หอสมุดกลาง สถาบันวิทยบริการ จุฬาลงกรณ์มหาวิทยาลัย

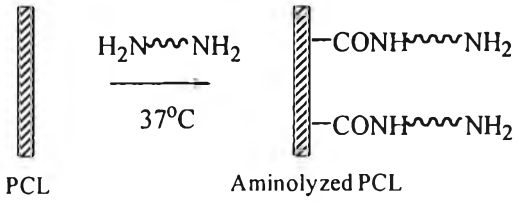
CHAPTER IV

RESULTS AND DISCUSSION

This chapter is divided into 2 sections. The first section is dedicated to surface modification of polycaprolactone (PCL) film to covalently introduce amino and carboxyl groups on the surface *via* aminolysis and graft copolymerization, respectively, followed by an activation of surface amino and carboxyl group into active *N*-hydroxysuccinimide (NHS) ester and an immobilization of biomolecules, collagen (marine, type I and type IV) or chitosan (MW 15,000 and 83,000). The second section is an investigation of cytocompatibility of surface-modified PCL films against skin-mediated cell lines.

4.1 Surface Modification of PCL Film *via* Aminolysis

Amino groups were covalently introduced onto the PCL surface by the reaction between 1,6-hexamethylenediamine and the ester groups of PCL at 37°C. The effect of aminolyzing time and amine concentration was investigated. The quantitative NH_2 amount on aminolyzed PCL film surface was measured by the ninhydrin method.



4.1.1 Effect of Aminolyzing Time

For investigating the influence of aminolyzing time, the concentration of 1,6-hexamethylenediamine/IPA solution was fixed at 1 M and the reaction time was varied from 1 to 24 h. According to Figure 4.1, the amino concentration per surface area increased rapidly as a function of reaction time within the first 8 h. Using a

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longer reaction time, the density of amino groups did not rise much further suggesting that the surface aminolysis was almost complete.

The alteration of surface wettability of PCL film after aminolysis expressed in terms of advancing/receding water contact angles was also shown in Figure 4.1. The water contact angle decreased with aminolyzing time due to the introduction of hydrophilic NH_2 group from 85°/50° of virgin PCL film to the minimum of 75°/43° at 8 h of aminolysis and then increased slightly. This increase of contact angle after 8 h may possibly be due to the partial surface degradation of PCL films with longer reaction time resulting in more surface roughness and higher advancing contact angle value [8].

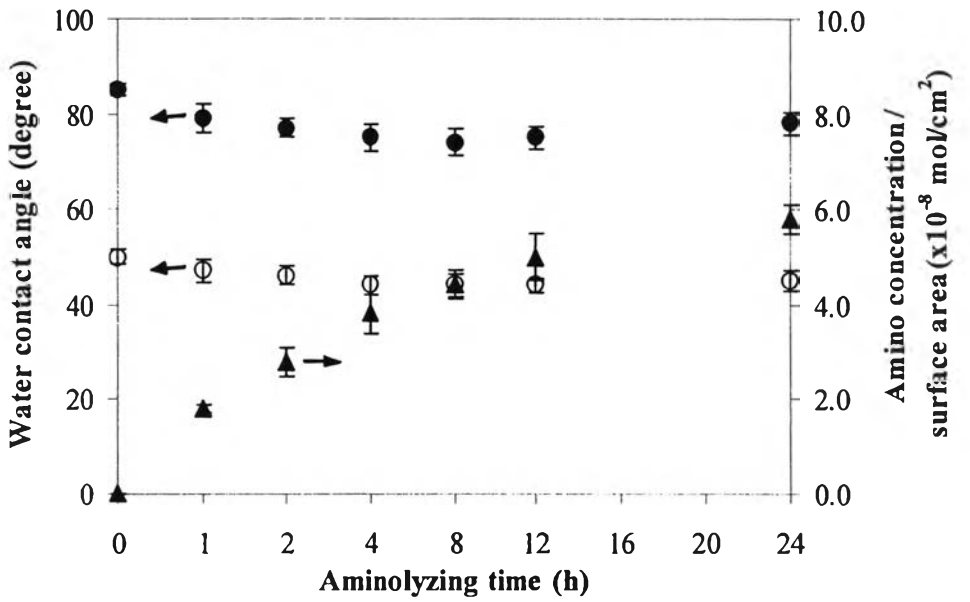


Figure 4.1 Water contact angle (advancing (●) and receding (○)) and amount of NH_2 group (▲) of PCL films after reaction with 1M 1,6-hexamethylenediamine/IPA as a function of reaction time

4.1.2 Effect of Amine Concentration

By choosing the aminolyzing time of 8 h, the concentration of 1,6-hexamethylenediamine/IPA between 0.25 and 3 M was used to study the effect of

amine concentration. As shown in Figure 4.2, the amount of NH_2 groups on the PCL film increased linearly with 1,6-hexamethylenediamine concentration.

The water contact angle decreased with the increasing of amine concentration and reached a minimum of 73°/42° at 1.5 M and remained almost unchanged throughout the rest of reaction time, implying that the amine concentration at 1.5 M was high enough for the surface aminolysis to reach the completion.

Compared the NH_2 concentration per surface area with the water contact angle, the inconsistency between these data was found. This is due to the fact that the water contact angle reflects the surface property of a material. Hence, the existence of hydrophilic–hydrophobic domains on a material surface will have a great influence on its value. On the other hand, ninhydrin method reflects the total amount of NH_2 groups existed not only on the surface, but also in the entire bulk materials. These results presumably suggested that increasing of amine concentration to 2 and 3 M will push the reaction to occur in deeper depth of the film.

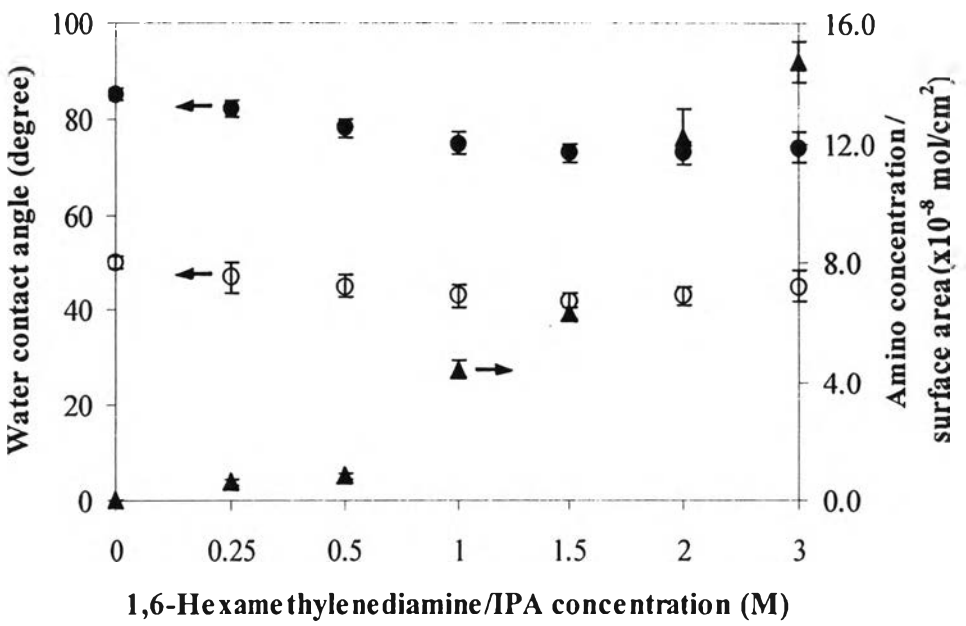


Figure 4.2 Water contact angle (advancing (●) and receding (○)) and amount of NH_2 group (▲) of PCL films after reaction with varied concentration of 1,6-hexamethylenediamine/IPA for 8 h

The presence of NH_2 group on the aminolyzed PCL was also detected by ATR-FTIR analysis (Figure 4.3). There was a major absorption peak assigned to the ester carbonyl of virgin PCL appeared at 1720 cm^{-1}. No signals from N-H stretching of NH_2 or carbonyl stretching of amide group were observed on aminolyzed PCL. This might be the result of the extremely low concentration of NH_2 present within the sampling depth of ATR-FTIR (1-2 μm).

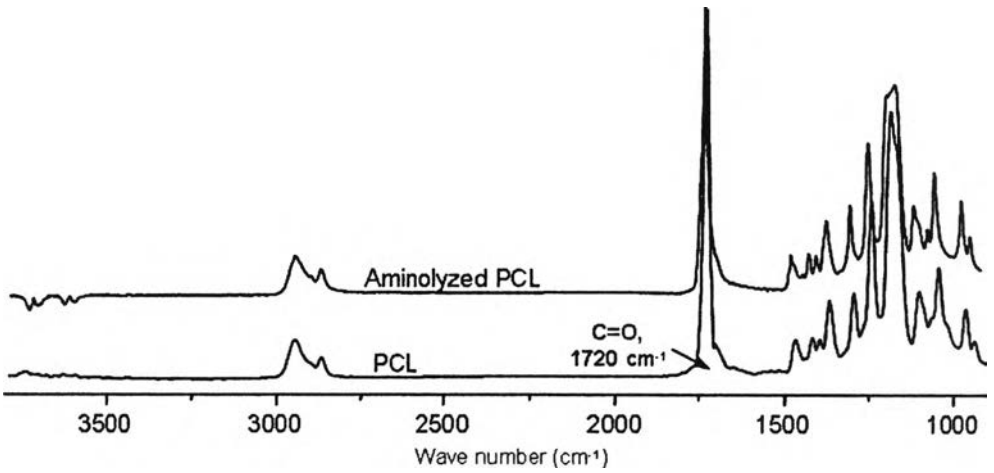
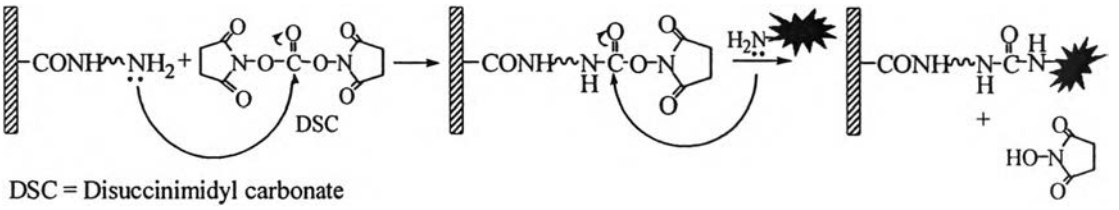


Figure 4.3 ATR-FTIR spectra of PCL film before and after aminolysis by 1.5 M 1,6-hexamethylenediamine/IPA solution for 8 h

4.2 Activation of Aminolyzed PCL Film and Immobilization of Biomolecules

In this work, to achieve the covalent coupling of biomolecules, the surface amino group of aminolyzed PCL was first activated with *N,N'*-disuccinimidyl carbonate (DSC) to form a succinimidyl ester and then immobilized with selective biomolecules, collagen or chitosan. The mechanism of the activation of amino groups on the surface followed by the coupling reaction with biomolecules is shown in Scheme 4.1.



Scheme 4.1 Mechanism of an activation of surface amino group followed by a coupling reaction with biomolecules

4.2.1 Activation of Amino Group on Aminolyzed PCL Film

In the activation step, the effect of activation time was investigated. The activation reaction was carried out using 0.1 M *N,N'*-disuccinimidyl carbonate (DSC)/dimethylsulfoxide (DMSO) in the presence of 0.1 M triethylamine at room temperature. According to Figure 4.4, the water contact angle increased from 73°/42° (aminolyzed PCL) to 89°/45° (activated aminolyzed PCL) after the activation for 1 h and remained constant for the rest of activation time implying that the highest extent of activation in the surface region has been achieved. This increasing of contact angle suggests that the hydrophilic amino groups have been converted to hydrophobic *N*-succinimidyl groups.

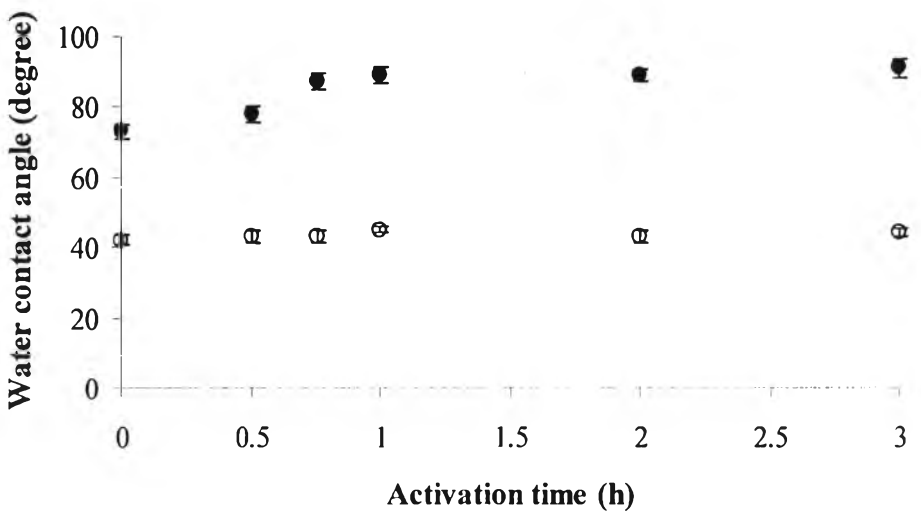


Figure 4.4 Water contact angle of aminolyzed PCL films after the reaction with 0.1 M DSC/DMSO: advancing (●) and receding (○)

4.2.2 Immobilization of Biomolecules on Activated Aminolyzed PCL Film

By choosing the activation time of 1 h and 0.1 M DSC/DMSO solution, the influence of immobilization time and biomolecule concentration was studied. In case of collagen, only marine collagen was used for this investigation.

4.2.2.1 Effect of Immobilization Time

Activated aminolyzed PCL was immersed in 10 mg/mL collagen/PBS solution at room temperature for 4 to 48 h. From Figure 4.5, the advancing water contact angle decreased from 89° of activated aminolyzed PCL to 59° after immobilized with collagen for 24 h while the amount of NH_2 groups on the surface reached the maximum value of 1.31×10^{-8} mol/cm^2 at the same period of time. The contact angle did not decrease any further after 24 h implying that the highest extent of immobilization in the surface region has been achieved.

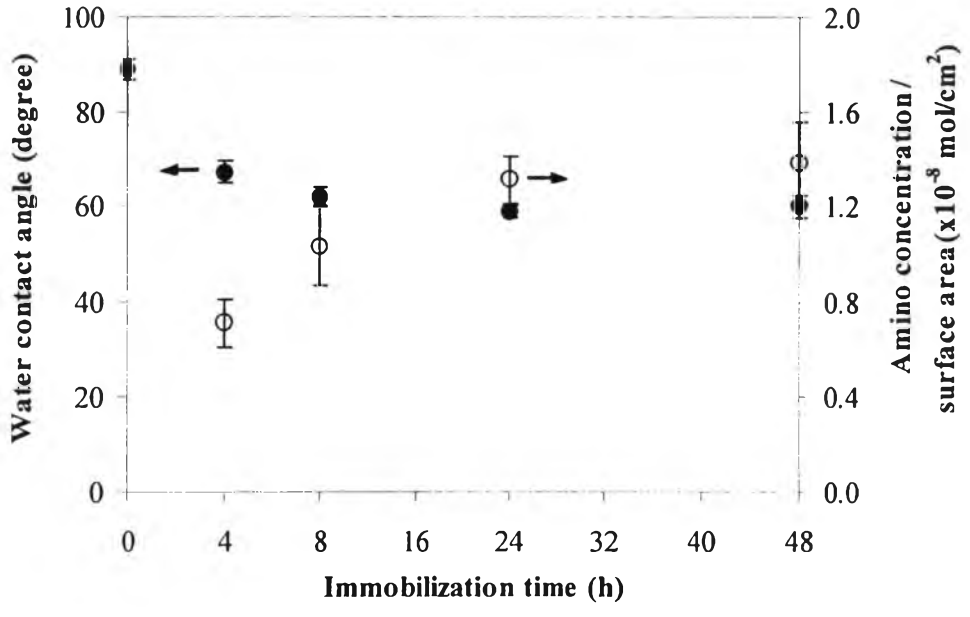


Figure 4.5 Advancing water contact angle (●) and amount of NH_2 group (○) of activated aminolyzed PCL films after collagen immobilization as a function of immobilization time

4.2.2.2 Effect of Biomolecule Concentration

Effect of biomolecule concentration on the extent of immobilization on the activated aminolyzed PCL film was determined by varying the collagen and chitosan concentration from 1 to 10 mg/mL using the immobilization time of 24 h at room temperature.

Collagen

As displayed in Figure 4.6, the advancing water contact angle decreased as a function of collagen concentration within 5 mg/mL and seemed to level off afterwards. Surface became more hydrophilic when compared to the control PCL (85°/50°). The water contact angle decreased from 89°/45° of activated aminolyzed PCL to 61°/0° of aminolyzed PCL-collagen due to the conversion of hydrophobic active ester to hydrophilic biomolecule. The amount of NH_2 group corresponding to the quantity of collagen immobilized on the surface increased with the increasing of collagen concentration. The highest amount of NH_2 group is 1.31×10^{-8} mol/cm^2 at 10 mg/mL collagen concentration.

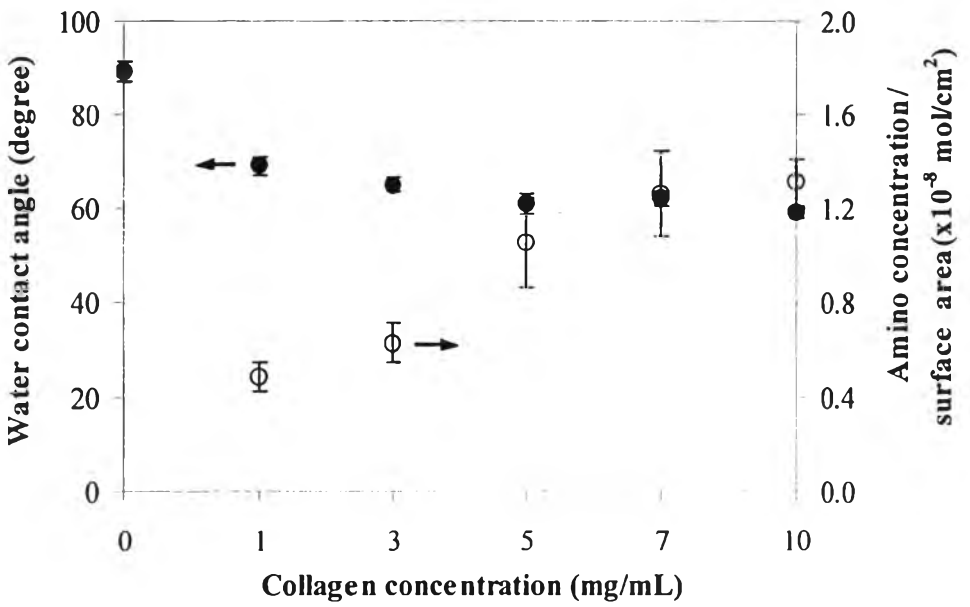


Figure 4.6 Advancing water contact angle (●) and amount of NH_2 group (○) of activated aminolyzed PCL films after immobilization with varied concentration of collagen

Chitosan

Two chitosans having different molecular weight (MW): 15,000 and 83,000, were used in this work. As illustrated in Figure 4.7, the water contact angle of the immobilized films decreased as a function of chitosan concentration up to 3 mg/mL and tended to level off afterwards for both MW. The NH_2 group content increased when the concentration of chitosan increased. As expected, at the same concentration of chitosan, the aminolyzed PCL-chitosan 83000 films had higher NH_2 density than the aminolyzed PCL-chitosan 15000 due to the fact that the number of repeat unit containing NH_2 group in chitosan MW 83,000 molecule is greater than that in chitosan MW 15,000 molecule. At 3 mg/mL chitosan concentration, aminolyzed PCL-chitosan 15000 and 83000 yielded 2.3×10^{-9} and 2.7×10^{-9} mol/cm^2 of NH_2 groups, respectively.

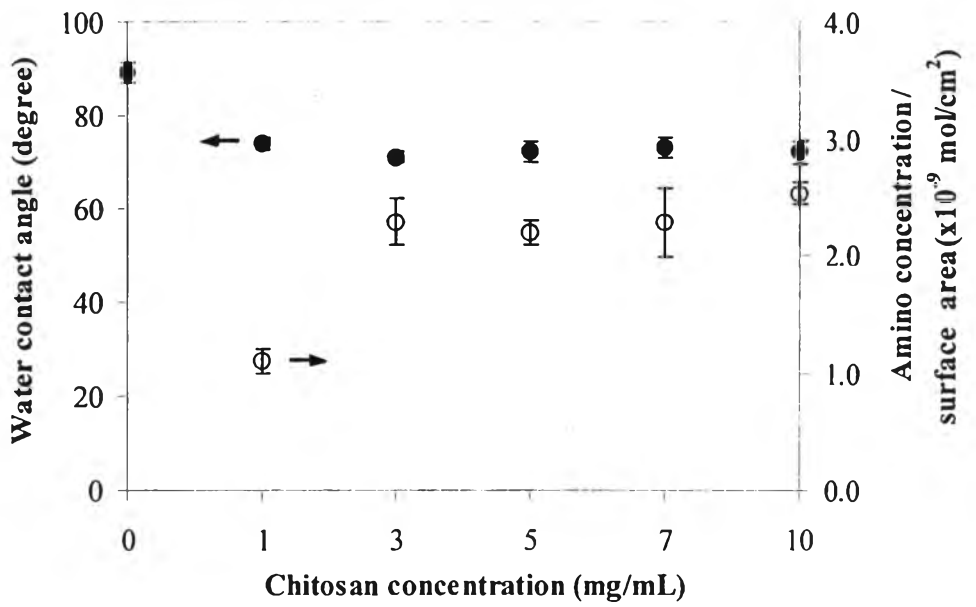


Figure 4.7 Advancing water contact angle (●) and amount of NH_2 group (○) of activated aminolyzed PCL films after immobilization with varied concentration of chitosan MW 15000

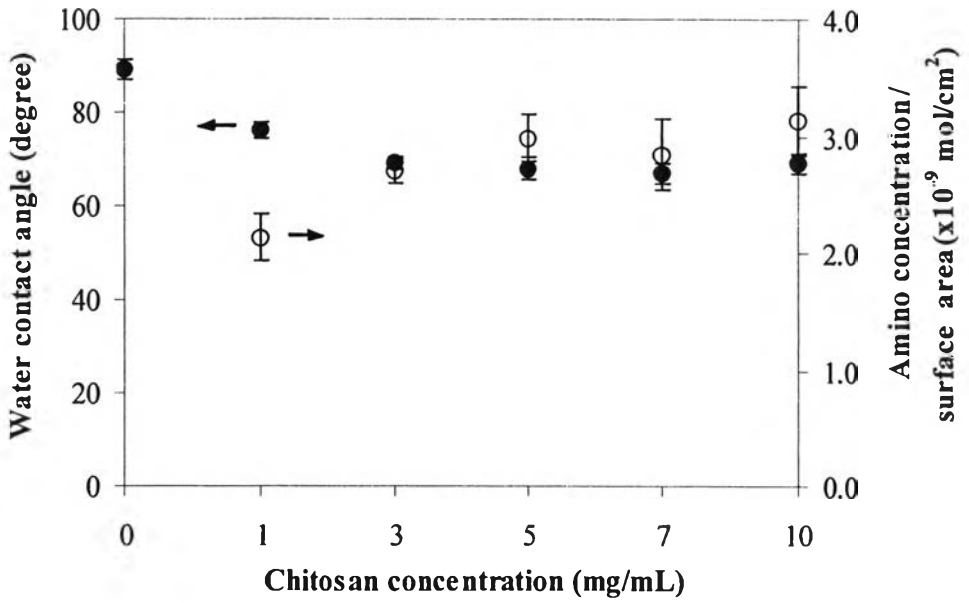


Figure 4.8 Advancing water contact angle (●) and amount of NH_2 group (○) of activated aminolyzed PCL films after immobilization with varied concentration of chitosan MW 83000

When comparing the water contact angle and NH_2 density on the surface of collagen and chitosan immobilized films, it was observed that the water contact angles of the chitosan MW 15000- and 83000-immobilized films were higher and the amount of NH_2/surface area were lower than the collagen-immobilized films. This might be due to the larger structure and the water insoluble properties of chitosan leading to its lower extent of immobilization in comparison with collagen.

The absorption peaks from ATR-FTIR of PCL and modified PCL are shown in Figure 4.9. For the activated aminolyzed PCL spectrum, no C=O stretching of succinimidyl ester on the activated aminolyzed PCL was observed. Once again, this may be regarded as a result of the amount of NH_2 group and its corresponding activated succinimidyl ester group being lower than the detection limit of the technique. Nonetheless, after biomolecule immobilization, C=O stretching peak at 1640 cm^{-1} (amide I) and N–H bending peak at 1560 cm^{-1} (amide II) evidently appeared in the spectra of aminolyzed PCL-collagen, aminolyzed PCL-chitosan 15000, and aminolyzed PCL-chitosan 83000. Moreover, the broad peak in the range

of 3000-3600 cm^{-1} corresponding to N-H stretching of NH_2 in collagen and chitosan was observed indicating that collagen and chitosan have been successfully immobilized on the amino-containing PCL films.

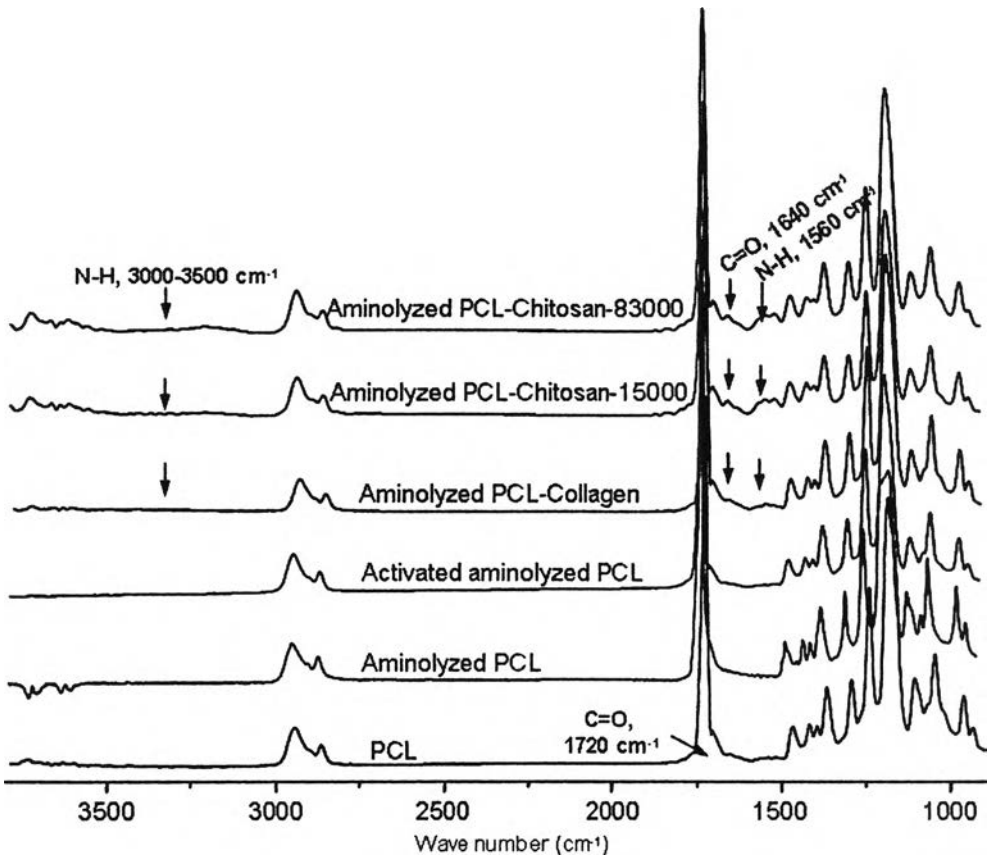


Figure 4.9 ATR-FTIR spectra of virgin PCL film and aminolyzed PCL films before and after activation and biomolecule immobilization

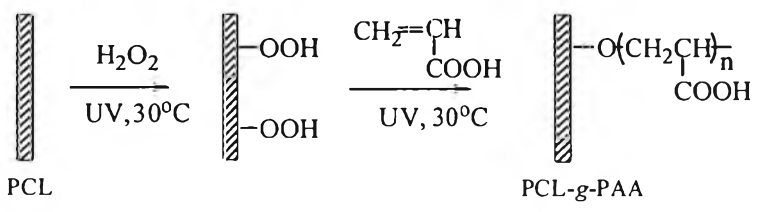
Furthermore, the success of aminolysis followed by immobilization of biomolecules on PCL was also confirmed by the result from XPS analysis as shown in Table 4.1. After aminolysis of PCL, the N_{1s} peak was detected and the N/C ratio was increased from 0 to 0.02. The N/C ratio of aminolyzed PCL increased after immobilization of biomolecules due to the large amount of NH_2 group in biomolecule structure was additionally introduced. It can be seen that the aminolyzed PCL-collagen had higher N/C ratio than aminolyzed PCL-chitosan 83000. These data are in accord with the amount of NH_2 determined by the ninhydrin method.

Table 4.1 Atomic composition of virgin PCL film and aminolyzed PCL films before and after biomolecule immobilization

Sample	Atomic composition (%)			N/C ratio
	C	O	N	
PCL	71.0	29.0	-	0
Aminolyzed PCL	71.7	26.7	1.6	0.02
Aminolyzed PCL-collagen	70.0	26.6	3.4	0.05
Aminolyzed PCL-chitosan 83000	70.4	27.4	2.2	0.03

4.3 Surface Modification of PCL Film *via* Photo-oxidation and Graft Copolymerization

Introducing carboxyl group on the surface is another route to give PCL film the ability to immobilize biomolecules. PCL film was modified by graft copolymerization of acrylic acid (AA) initiated under UV irradiation. Photo-oxidizing PCL film in hydrogen peroxide solution firstly generates macromolecular hydroperoxide groups (P–OOH, where P represents the PCL polymer chain) on its surface. Under UV irradiation, the hydroperoxide groups decompose into macromolecular oxygen radicals (P–O•) that have the ability to initiate the grafting copolymerization of acrylic acid (AA) on the film surface, and hydroxyl radicals (H–O•) which may initiate homopolymerization of AA in solution. A large amount of water was necessarily used to dissolve the homopolymer, yielding a PAA grafted PCL (PCL-*g*-PAA). The effect of photo-oxidation time, grafting time and monomer concentration was investigated. The quantitative density of COOH groups grafted on PCL membrane was determined by the reaction with toluidine blue O.



4.3.1 Effect of Photo-oxidation Time

The graft copolymerization was conducted in 10% AA solution at 30°C for 1 h after photo-oxidation. As shown in Figure 4.10, the density of COOH groups on the PCL-*g*-PAA film increased rapidly within the first 30 min of photo-oxidation giving the highest value at 9.1×10^{-8} mol/cm^2. The content of COOH groups was, however, decayed as the photo-oxidation proceeded beyond 30 min. Two possible explanations may account for such a result: (1) The photo-oxidized layer of the PCL surface was somewhat degraded, (2) Extensive quantity of generated radical and monomer favors initiation, chain transfer, and termination, but not propagation.

To compare the hydrophilicity of the modified and unmodified film surfaces, water contact angles were measured. As a consequence of graft copolymerization of AA onto the film surface, the water contact angle decreased sharply from 85°/50° to 45°/0° after 30 min of photo-oxidation and then increased to 70°/34° after 2 h. The contact data evidently coincide with the COOH concentration per surface area data.

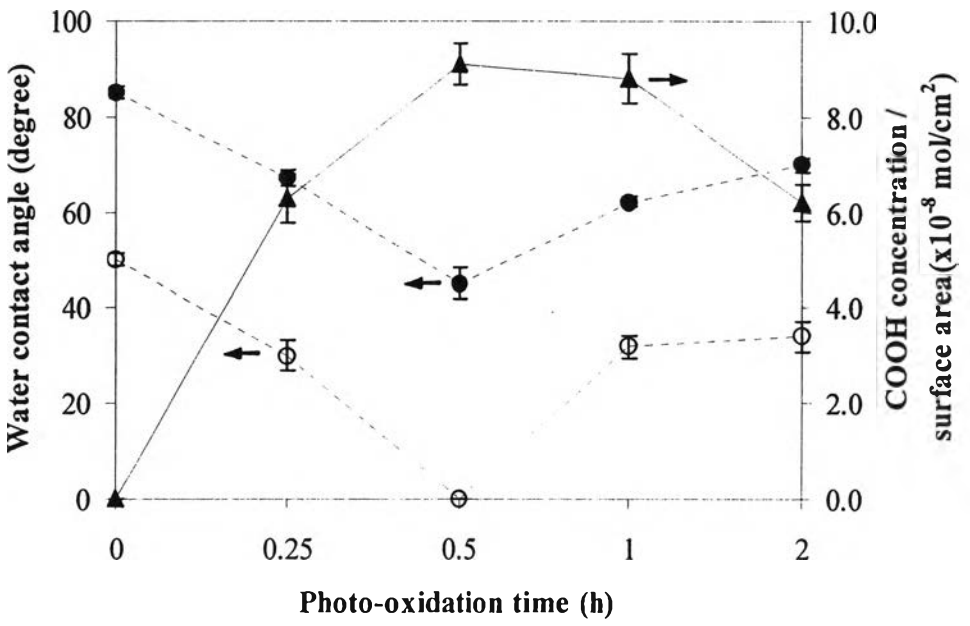


Figure 4.10 Water contact angle (advancing (●) and receding (○)) and amount of COOH group (▲) of PCL-*g*-PAA films as a function of photo-oxidation time. The graft copolymerization was conducted in 10% AA solution at 30°C for 1 h

4.3.2 Effect of Grafting Time

Using a chosen photo-oxidation time of 30 min and a given concentration of 10% AA solution, the influence of grafting time on the COOH density and water contact angle on the grafted film was investigated. The results are shown in Figure 4.11. The concentration of COOH groups on PCL-*g*-PAA film increased along with the grafting time within the first 1h, and then dropped after the longer grafting time was used. It is also suspected that the chain transfer and termination overshadowed the initiation and propagation towards the end of reaction at which the AA monomer was mostly consumed and the solution became quite viscous in the presence of free homopolymer. The water contact angles plotted in the same figure are in good agreement with the COOH density data. The lowest contact angle obtained after 1h grafting was 45°/0°.

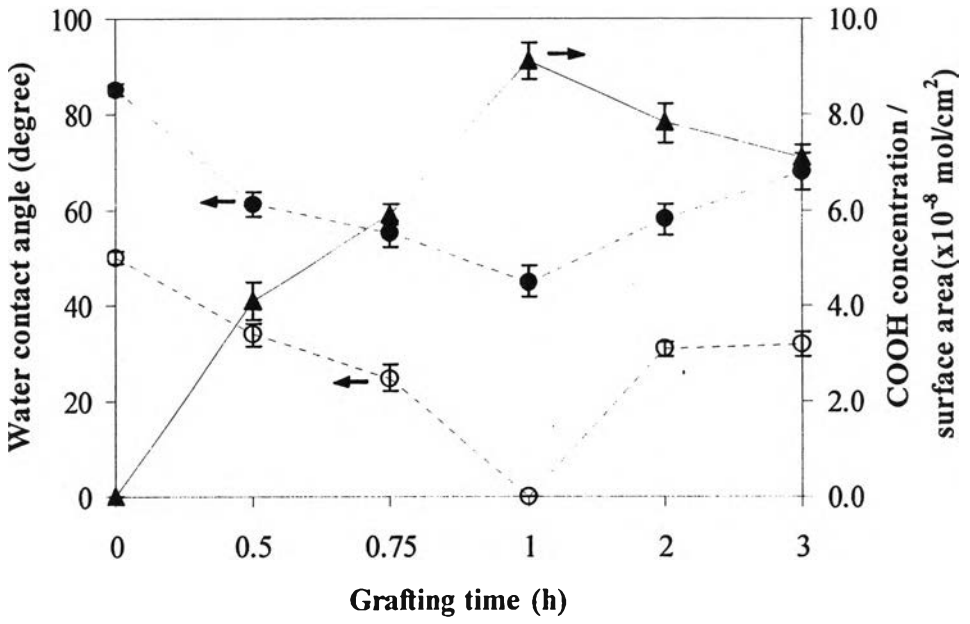


Figure 4.11 Water contact angle (advancing (●) and receding (○)) and amount of COOH group (▲) of PCL-*g*-PAA films as a function of grafting time. The graft copolymerization was conducted in 10% AA solution at 30°C after photo-oxidation for 30 min

4.3.3 Effect of Monomer Concentration

To determine the effect of monomer concentration on the extent of graft copolymerization, the photo-oxidation time and grafting time were fixed at 30 min and 1h, respectively. The monomer concentration was varied in the range of 5-15%. As can be seen from Figure 4.12, the COOH concentration per surface area increased with the AA concentration up to 10% and then dropped down when the monomer concentration was 15%. It is believed that the high concentration of monomer in the solution would favor the homopolymerization over the graft copolymerization. The contact angle data plotted in the same figure also reflected the same tendency.

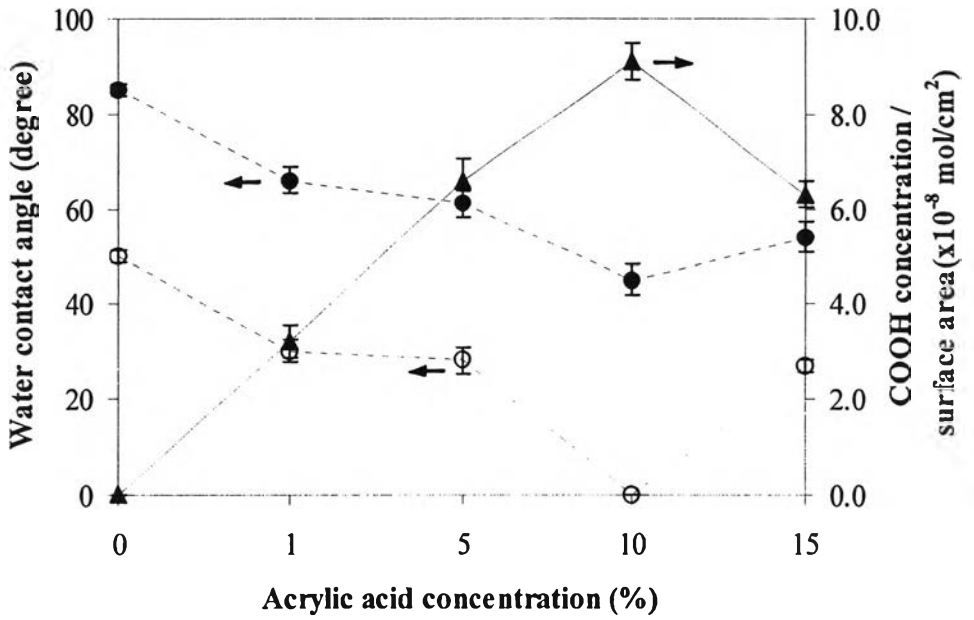


Figure 4.12 Water contact angle (advancing (●) and receding (○)) and amount of COOH group (▲) of PCL-*g*-PAA films as a function of acrylic acid concentration. The graft copolymerization was conducted at 30°C for 1 h after photo-oxidation for 30 min

An optimum condition for the preparation of PCL-*g*-PAA film that was deduced is to use 10% AA and carrying out the photo-oxidation and graft copolymerization for 30 min and 1 h, respectively.

The structure of PCL-*g*-PAA surface was studied by ATR-FTIR analysis. According to Figure 4.13, the absorption peak assigned to the C=O stretching in the spectrum of PCL-*g*-PAA film was almost indistinguishable from that in the spectrum of virgin PCL film. This is due mainly to the fact that the C=O stretching of acid group in PAA generally appears at 1718 cm^{-1} which is in a close proximity to the C=O stretching of ester in PCL at 1720 cm^{-1}. Nonetheless, there was a very weak and broad signal appearing in the range of 3000-3500 cm^{-1} that can be assigned to the O-H stretching of acid group in PAA.

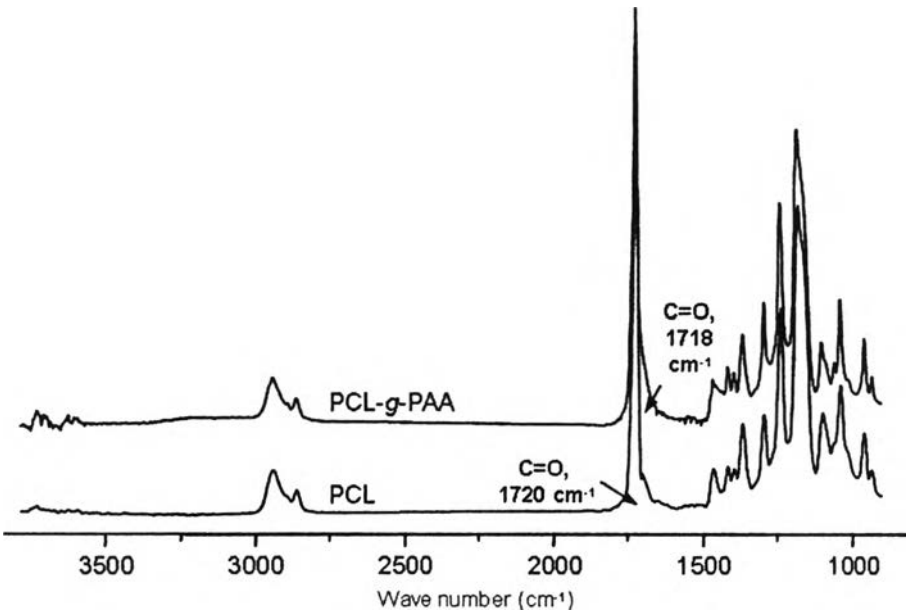
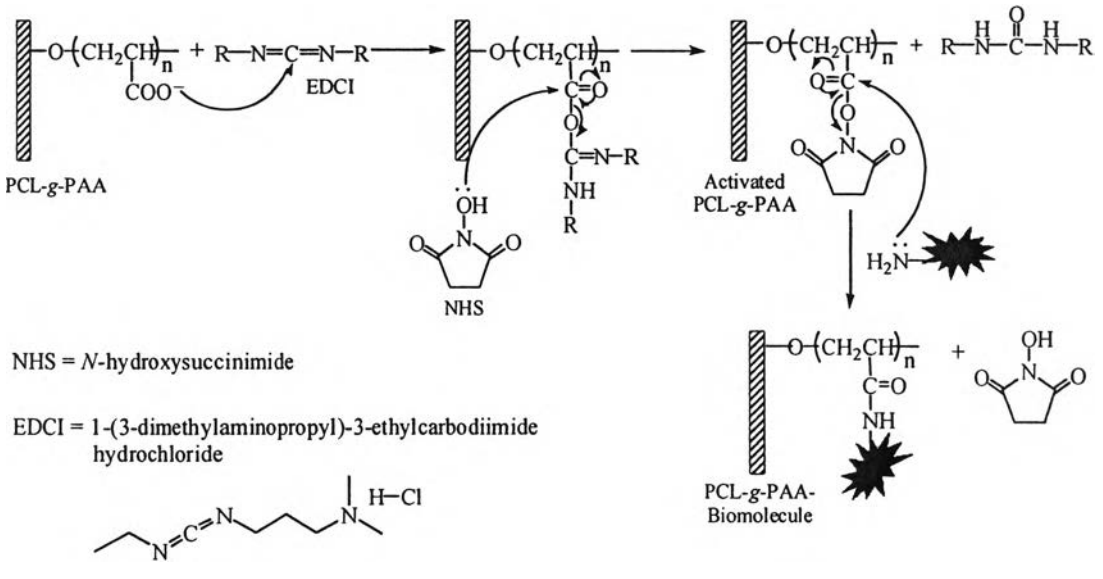


Figure 4.13 ATR-FTIR spectra of PCL and PCL-*g*-PAA films (photo-oxidation for 30 min and graft copolymerization in 10% AA for 1h)

4.4 Activation of PCL-g-PAA Film and Immobilization of Biomolecules

The activation of the carboxyl group with a water-soluble carbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) alone is possible but not quite practical since the activated group often suffers from hydrolysis and is easily deactivated. For this reason, an alternative method of using *N*-hydroxysuccinimide (NHS) in combination with EDCI was selected. The *N*-hydroxysuccinimide esters resulting from the NHS/EDCI activation are known to be

more stable and can react spontaneously with amines and other nucleophilic groups. The stepwise activation of carboxyl group by NHS/EDCI followed by the immobilization of biomolecule is mechanistically shown in Scheme 4.2.



Scheme 4.2 Mechanism of an activation of surface carboxyl group followed by a coupling reaction with biomolecules

4.4.1 Activation of PCL-*g*-PAA Film

In the activation step, the effect of activation time was investigated. The activation reaction was carried out using 0.1 M NHS in the presence of 0.4 M EDCI at room temperature. According to Figure 4.14, the water contact angle increased from 45°/0° (PCL-*g*-PAA) to 70°/38° (activated PCL-*g*-PAA) after the activation for 1 h and remained unchanged afterwards implying that the highest extent of activation in the surface region has been achieved. This increasing of contact angle suggests that the hydrophilic carboxyl groups have been converted to hydrophobic *N*-succinimidyl groups.

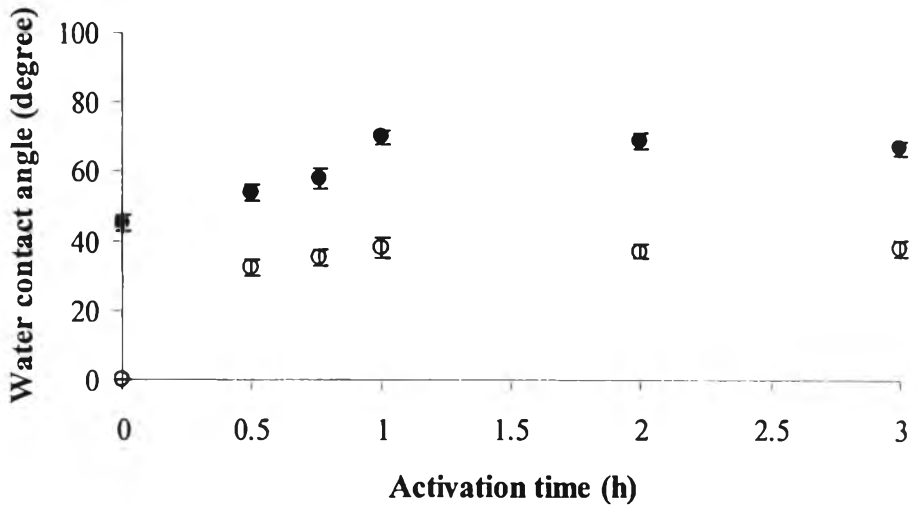


Figure 4.14 Water contact angle of PCL-*g*-PAA films after the reaction with aqueous solution of 0.1 M NHS/0.4 M EDCI: advancing (●) and receding (○) water contact angle

4.4.2 Immobilization of Biomolecules on Activated PCL-*g*-PAA Film

By choosing the activation time of 1 h and 0.1 M NHS/0.4 M EDCI aqueous solution, the influences of immobilization time and biomolecule concentration on the extent of immobilization were studied. In case of collagen, only marine collagen was used for this investigation.

4.4.2.1 Effect of Immobilization Time

Activated PCL-*g*-PAA was immersed in 10 mg/mL collagen/PBS solution at room temperature for 4 to 48 h. From Figure 4.15, the advancing water contact angle decreased from 70° of activated PCL-*g*-PAA to 34° after immobilization with collagen for 24 h while the amount of NH_2 groups (from immobilized collagen) on the surface simultaneously reached the maximum value of 1.77×10^{-8} mol/cm^2. The contact angle did not decrease any further after 24 h implying that the highest extent of immobilization in the surface region has been achieved.

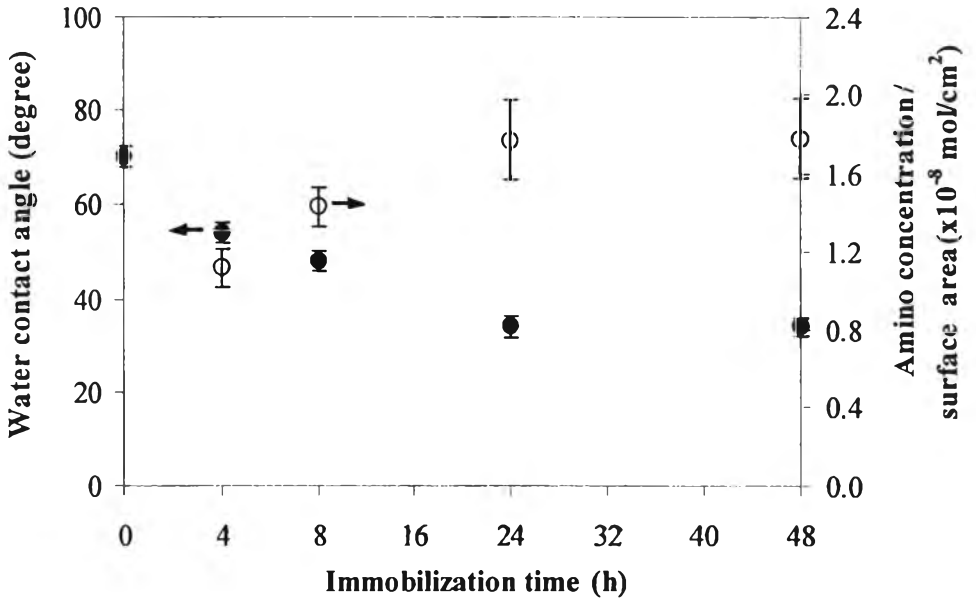


Figure 4.15 Advancing water contact angle (●) and amount of NH_2 group (○) of activated PCL-*g*-PAA films after collagen immobilization as a function of immobilization time

4.4.2.2 Effect of Biomolecule Concentration

Effect of biomolecule concentration on the extent of immobilization of the activated PCL-*g*-PAA film was determined by varying the biomolecule concentration from 1 to 10 mg/mL using the immobilization time of 24 h at room temperature.

Collagen

As displayed in Figure 4.16, the advancing water contact angle decreased as a function of collagen concentration within 5 mg/mL and seemed to level off afterwards. Increasing the concentration of collagen resulted in an increase of the amino group content on the surface. The highest amount of NH_2 group is 1.77×10^{-8} mol/cm^2 at 10 mg/mL collagen concentration.

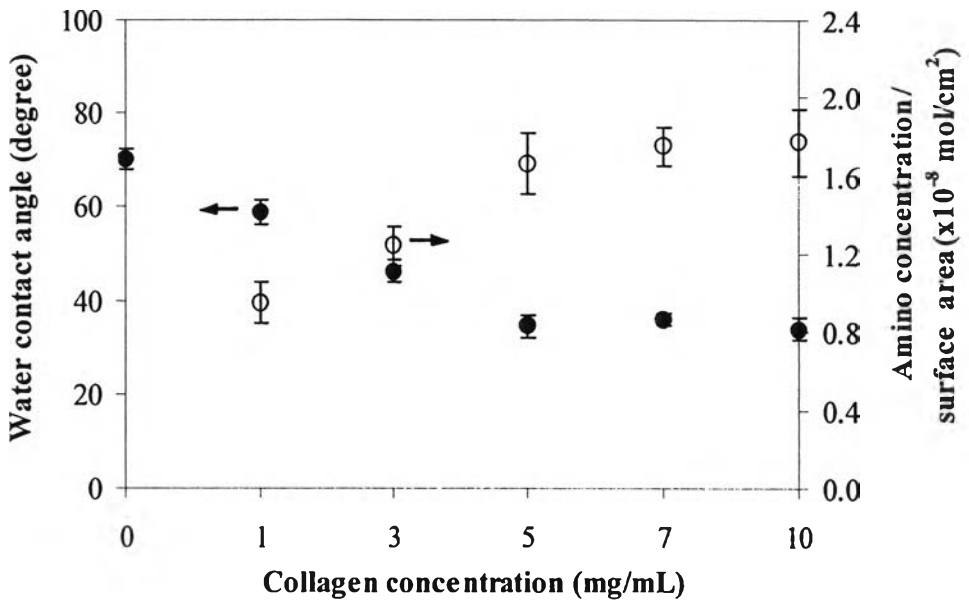


Figure 4.16 Advancing water contact angle (●) and amount of NH_2 group (○) of activated PCL-*g*-PAA films after the immobilization with varied concentration of collagen for 24 h

Chitosan

To determine an effect of chitosan concentration on the extent of immobilization, the reaction was carried out by using the same immobilization time as collagen, 24 h. The water contact angle of the chitosan-immobilized film decreased as a function of chitosan concentration up to 3 mg/mL and tended to level off afterwards for both MW (Figures 4.17 and 4.18). The NH_2 content/surface area corresponded quite well with the contact angle data. At 3 mg/mL chitosan concentration, PCL-*g*-PAA-chitosan 15000 and 83000 yielded 7.8×10^{-9} and 8.7×10^{-9} mol/cm^2 of NH_2 groups, respectively.

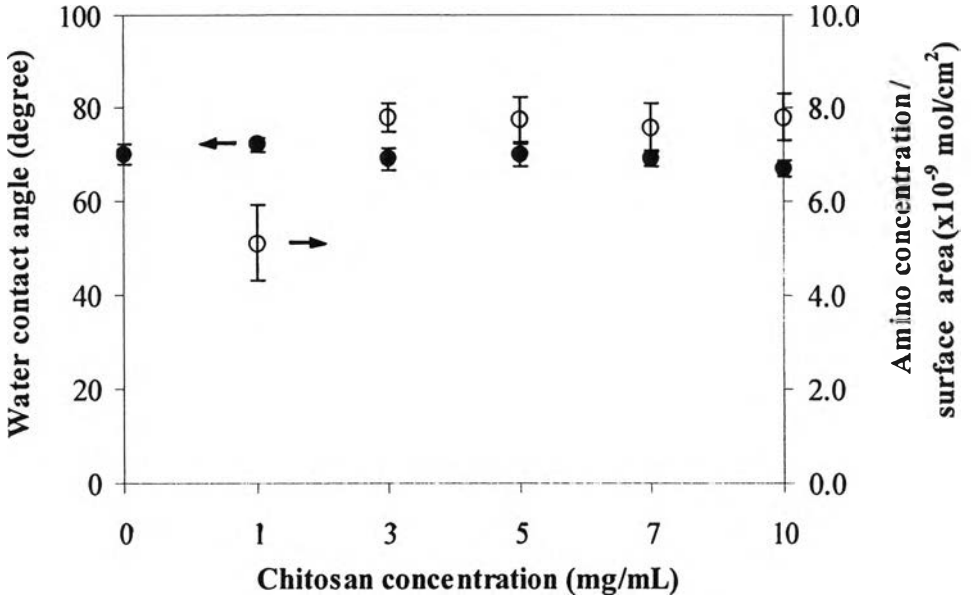


Figure 4.17 Advancing water contact angle (●) and amount of NH_2 group (○) of activated PCL-*g*-PAA films after the immobilization with varied concentration of chitosan MW 15000

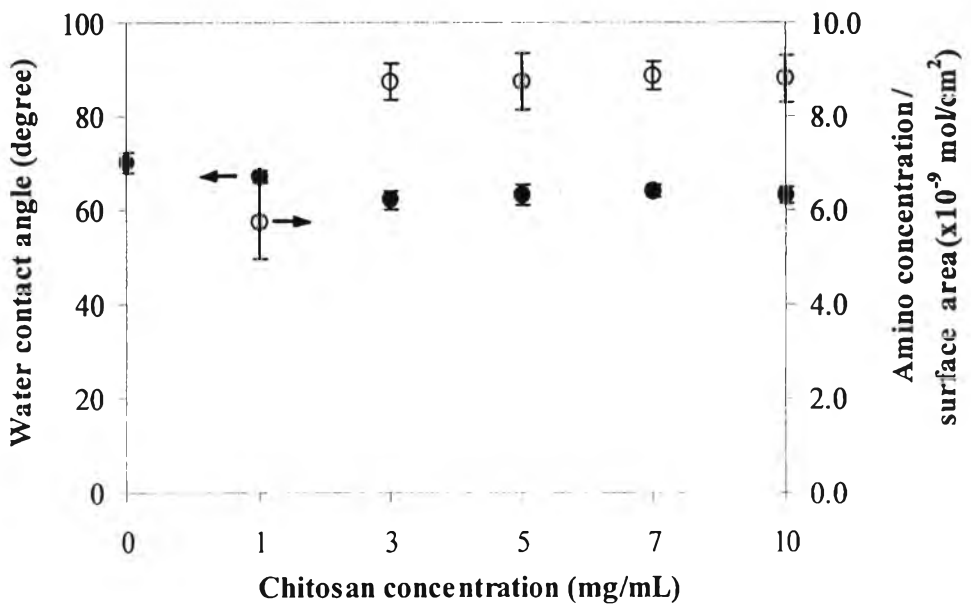


Figure 4.18 Advancing water contact angle (●) and amount of NH_2 group (○) of activated PCL-*g*-PAA films after the immobilization with varied concentration of chitosan MW 83000

ATR-FTIR spectra of PCL and PCL-g-PAA both before and after activation and biomolecule immobilization are shown in Figure 4.19. For the activated PCL-*g*-PAA, an emerging weak shoulder at 1808 cm^{-1} observed in the spectrum can be assigned to the C=O stretching of succinimidyl ester. After biomolecule immobilization, C=O stretching peak at 1640 cm^{-1} (amide I) and N–H bending peak at 1560 cm^{-1} (amide II) evidently appeared in the spectra of PCL-*g*-PAA-collagen, PCL-*g*-PAA-chitosan 15000 and PCL-*g*-PAA-chitosan 83000. Moreover, the broad peak in the range of 3000-3600 cm^{-1} corresponding to N-H stretching of NH_2 in collagen and chitosan was observed indicating that collagen and chitosan have been successfully immobilized on the carboxyl-containing PCL films.

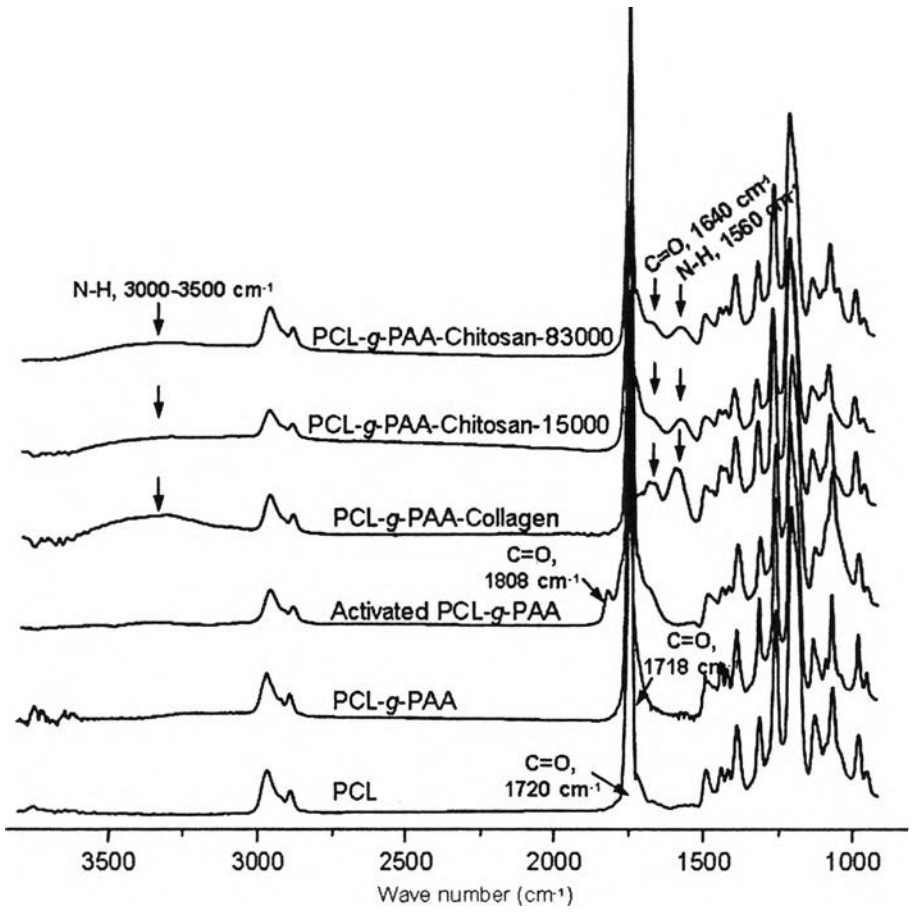


Figure 4.19 ATR-FTIR spectra of virgin PCL film and PCL-*g*-PAA films before and after activation and biomolecule immobilization

Surface atomic compositions of the PCL-*g*-PAA films before and after biomolecule immobilization as characterized by XPS are shown in Table 4.2. After immobilizing with collagen and chitosan, the N_{1s} peak corresponded to the NH_2 in both molecules was detected. The N/C ratio of PCL-*g*-PAA-collagen was higher than that of chitosan MW 83000. These data are in accord with the amount of NH_2 determined by the ninhydrin method.

Table 4.2 Atomic composition of virgin PCL film and PCL- *g*-PAA films before and after biomolecule immobilization

Sample	Atomic composition (%)			N/C ratio
	C	O	N	
PCL	71.0	29.0	-	0
PCL-*g*-PAA	72.5	27.5	-	0
PCL-*g*-PAA-collagen	65.6	28.3	6.1	0.09
PCL-*g*-PAA-chitosan 83000	66.8	29.7	3.5	0.05

4.5 Cell Culture

In vitro cytocompatibility can be used as a primary indication of how cells response to a surface of interest. It is usually expressed in terms of cell adhesion and proliferation. As mentioned in Chapter II, keratinocytes and fibroblasts are the main cells in epidermis and dermis layer, respectively. In order to render biomolecule-immobilized PCL suitable material for artificial skin application, human keratinocyte (HEK001) and fibroblast (L929) cell lines were used for this investigation. Cell adhesion and proliferation was evaluated by using MTT method [34] and the value of cell adhesion and proliferation ratio is reported as the viability of cells attached to a surface in proportion to the viability of cells attached to tissue culture polystyrene (TCPS) plate in the same culture media. All of the modified PCL films from each optimized condition were chosen for the investigation of cytocompatibility (Table 4.3).

Table 4.3 Optimized conditions used for the modification of PCL films that were chosen for cytocompatibility investigation

Sample	Condition
Aminolyzed PCL	Aminolysis in 1.5 M 1,6-hexamethylenediamine/ IPA for 8 h
PCL-*g*-PAA	Photo-oxidation for 30 min followed by graft copolymerization in 10% AA for 1h
Aminolyzed PCL-collagen Aminolyzed PCL-chitosan	Activation in 0.1 M DSC for 1 h followed by immobilization in 5 mg/mL collagen solution or 3 mg/mL chitosan solution for 24 h
PCL-*g*-PAA-collagen PCL-*g*-PAA-chitosan	Activation in 0.1 M NHS/0.4 M EDCI for 1 h followed by immobilization in 5 mg/mL collagen solution or 3 mg/mL chitosan solution for 24 h

4.5.1 Keratinocyte (HEK001) Cell Culture

The HEK001 cell adhesion ratio of PCL, aminolyzed PCL and aminolyzed PCL-biomolecules are shown in Figure 4.20. Having cell adhesion ratio of 61%, virgin PCL can be considered as a fair substrate for cell adhesion as compared to 100% of TCPS. Evidently, the alteration of surface functionality had a significant impact on HEK001 adhesion and proliferation. Introducing NH_2 group on PCL surface via aminolysis increased the cell adhesion ratio of the virgin PCL film. After immobilization of biomolecules, collagen and chitosan, which possess the biocompatibility, the modified PCL films became more favorable for HEK001 adhesion. According to HEK001 proliferation ratio shown in Figure 4.21, all of the modified films became relatively good substrates for cell proliferation. Although the cytocompatibility of all modified PCL films were superior to the virgin PCL film, none can compete with the TCPS.

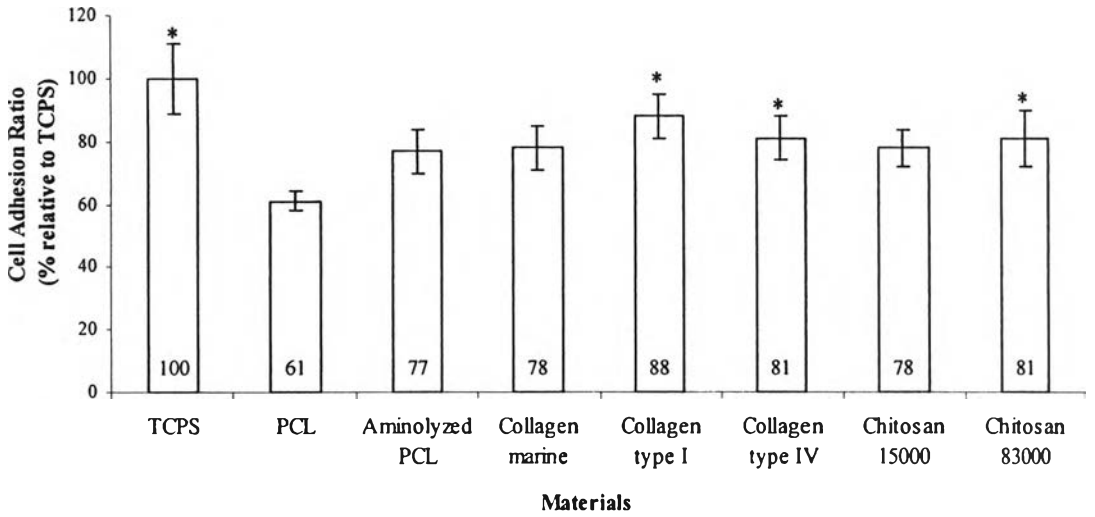


Figure 4.20 *In vitro* cell adhesion ratio of HEK001 on aminolyzed PCL before and after biomolecule immobilization in comparison with TCPS and virgin PCL * indicates that the sample was significantly different from PCL with $p<0.01$

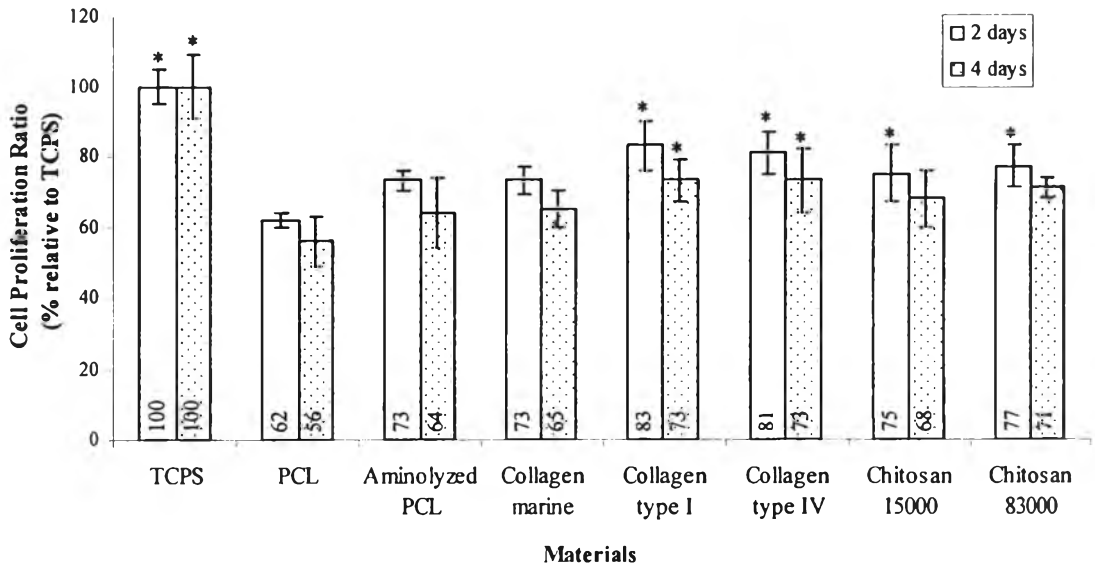


Figure 4.21 *In vitro* cell proliferation ratio of HEK001 on aminolyzed PCL before and after biomolecule immobilization in comparison with TCPS and virgin PCL * indicates that the sample was significantly different from PCL with $p<0.01$

The HEK001 cell adhesion and proliferation ratio of PCL, PCL-*g*-PAA and PCL-*g*-PAA-biomolecules are shown in Figures 4.22 and 4.23, respectively. Evidently, the alteration of surface functionality and hydrophilicity had a significant impact on HEK001 adhesion and proliferation. Introducing COOH group on PCL surface via graft copolymerization of acrylic acid markedly increased the cell adhesion and proliferation ratio of the virgin PCL film. After immobilization of biomolecules, collagen and chitosan, the surface-modified PCL films became more favorable for both adhesion and proliferation. It was observed that the adhesion and proliferation ratio of PCL-*g*-PAA-collagen type I were comparable to that of TCPS. Evidently, the series of PCL-*g*-PAA and PCL-*g*-PAA-biomolecules yielded superior cytocompatability to the series of aminolyzed PCL and aminolyzed PCL-biomolecules. This issue will be later discussed in terms of the extent of surface modification and biomolecule immobilization.

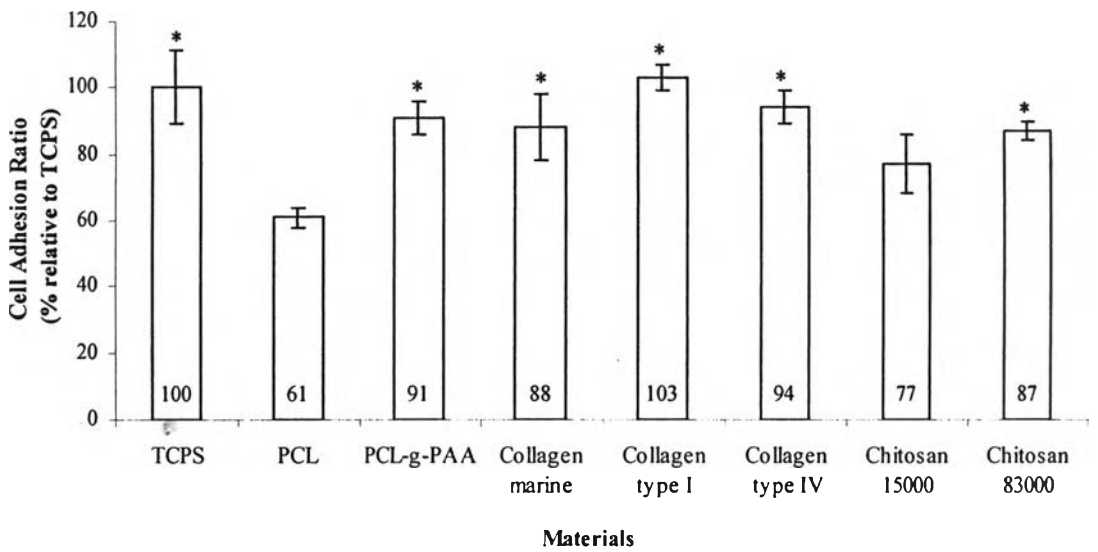


Figure 4.22 *In vitro* cell adhesion ratio of HEK001 on PCL-*g*-PAA before and after biomolecule immobilization in comparison with TCPS and virgin PCL
* indicates that the sample was significantly different from PCL with $p<0.01$

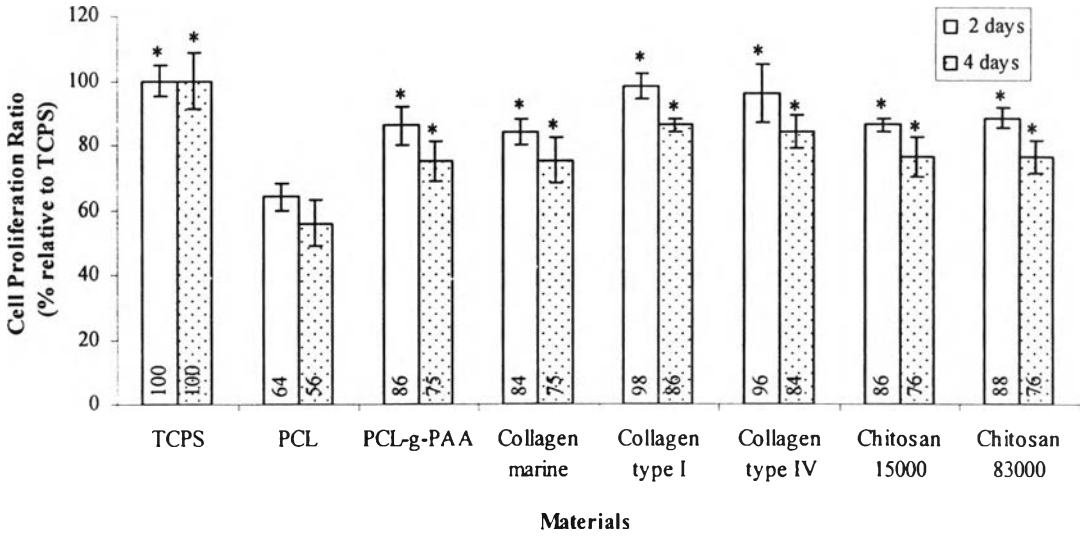


Figure 4.23 *In vitro* cell proliferation ratio of HEK001 on PCL-*g*-PAA before and after biomolecule immobilization in comparison with TCPS and virgin PCL * indicates that the sample was significantly different from PCL with $p<0.01$

4.5.2 Fibroblast (L929) Cell Culture

In the case of fibroblast L929, PCL had cell adhesion ratio at 69%, slightly better than the value observed for HEK001. The cell adhesion ratio and proliferation ratio of PCL, aminolyzed PCL and aminolyzed PCL-biomolecules shown in Figures 4.24 and 4.25, respectively, followed a similar trend which has been previously observed for HEK001. The surface modification and biomolecule immobilization, however, seem to have a more pronounced effect on the cytocompatibility towards L929 fibroblasts than HEK001. Aminolyzed PCL-collagen type I which is the best substrate possessed as high as 99% adhesion ratio of L929 whereas 88% cell adhesion ratio was observed in the case of HEK001.

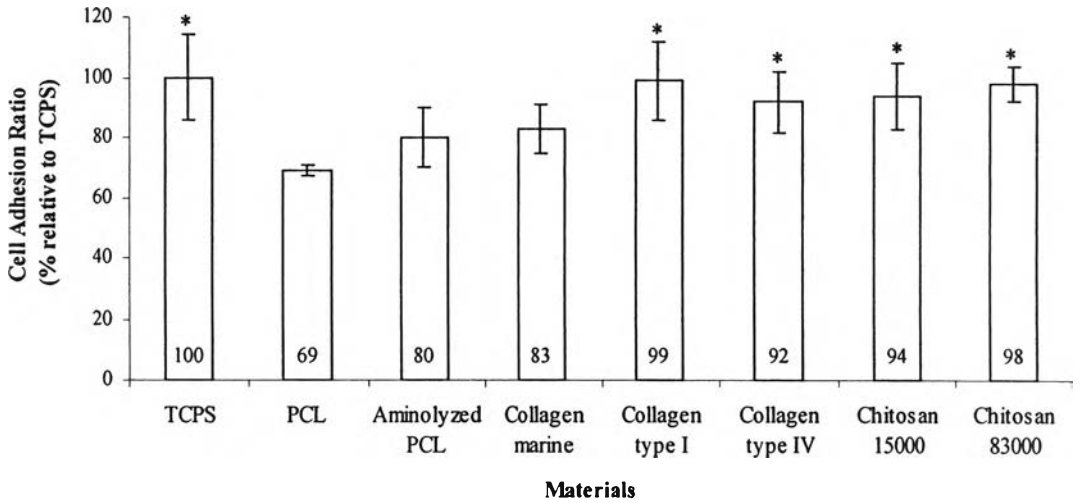


Figure 4.24 *In vitro* cell adhesion ratio of L929 on aminolyzed PCL before and after biomolecule immobilization in comparison with TCPS and virgin PCL * indicates that the sample was significantly different from PCL with $p<0.01$

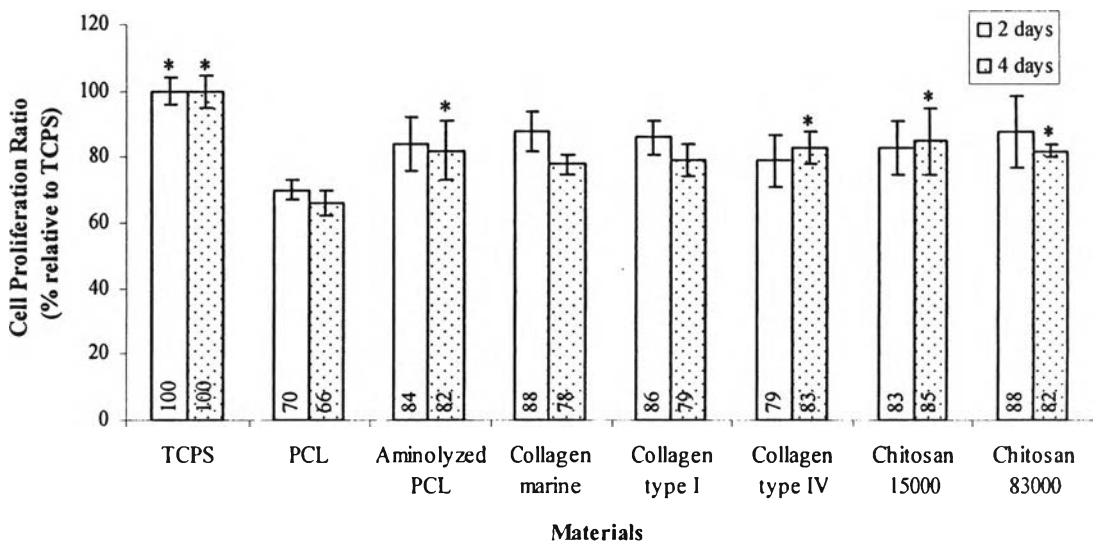


Figure 4.25 *In vitro* cell proliferation ratio of L929 on aminolyzed PCL before and after biomolecule immobilization in comparison with TCPS and virgin PCL * indicates that the sample was significantly different from PCL with $p<0.01$

The L929 cell adhesion and proliferation ratio of PCL, PCL-*g*-PAA and PCL-*g*-PAA-biomolecules are shown in Figures 4.26 and 4.27. The results conformed to what previously described for HEK001 cell line. Most of the surface-modified PCL films sustained as high as 90% cell adhesion ratio. The ratio of some films exceeded 100% of TCPS. In terms of both cell adhesion ratio and cell proliferation ratio, PCL-*g*-PAA-collagen type I seems to be the best substrate not only for HEK001, but also for L929.

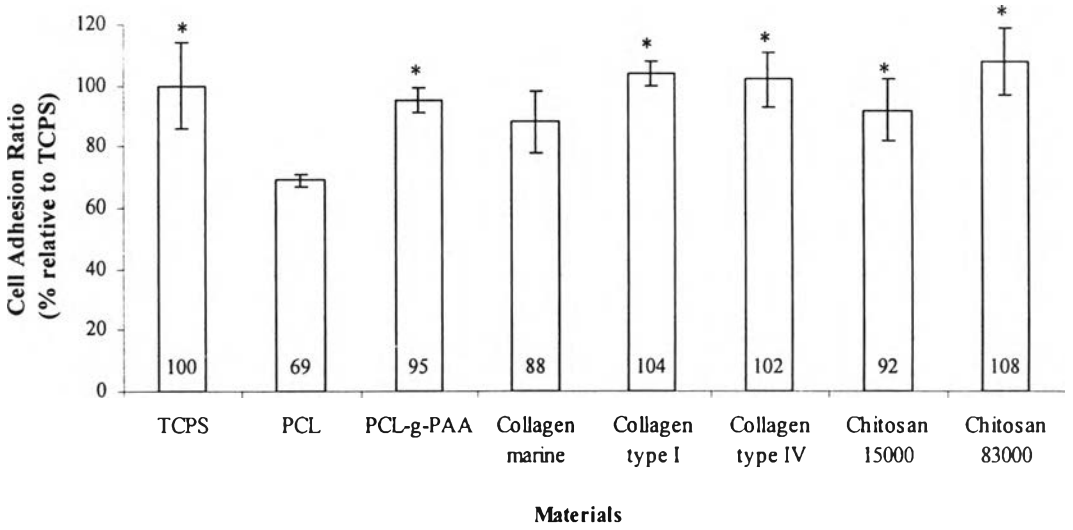


Figure 4.26 *In vitro* cell adhesion ratio of L929 on PCL-*g*-PAA before and after biomolecule immobilization in comparison with TCPS and virgin PCL
* indicates that the sample was significantly different from PCL with $p<0.01$

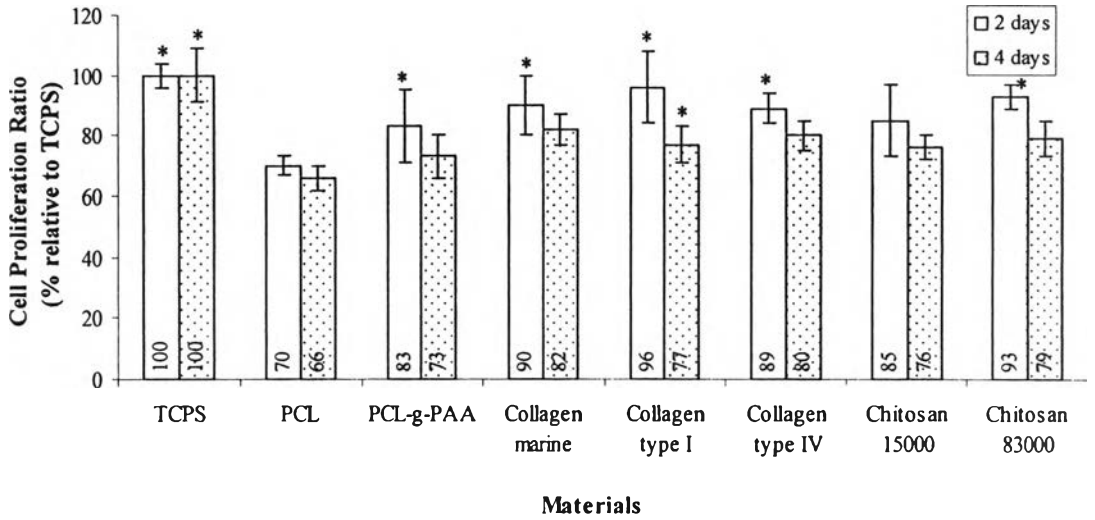


Figure 4.27 *In vitro* cell proliferation ratio of L929 on PCL-*g*-PAA before and after biomolecule immobilization in comparison with TCPS and virgin PCL
* indicates that the sample was significantly different from PCL with $p<0.01$

According to the study on the compatibility between cells and materials; the water contact angle (hydrophobicity-hydrophilicity), surface functional group, surface functional moieties and surface charge are the most important factors in the adhesion of cells [19]. The better cell adhesion and proliferation of aminolyzed PCL and PCL-*g*-PAA might be explained by the increase in hydrophilicity. In particular, it has been also reported that placing N-containing groups such as amine on the surface is advantageous to cell adhesion due to the fact that a fraction of N-containing groups might be positively charged at physiological pH because of the protonation. As a result, this would enhance the interaction between the surface and the cells which carried negative charge [1].

Being the main component protein in extracellular matrix (ECM), collagen type I has the influence on several cellular responses such as attachment and proliferation. This perhaps accounts for the high efficiency of collagen type I in enhancing cytocompatibility of PCL films against both cell lines. Considering the data listed in Table 4.4, it is rather unfair to comparatively judge the effectiveness of each biomolecule based on the fact that its immobilized quantity, expressed in term

of NH_2 concentration/surface area, was varied. Obviously, the extent of biomolecule immobilization depends largely on the density of active functional group (NH_2 or COOH) obtained after surface modification (aminolysis or graft copolymerization). This explained why PCL-g-PAA and PCL-g-PAA-biomolecule possessed superior cytocompatibility to aminolyzed PCL and aminolyzed PCL-biomolecule. Despite their low immobilized density in comparison with the collagen family, chitosan is a reasonably good candidate from economical perspective. Cellular responses of some of PCL films immobilized with chitosan were comparable with or even better than some of PCL films immobilized with collagen, especially marine collagen.

Table 4.4 Amount of active functional groups (NH_2 or COOH) obtained after surface modification and NH_2 density on the surface of biomolecule-immobilized PCL films

Surface modification route	Active functional group (mol/cm^2)	NH_2 concentration/surface area (mol/cm^2) after biomolecule immobilization				
		Collagen Marine	Collagen type I	Collagen type IV	Chitosan MW 15000	Chitosan MW 83000
Aminolysis	6.30×10^{-8}	1.05×10^{-8}	0.94×10^{-8}	0.79×10^{-8}	0.23×10^{-8}	0.27×10^{-8}
Graft copolymerization	9.10×10^{-8}	1.66×10^{-8}	1.22×10^{-8}	1.10×10^{-8}	0.78×10^{-8}	0.87×10^{-8}