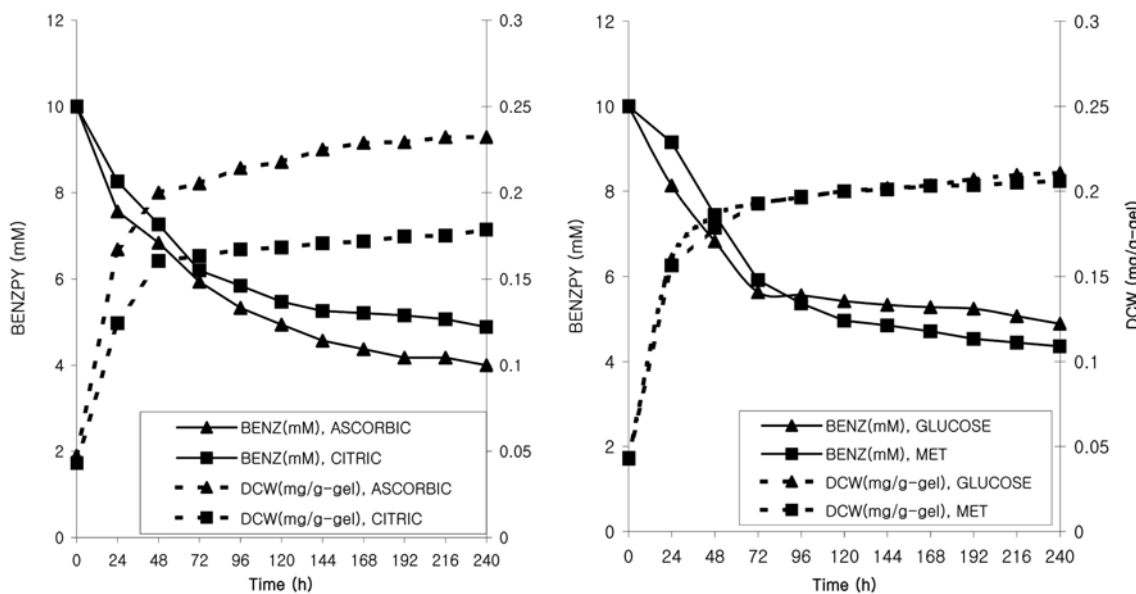


Fig. 15. Time-course curves of benzo(e)pyrene degradation and the corresponding microbial growth in the PVA hydrogels incorporating the four representative additives.

form better in degradation regardless of its cell mass' inferiority to citric acid. One probable reason for this is that methionine, an amino acid would be a vital component for cells to secrete the responsible enzymes for PAH degradation with meanwhile citric acid as an inert would not. The total enzymes were analyzed in an SDS-PAGE system (not shown in here). That figure depicted a stronger protein band for methionine even if any reasonable metabolic pathways were yet unknown.

4-3. Benzo(e)pyrene

As shown in phenanthrene and pyrene, L-ascorbic acid gels were the highest performing for benzo(e)pyrene among the four (Fig. 15). The cell masses obtained with benzo(e)pyrene were as similar as those with the other PAHs but resulted in less efficiency (51-60%).

4-4. Summary

Fig. 16 shows a comparison of the four gels for three PAHs in terms of degradation efficiency and rate on a basis of mass of gel. The degradation rates and efficiencies decreased linearly with any additional aromatic rings. Ascorbic acid and DL-methionine hydrogels served as good media candidates for microbial decomposition of 3-5 ring PAHs overall. Table 3 summarizes the maximum degradation performances achieved by the four selective biohydrogels.

CONCLUSION

PVA hydrogels with additives for better gel characteristics, prepared by a CGA technique were analyzed in terms of water con-

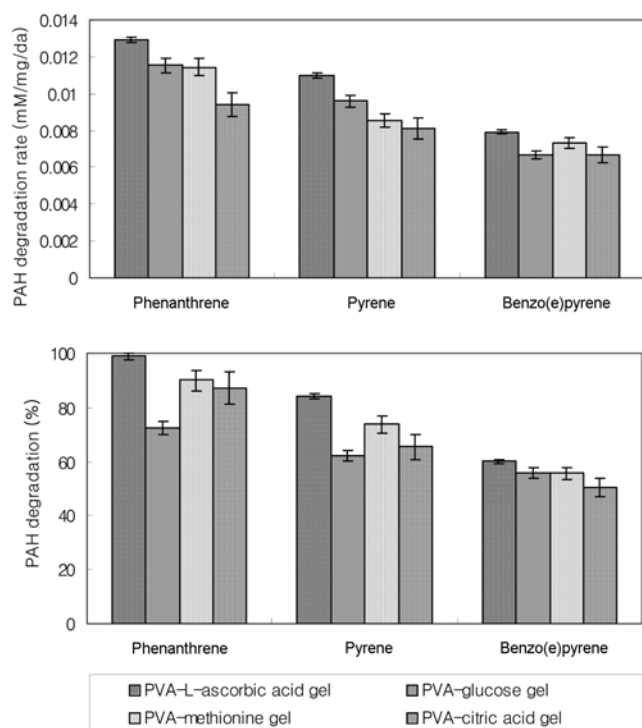


Fig. 16. Comparison between degradation efficiencies and rates of PAHs in four major PVA-hydrogels.

tent, swelling ratio, SEM photography and microbial housing media. The gels with amino acids showed the highest swelling ratios, probably because the amide and carboxylic functional groups played a significant role in formation of hydration and/or hydrogen bonding, leading to a dense but flexible network structure. Boric acid and starch induced a rapid dehydration to result in lower water content and less elastic, hardened gels. Bulky compounds such as ascorbic acid and starch or hydrophobic substances like nicotinamide tended to expand the pores due to their size or water expelling capability, respectively. In summary, hydrophobicity, functional groups and bulkiness of the additives were more critical than mere molecular weights. The pores were larger in order of citric acid, methionine, nicotinamide, ascorbic acid and starch (SEM observation). Elasticity, water content and swelling ratio were mutually well correlated.

Microbial decay tests using the gels revealed that L-ascorbic acid

gels were superior in PAH degradation rate, efficiency and corresponding microbial growth. Methionine, citric acid, and glucose followed in the order. The biomasses in gels were more or less proportional to the degradation rates for PAHs.

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Table 3. A typical data set for PAH degradation with different cell capturing types and gel additives

Cells on media	Additive	Phenanthrene		Pyrene		Benzo(e)pyrene	
		Deg. percentage (%)	Cell density (mg/g-gel)	Deg. percentage (%)	Cell density (mg/g-gel)	Deg. percentage (%)	Cell density (mg/g-gel)
Immobilization	Ascorbic acid	92	0.267	85	0.260	60	0.241
	Citric acid	88	0.241	66	0.204	51	0.185
	Glucose	73	0.200	62	0.223	56	0.219
	Methionine	90	0.248	74	0.230	56	0.215
Adhesion	Ascorbic acid	88	0.241	78	0.230	54	0.230
	Citric acid	71	0.215	64	0.193	49	0.182
	Glucose	55	0.174	52	0.215	48	0.204
	Methionine	74	0.219	70	0.215	55	0.204