CHAPTER IV

RESULTS AND DISCUSSION

The aim of this work is to covalently attach Arg-Gly-Asp (RGD)-containing peptides on poly(DTE-*co*-20%DT carbonate) surface, employing a two-step procedure. The first step involved an activation of surface carboxyl group into active *N*-hydroxysuccinimide (NHS) ester that is less prone to hydrolysis. The second step was a coupling of RGD-containing peptide. The mechanism of the activation of carboxyl groups on the surface followed by the coupling reaction with RGD-containing peptide is shown in Figure 4.1.

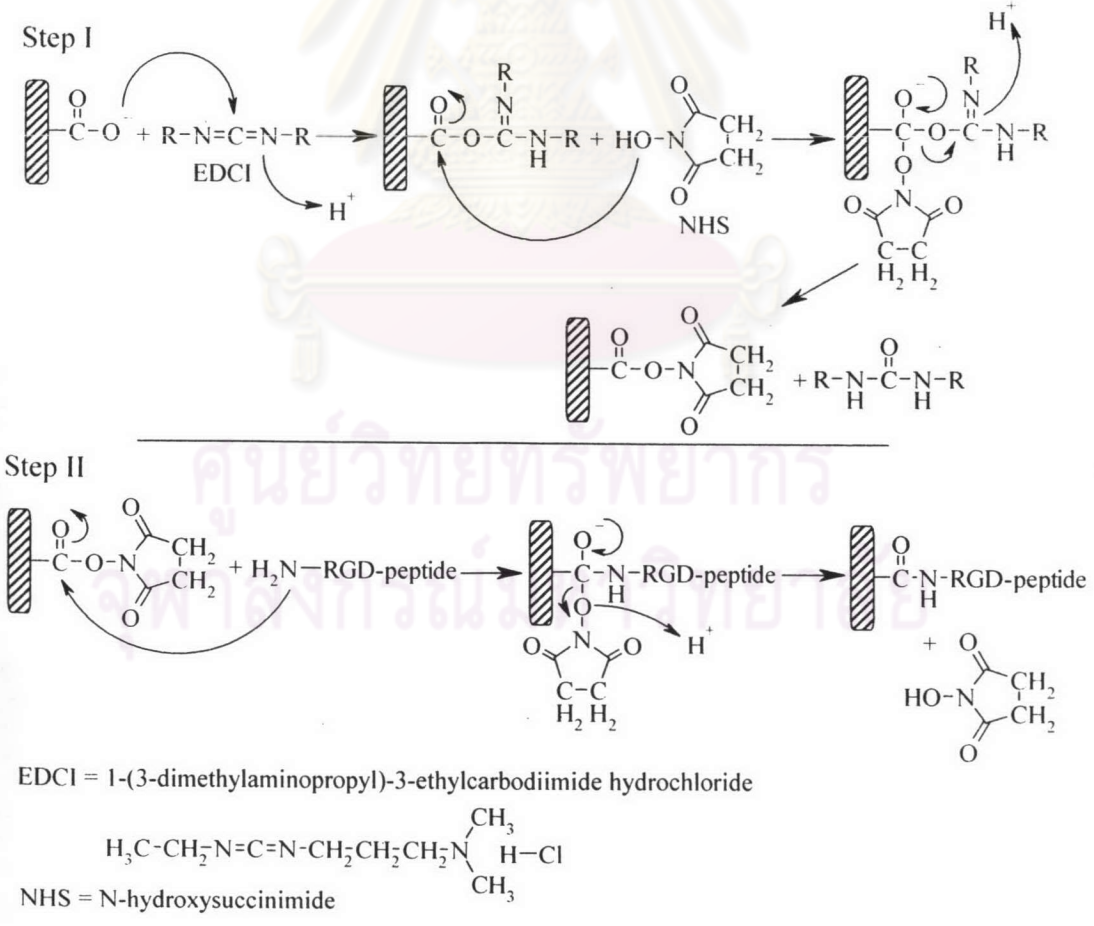
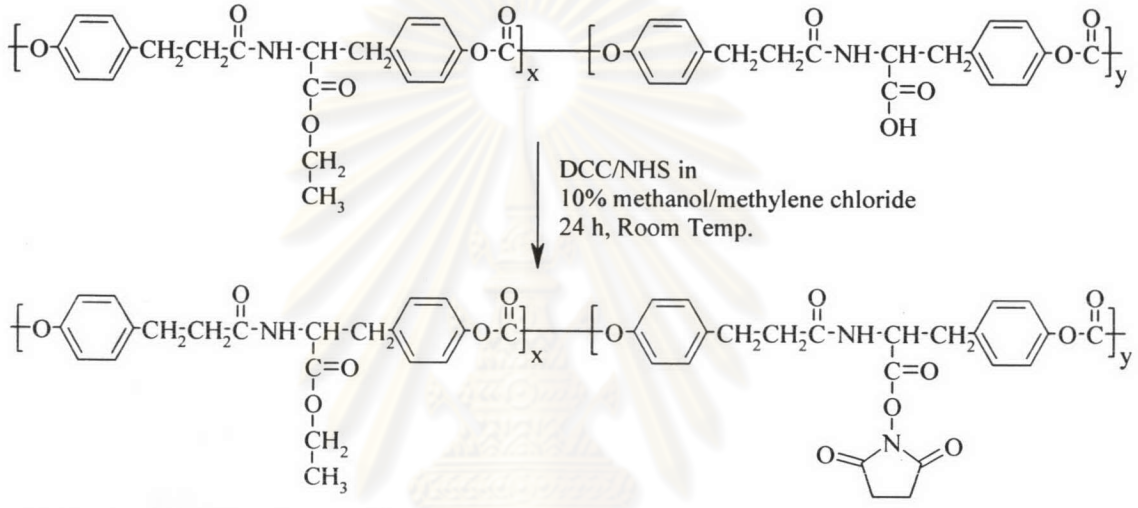


Figure 4.1 Mechanism of an activation of surface carboxyl group followed by a coupling reaction with RGD-containing peptide.

4.1 Activation of Poly(DTE-*co*-20%DT carbonate) with *N*-hydroxysuccinimide under Homogeneous Condition

The activation reaction was first conducted under homogeneous condition in order to determine a possibility to follow an extent of activation using ^{1}H NMR. Two solvent systems that can completely solubilize poly(DTE-*co*-20%DT carbonate) were chosen: 10% (v/v) methanol/methylene chloride and tetrahydrofuran.



poly(DTE-*co*-20%DT carbonate), [y/(x+y)] x 100 = 20%

The activation reaction was carried out using 0.25 M *N*-hydroxysuccinimide (NHS) in the presence of 0.25 M dicyclohexylcarbodiimide (DCC) at room temperature for 24 h. According to Figure 4.2, there were significant changes of signals from ^{1}H NMR spectrum of virgin poly(DTE-*co*-20%DT carbonate) to the activated ones. A group of new peaks appearing in the range of 3.4-3.8 ppm and in the range of 1.8-2.0 can be assigned to dicyclohexylurea (DCU) which is a solid by-product of reaction. The urea precipitate cannot be completely removed even after filtration through PTFE membrane having 1.0 μm pore diameter. A signal at 2.85 ppm which is not present in ^{1}H NMR spectrum of virgin poly(DTE-*co*-20%DT carbonate) was labeled as protons of *N*-succinimide esters attached to carboxyl groups of the polymer. While a signal at 2.76 ppm corresponded to the unreacted NHS that was trapped inside the polymer. This study has demonstrated that it is possible to follow the extent of activation using ^{1}H NMR analysis since the signal of reacted NHS was well separated from the one of unreacted NHS. The extent of activation in

term of % substitution can be calculated from the relative peak area at 2.85 ppm belonging to the protons of *N*-succinimide and those binding to the α-carbon of tyrosine in the range of 4.76-4.84 ppm according to the following equation

$$\% \text{ Substitution} = \frac{\text{Integration of protons at 2.85 ppm}/4}{\text{Integration of protons in range of 4.76-4.84 ppm}} \times \frac{100}{\%DT}$$

The fact that the relative ratio of integration of protons at 2.85 ppm and the one of protons at 2.76 ppm obtained from 10% (v/v) methanol/methylene chloride is quite similar to the one obtained from tetrahydrofuran implies that the activated carboxyl groups in the form of *N*-succinimide esters are quite stable even in the presence of methanol which is a protic solvent.

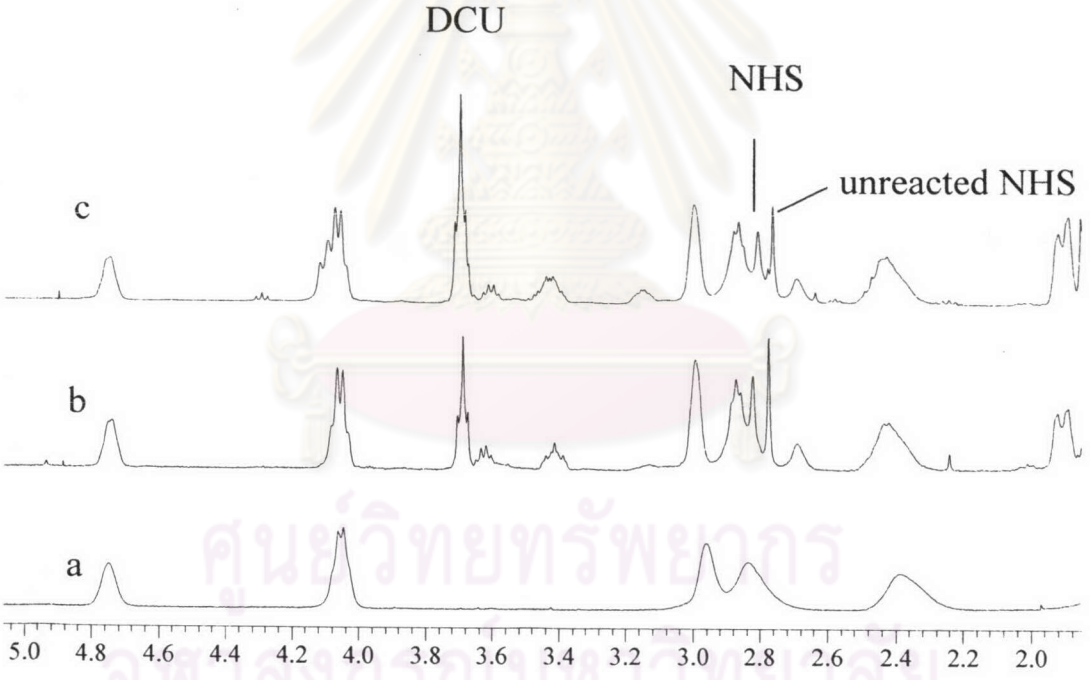
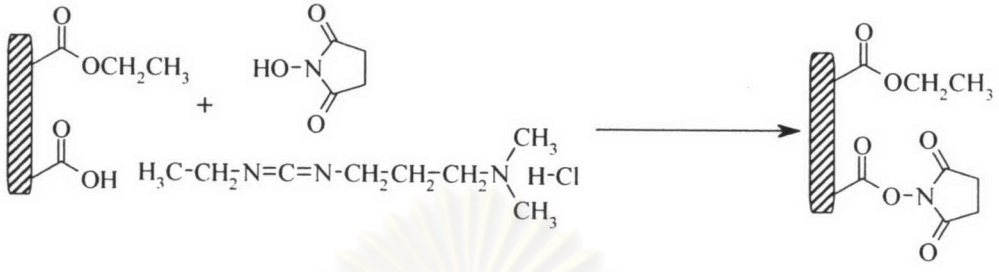


Figure 4.2 ^{1}H NMR spectra of (a) poly(DTE-*co*-20%DT carbonate) and poly(DTE-*co*-20%DT carbonate) after reacted with *N*-hydroxysuccinimide in (b) tetrahydrofuran and (c) 10% (v/v) methanol/methylene chloride.

4.2 Activation of Poly(DTE-*co*-20%DT carbonate) with *N*-hydroxysuccinimide under Heterogeneous Condition



In light of the success of the activation conducted under homogeneous condition, the activation of carboxyl groups on the surface of poly(DTE-*co*-20%DT carbonate) with *N*-hydroxysuccinimide under heterogeneous condition was then pursued. In stead of using DCC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) was used together with NHS for the heterogeneous reaction. Effects of solvent, concentration of coupling agent (NHS and EDCI) and reaction time on the extent of activation were investigated. ^{1}H NMR was used as a major tool for characterization while water contact angle measurement and ATR-FTIR were used in some cases. A series of experiments was conducted in order to determine the optimum condition for the activation of carboxyl groups on the surface of poly(DTE-*co*-20%DT carbonate) prior to an attachment with RGD-containing peptides.

4.2.1 Effect of Solvent

In principle, the success of chemical reaction at the polymer/solution interface is determined by the ability of solvent to wet and/or swell the polymer surface. According to literatures, the covalent attachment of RGD-containing peptides are generally performed in aqueous solution due to the fact that water can dissolve peptides as well as coupling reagents, NHS/EDCI in this particular case, and also wet the hydrophilic polymer substrate to some extent. For this reason, water was selected as the first solvent to be investigated. According to Figure 4.3, the water contact angle has increased from 71.5°/29.8° of virgin poly(DTE-*co*-20%DT carbonate) film to the maximum of ~81.7°/32.6° of the activated film after 150 min of reaction in aqueous solution of 0.1 M NHS/EDCI. The contact angle did not increase any further after 150 min implying that the highest extent of activation in the surface region has been

achieved. This elevation of contact angle suggests that the hydrophilic carboxyl groups have been converted to hydrophobic *N*-succinimide groups.

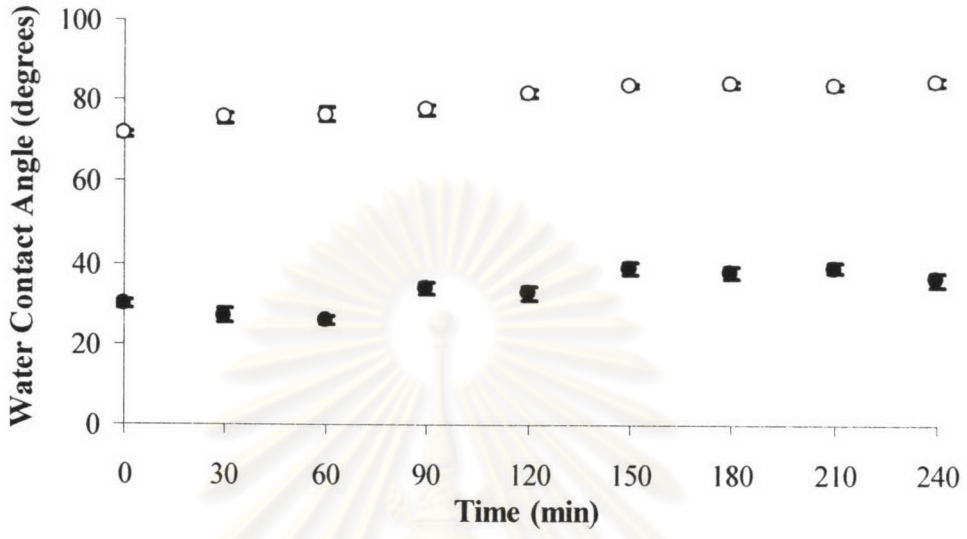


Figure 4.3 Water contact angle of poly(DTE-*co*-20%DT carbonate) film after the reaction with aqueous solution of 0.1 M NHS/EDCI: advancing (○) and receding (●) water contact angle.

Activated poly(DTE-*co*-20%DT carbonate) film obtained after the reaction between poly(DTE-*co*-20%DT carbonate) film and aqueous solution of 0.1 M NHS/EDCI was also characterized by ATR-FTIR. An emerging weak shoulder at 1825 cm^{-1} observed in the spectrum of activated poly(DTE-*co*-20%DT carbonate) can be assigned to the the C=O stretching of succinimidyl ester (Figure 4.4). This result implied that the activation in aqueous solution has in fact proceeded at least to the sampling depth of ATR-FTIR (1-2 μm).

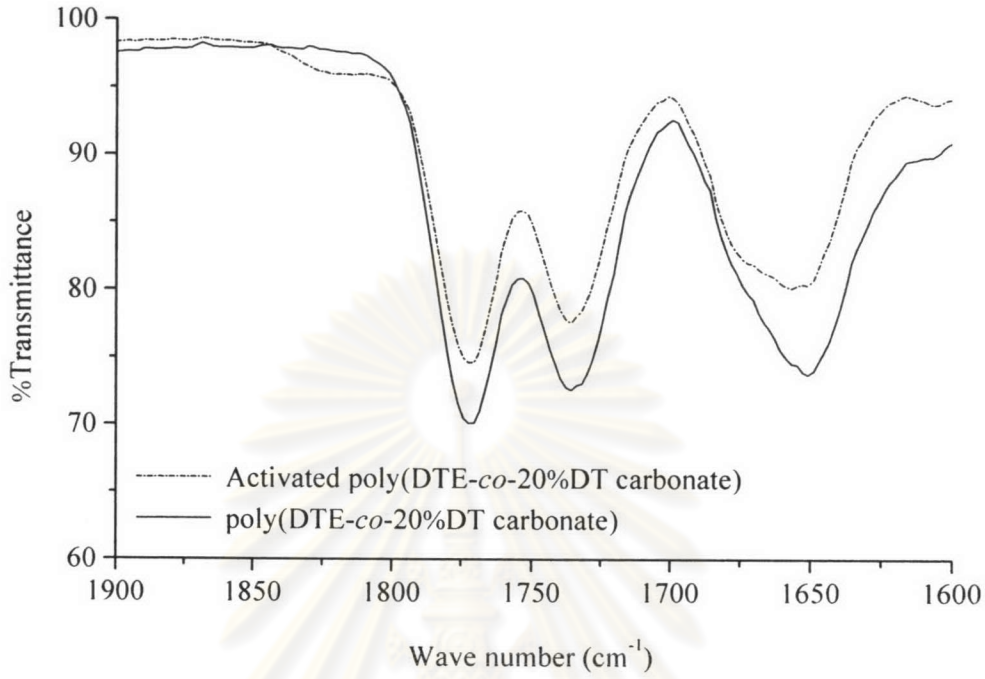


Figure 4.4 ATR-FTIR spectra of poly(DTE-*co*-20%DT carbonate) film and activated poly(DTE-*co*-20%DT carbonate) film

In order to quantitatively determine the extent of activation, the activated film was analyzed by ^{1}H NMR analysis. From Figure 4.5 (a), it turned out that there was no signal at 2.85 ppm which represents the protons of *N*-succinimide groups attached to the polymer. This may be explained by the fact that ^{1}H NMR analysis is a bulk characterization technique so it cannot assess any changes of functionality unless the activation has proceeded to a sufficient depth. In addition, water may not be able to swell the surface of poly(DTE-*co*-20%DT carbonate) film that well. As a consequence, the depth of activation was quite thin. For these reasons, ethanol was proportionally added to the aqueous solution of NHS/EDCI in order to increase swelling of poly(DTE-*co*-20%DT carbonate).

The ^{1}H NMR spectra shown in Figure 4.5 (b-d) indicated that an extensive swelling of poly(DTE-*co*-20%DT carbonate) was necessary for the reaction to proceed deep enough so that the success of surface activation can be followed by ^{1}H

NMR. This is the reason why the signal at 2.85 ppm was only detected when ethanol was used as a solvent since it can swell the polymer much more extensively than the solvent mixtures between water and ethanol. Even though water contact angle and ATR-FTIR data can be used as indications of surface activation, they are not suitable for quantitative analysis. For the subsequent investigation, ethanol was thus used as a solvent to assure that the surface activation can proceed deep enough in the polymer film. The fact that the peak at 2.85 ppm previously assigned to the unreacted NHS was absent help demonstrating an advantage of using heterogeneous condition for activation. Since the reaction only took place on the surface, there was less chance for DCU or unreacted NHS to be trapped inside the polymer. If there were some, they should be insignificantly small and can be easily removed.

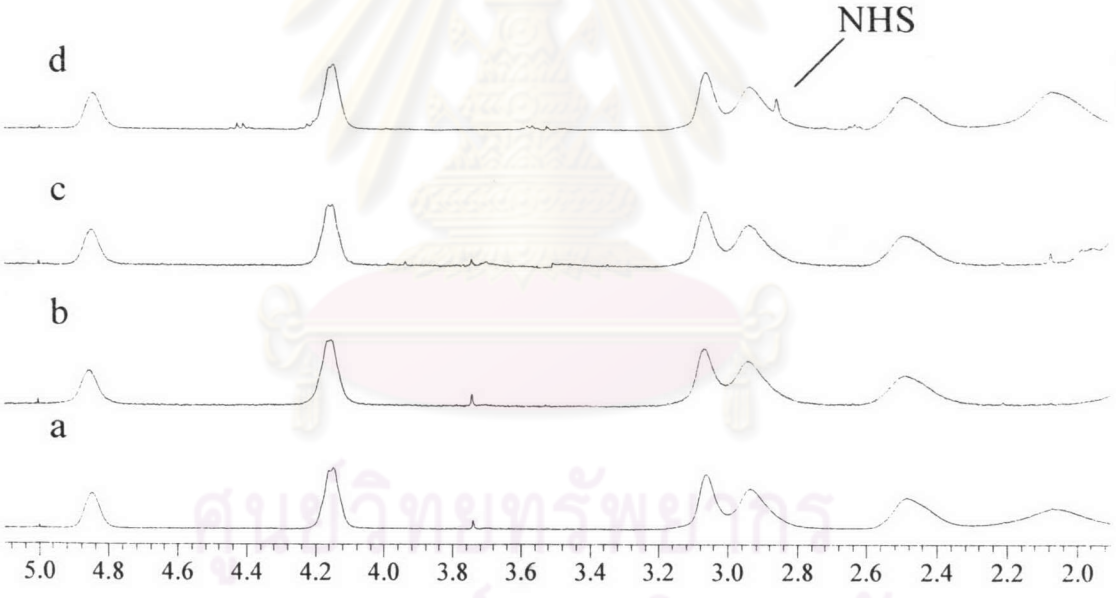


Figure 4.5 ^{1}H NMR spectra of poly(DTE-*co*-20%DT carbonate) film after the reaction with 0.1 M NHS/EDCI in (a) water, (b) 20% ethanol/water, (c) 50% ethanol/water and (d) ethanol for 2 h.

4.2.2 Effect of NHS/EDCI Concentration

Using ethanol as a solvent, %yield of activation calculated from the relative integration of the peak at 2.85 ppm corresponding to the proton of *N*-hydroxysuccinimide and the integration of the peak in the range of 4.76-4.84 ppm

corresponding to the α-carbon of tyrosine was linearly increased as a function of NHS/EDCI concentration (Figure 4.6). It is worth noting that the calculated % substitution was an average yield throughout the bulk because ^{1}H NMR analysis is a bulk characterization technique. It may not represent an actual reaction yield at the surface which could be considerably higher. It is suspected that increasing the NHS/EDCI concentration should promote the activation to proceed much deeper into the bulk resulting in higher overall yield which may or may not be relevant to the surface yield. Therefore, it is also possible that % substitution of activation in the surface region may reach its maximum without having to use a high concentration of NHS/EDCI.

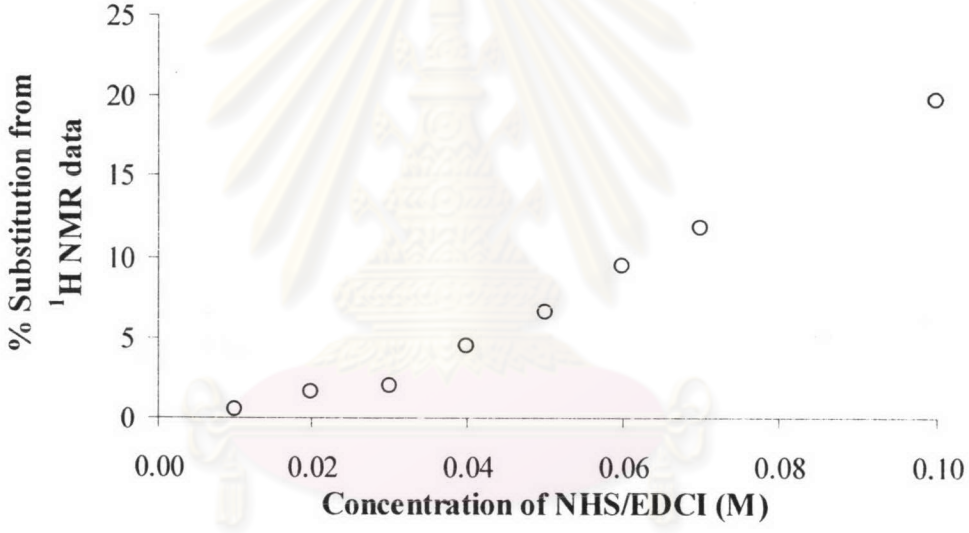


Figure 4.6 % Substitution of activation of poly(DTE-*co*-20%DT carbonate) film after reaction with NHS/EDCI solution in ethanol for 2 h as a function of NHS/EDCI concentration

4.2.3 Effect of Reaction Time

Choosing the moderate (0.05 M) and highest (0.1 M) concentration of NHS/EDCI used in the previous section, an effect of reaction time on % substitution of activation was investigated. Shown in Figure 4.7, % substitution of reaction was increased as a function of reaction time within the first 2 h of reaction for both cases.

The maximum substitution of 8 % and 20% were obtained using the NHS/EDCI concentration of 0.05 M and 0.1 M, respectively.

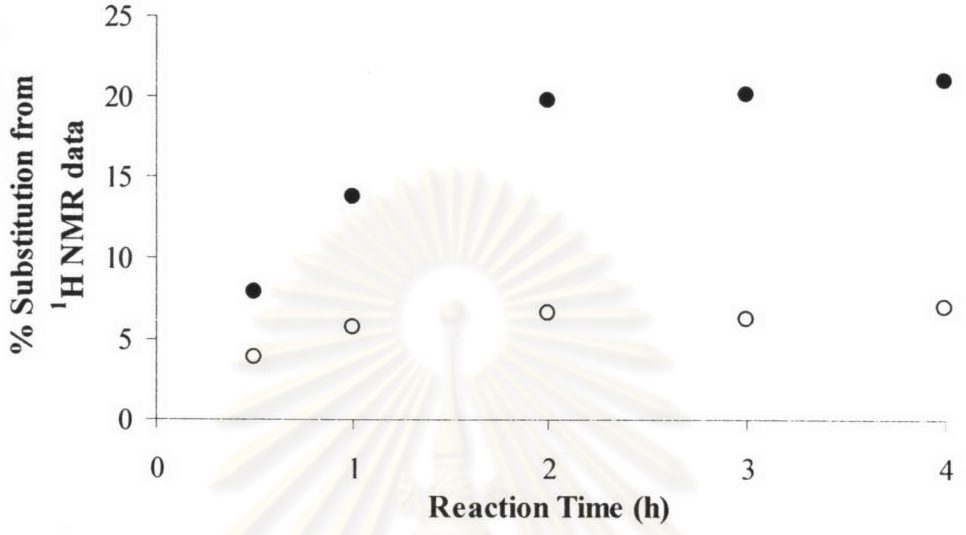


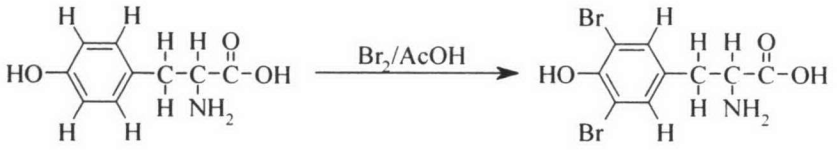
Figure 4.7 % Substitution of activation of poly(DTE-*co*-20%DT carbonate) film after reaction with NHS/EDCI solution in ethanol as a function of reaction time: 0.1 M NHS/EDCI (●) and 0.05 M NHS/EDCI (○).

An optimum condition for the preparation of NHS-activated poly(DTE-*co*-20%DT carbonate) film that can be deduced from ^{1}H NMR data is to use 0.1 M NHS/EDCI in ethanol and carrying out the activation for 2h

4.3 Reaction of Activated Poly(DTE-*co*-20%DT carbonate) Film with L-3,5-Dibromotyrosine

Prior to immobilization of RGD-containing peptides, the activity of *N*-succinimide groups on the polymer surface towards the substitution of amino groups of a model compound was first tested. L-3,5-Dibromotyrosine was selected as a model compound for peptide. Bromine was introduced to L-tyrosine as a tag element so the success of model compound attachment can be followed by X-ray photoelectron spectroscopy (XPS), a surface characterization technique that can probe an atomic composition of surface within a sampling depth of up to 50 Å

4.3.1 Synthesis of L-3,5-Dibromotyrosine



As a consequence of *ortho* and *para*-directing activation of hydroxyl group attached to an aromatic ring of L-tyrosine, the reaction between L-tyrosine and excess Br_2 *via* electrophilic aromatic substitution yielded disubstituted product of L-3,5-dibromotyrosine. The substitution only occurred at two *ortho* positions of the hydroxyl group since the para position was not available. ^{1}H NMR spectrum of L-3,5-dibromotyrosine is shown in Figure 4.8. Two doublet signals from aromatic ring of L-tyrosine at 6.90 ppm and 7.19 ppm were replaced by a singlet signal at 7.18 ppm after bromination confirming the disubstitution of bromine in aromatic ring.

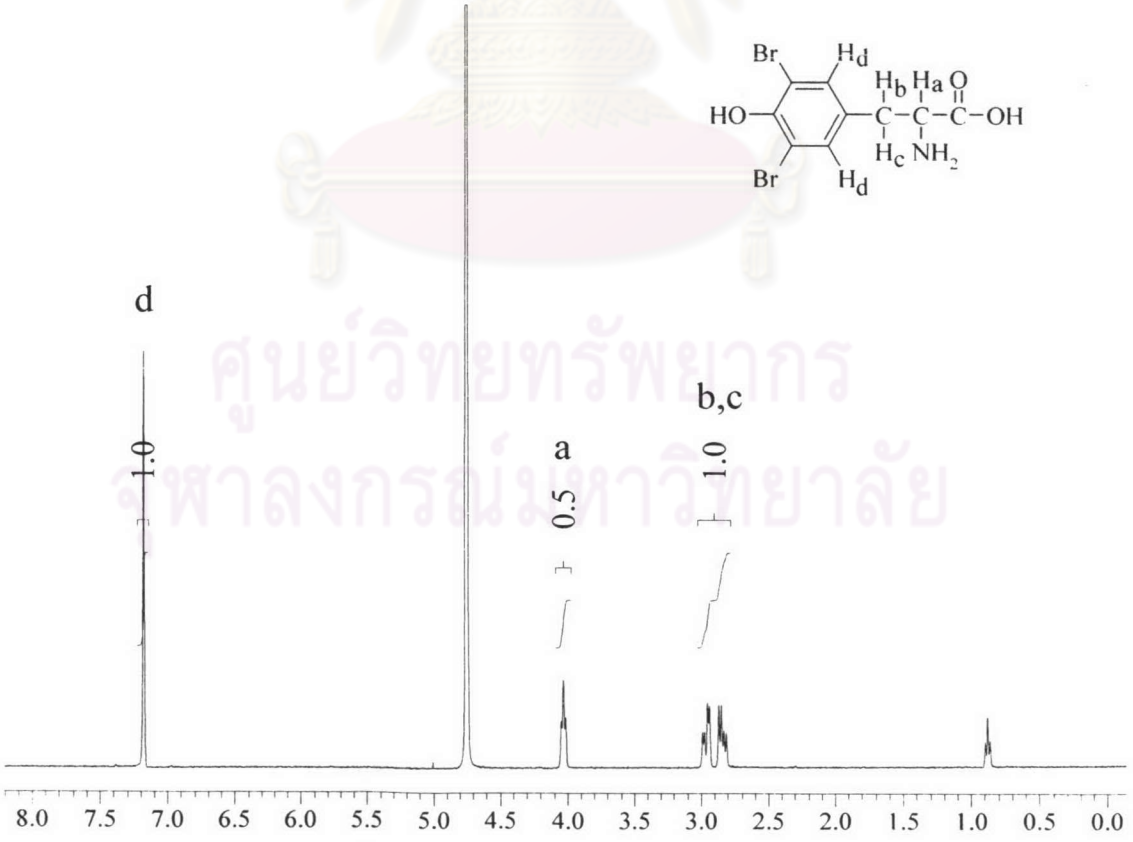
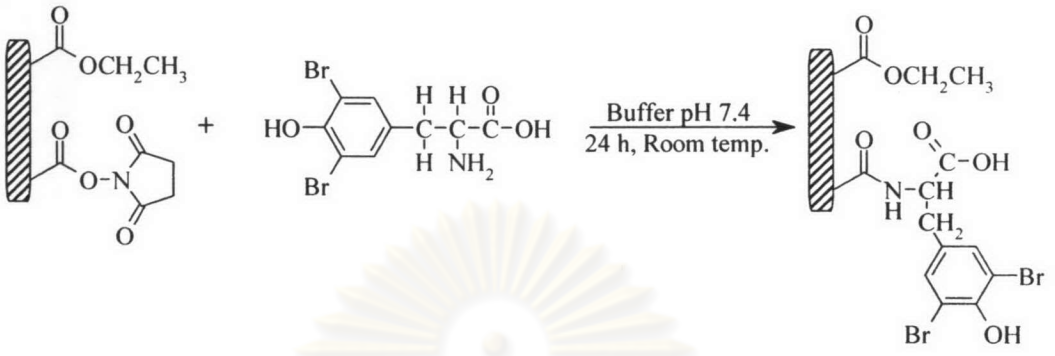


Figure 4.8 ^{1}H NMR spectrum of L-3,5-dibromotyrosine.

4.3.2 Attachment of L-3,5-Dibromotyrosine on the Surface of Activated Poly(DTE-*co*-20%DT carbonate) Film

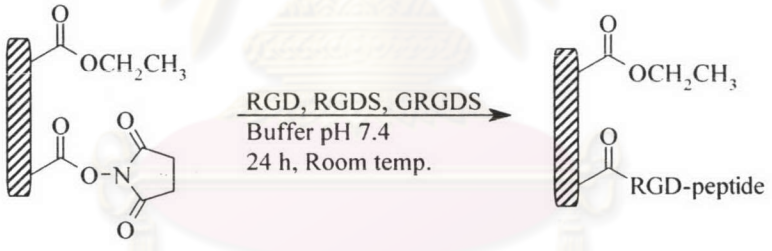


XPS was used to confirm the immobilization of L-3,5-dibromotyrosine on the activated poly(DTE-*co*-20%DT carbonate) surface. XPS data are outlined in Table 4.1. Theoretical atomic compositions calculated from atomic ratios of the chemical structures were also included for comparison. Controlled poly(DTE-*co*-20%DT carbonate) film is a virgin poly(DTE-*co*-20%DT carbonate) film that was exposed to the solution of L-3,5-dibromotyrosine simultaneously with the activated poly(DTE-*co*-20%DT carbonate) film. The fact that bromine composition was absent on the surface of controlled poly(DTE-*co*-20%DT carbonate) film indicated that the immobilization L-3,5-dibromotyrosine cannot take place without the activated succinimide group and there was no physisorption of L-3,5-dibromotyrosine on the surface. The atomic composition of the controlled film was quite consistent with the theoretical value. The theoretical percentage of bromine shown in the last row of the table was calculated based on the assumption that every single carboxyl group on the surface was converted to *N*-succinimide esters which later completely reacted with L-3,5-dibromotyrosine. % Substitution of L-3,5-dibromotyrosine on the surface calculated from XPS atomic composition was 92%. This outcome suggests that the activated carboxyl group in the form of *N*-succinimide ester was quite stable, yet reactive enough to interact with amino groups of the model peptide giving reasonably high % substitution. Such evidence also implies that the activated film should be able to react with RGD-containing peptides in a similar manner.

Table 4.1 Atomic composition of controlled poly(DTE-*co*-20%DT carbonate) film and NHS-activated poly(DTE-*co*-20%DT carbonate) film after L-3,5-dibromotyrosine attachment

Sample	Source	Atomic composition (%)			
		C	O	N	Br
Controlled poly(DTE-*co*-20%DT carbonate) film	XPS	71.4	24.2	4.4	-
	Theoretical	74.7	21.7	3.6	-
NHS-activated poly(DTE-*co*-20%DT carbonate) film after L-3,5-dibromotyrosine attachment	XPS	68.7	26.4	3.7	1.2
	Theoretical	73.5	21.2	4.0	1.3

4.4 Attachment of RGD-containing Peptides on the Surface of Activated Poly(DTE-*co*-20%DT carbonate) Film



Three RGD-containing peptides: RGD, RGDS, GRGDS were chosen for this study. Their structures are shown in Figure 4.9.

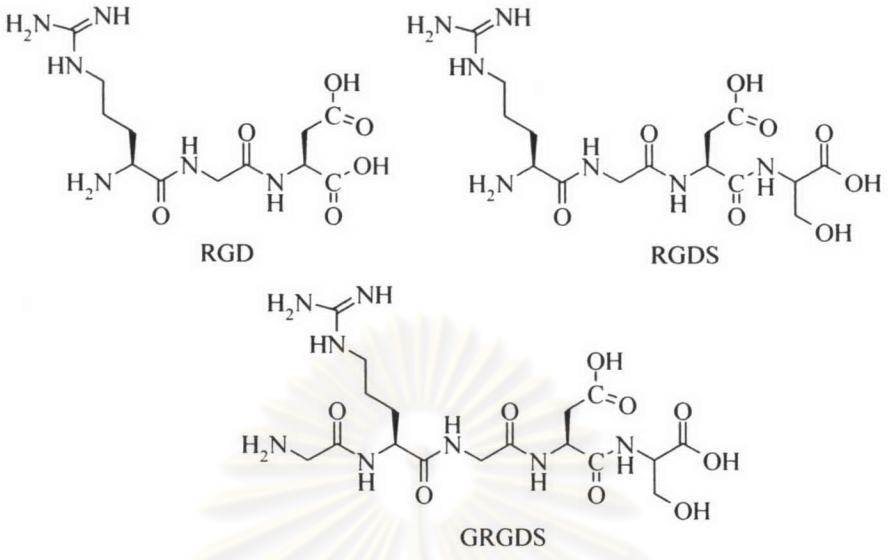


Figure 4.9 Chemical structures of RGD, RGDS and GRGDS

The amino group which is a part of peptide immobilized on the poly(DTE-*co*-20%DT carbonate) surface was quantified by ninhydrin method. The blue color which is a product of ninhydrin reacted with free amino groups (NH_2) has a maximum absorbance at 538 nm in 1,4-dioxane/2-propanol (1:1). Using 1,6-hexanediamine as a standard, a calibration curve was generated as shown in Figure 4.10.

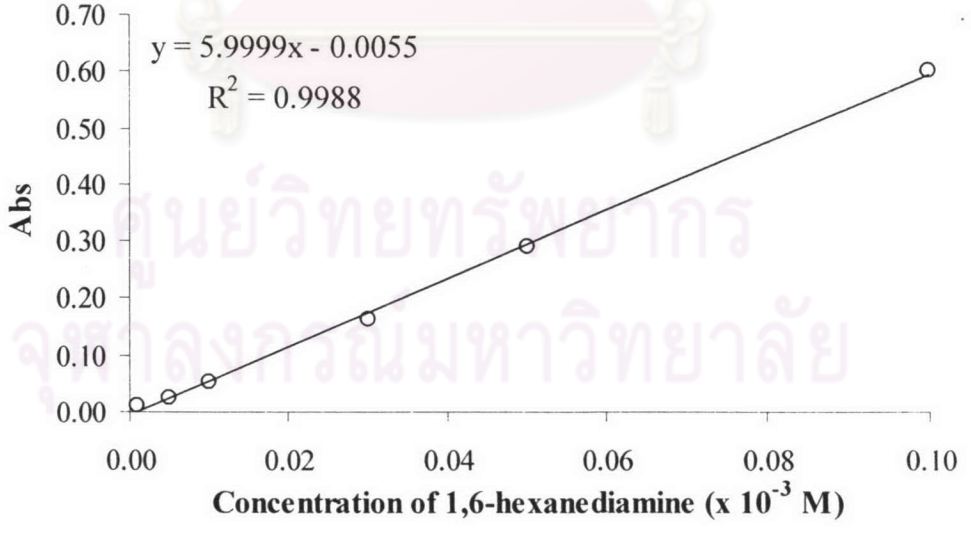


Figure 4.10 Calibration curve of UV absorbance as a function of 1,6-hexanediamine concentration using ninhydrin method.

4.4.1 Effect of Immobilization Time

Effect of reaction time on the immobilization of RGD on the surface of poly(DTE-*co*-20%DT carbonate) was determined by varying the reaction time from 2 to 48 h using the peptide concentration of 0.05 M. As displayed in Figure 4.11, the amino concentration per surface area was linearly increased as a function of reaction time within 24h and seemed to level off afterwards.

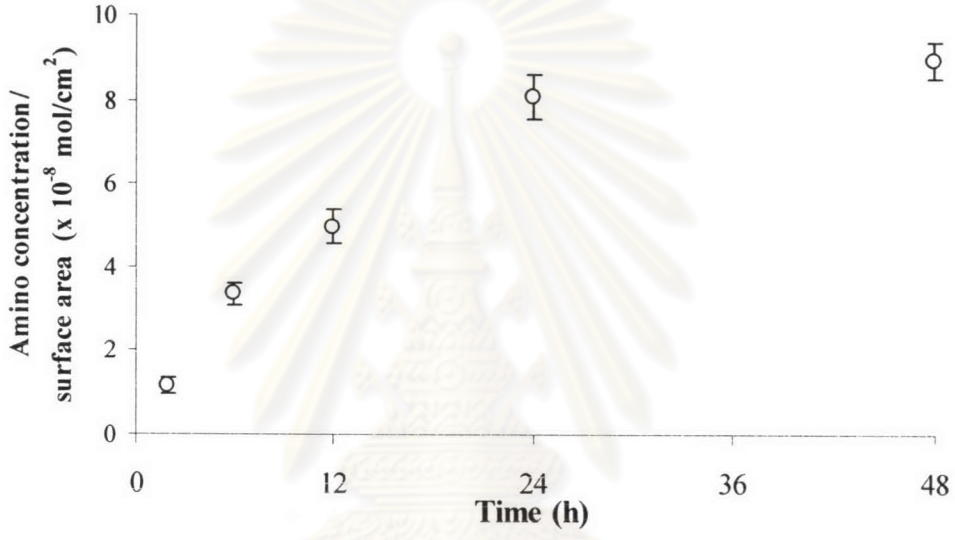


Figure 4.11 Amino concentration per surface area of immobilized RGD on poly(DTE-*co*-20%DT carbonate) surface as a function of immobilization time.

4.4.2 Effect of RGD-containing Peptide Concentration

The density of RGD-containing peptide or amino concentration/surface area on poly(DTE-*co*-20%DT carbonate) film was also influenced by RGD concentration. The data are illustrated in Figure 4.12. As the higher peptide concentration was used, the greater extent of peptide immobilization on the surface of poly(DTE-*co*-20%DT carbonate) was obtained. The density of immobilized RGD peptide of 16.1 x 10^{-8} mol/cm^2 was achieved after carrying the reaction using the RGD concentration of 0.05 M for 24 h.

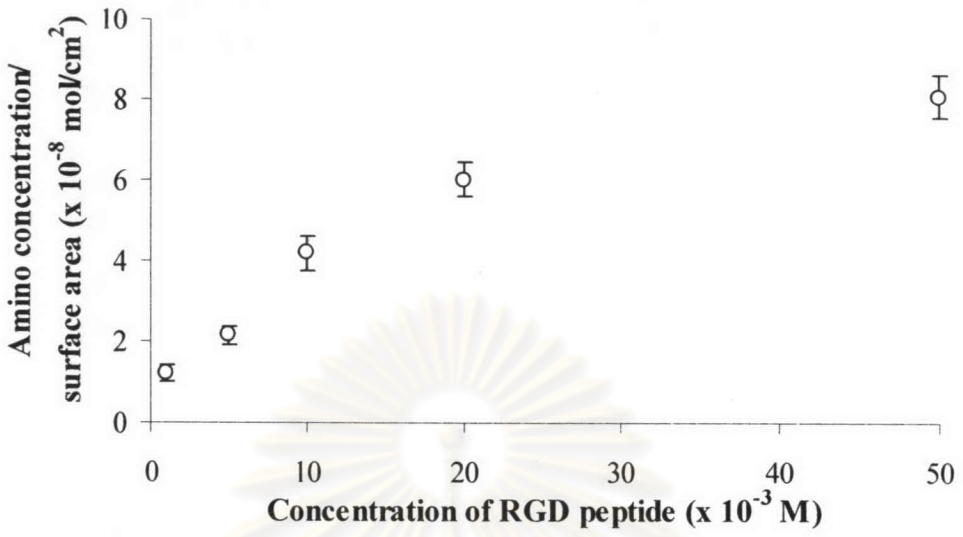


Figure 4.12 Amino concentration per surface area of immobilized RGD on poly(DTE-*co*-20%DT carbonate) surface as a function of peptide concentration.

4.4.3 Effect of RGD-containing Peptide

Using the concentration of peptide of 0.05 M and immobilization time of 24h, the extent of grafting of three RGD-containing peptides on the surface of activated poly(DTE-*co*-20%DT carbonate) were compared. As displayed in Figure 4.13, it seems that the longer peptide sequence, the lower the density of immobilized peptide. This trend may be explained by two reasons: (1) the bulkier structure of GRGDS in comparison with RGD and RGDS may lead to its lower reactivity to interact with the activated surface, (2) the limited solubility of the larger peptide in aqueous solution may account for the lower density of immobilized GRGDS in comparison with other two sequences.

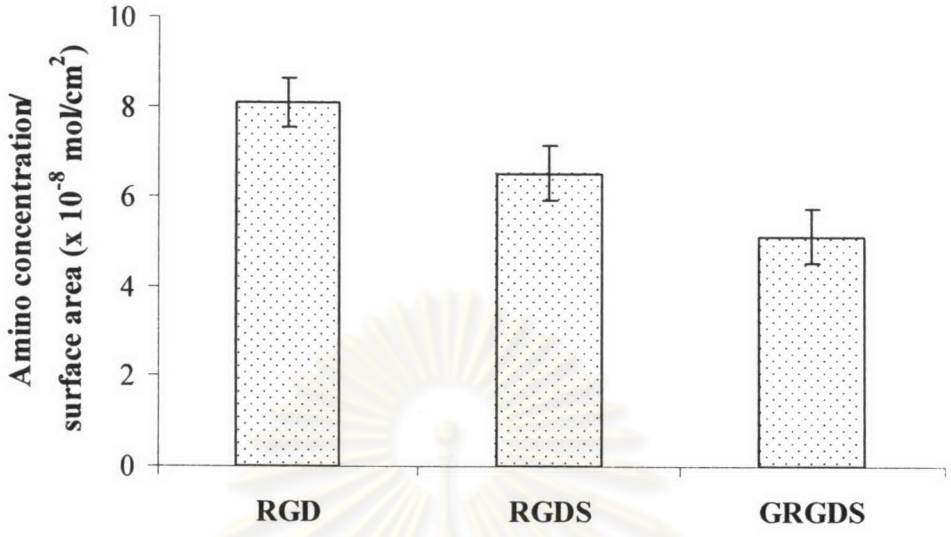
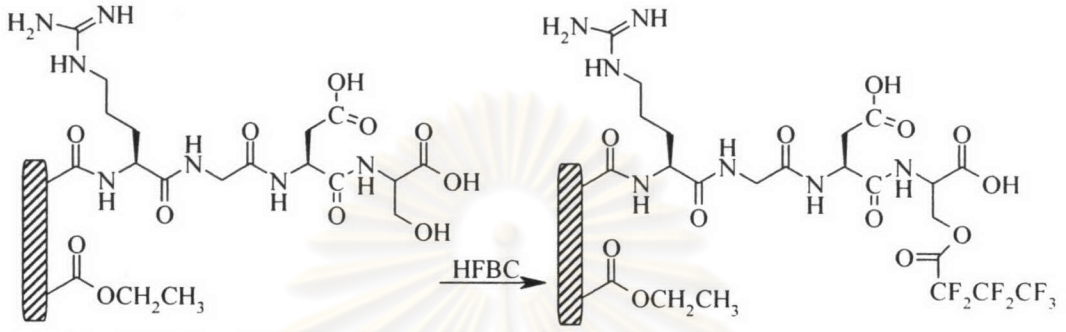


Figure 4.13 Amino concentration per surface area of immobilized RGD-containing peptides on poly(DTE-*co*-20%DT carbonate) surface using the peptide concentration of 0.05 M and immobilization time of 24h.

According to the ninhydrin analysis, the amino concentration per surface area which corresponds to the density of immobilized RGD-containing peptide is in the order of 10^{-8} mol/cm^2 in all cases. Such value is in fact much higher than 1 x 10^{-12} mol/cm^2 previously reported as a sufficient value that can promote the adhesion and spreading of fibroblast cells [26].

Since ninhydrin method is not a surface characterization technique, the calculated density of immobilized peptide may not reflect the actual density of immobilized peptide on the polymer surface. Therefore, XPS was additionally used to confirm the existence of RGD-containing peptide on the polymer surface. Taking into account that the virgin poly(DTE-*co*-20%DT carbonate) film, the activated poly(DTE-*co*-20%DT carbonate) films both before and after immobilization with RGD-containing peptide consist of the same basic elements (C, O, N), it is not possible to distinguish them by direct XPS analysis. In order to determine the success of peptide immobilization on the surface of the activated film, it is necessary to introduce a tag element that is different from those basic elements to the film after peptide immobilization before XPS analysis. Treating the film immobilized with RGD-containing peptide with heptafluorobutyryl chloride (HFBC) was chosen as a

route to introduce fluorine as a tag element. Heptafluorobutyryl chloride should be able to react with hydroxyl group which is a part of serine (S), an amino acid sequence at the end of GRGDS and RGDS.



Atomic compositions of poly(DTE-*co*-20%DT carbonate) films immobilized with RGD-containing peptides are tabulated in Table 4.2. Percentages of fluorine follow the same trend that was previously observed from ninhydrin analysis. GRGDS, the longer peptide sequence, tended to give lower density than RGDS. The theoretical percentages of fluorine was calculated based on the assumption that every single carboxyl groups on the surface was converted to *N*-succinimide esters which later completely reacted with the RGD-containing peptides. Percentages of substitution of RGDS and GRGDS on the surface calculated from XPS atomic composition were 75% and 30%, respectively. As expected, there was no signal of fluorine detected on the surface of poly(DTE-*co*-20%DT carbonate) film immobilized with RGD because there are no reactive sites on either the polymer or the peptide that can react with heptafluorobutyryl chloride. However, assuming that RGD is more reactive than RGDS, its %substitution should be more than or at least equal to 75%.

Table 4.2 Atomic composition RGD-immobilized poly(DTE-*co*-20%DT carbonate) film after labeling with heptafluorobutyryl chloride

Sample	Source	Atomic composition (%)			
		C	O	N	F
RGD-poly(DTE-*co*-20%DT carbonate) film	XPS	69.1	25.0	5.4	-
	Theoretical	71.5	21.7	6.8	-
RGDS-poly(DTE-*co*-20%DT carbonate) film	XPS	68.9	4.5	23.7	2.9
	Theoretical	68.2	6.7	21.2	3.9
GRGDS- poly(DTE-*co*-20%DT carbonate) film	XPS	67.6	26.9	4.4	1.1
	Theoretical	67.8	21.3	7.1	3.8

4.5 Cell Study

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. Fibroblast cell line (B95) was used for this investigation. The cell adhesion ratio (CAR) is shown in Figure 4.14. The value of CAR is reported as a number of cells attached to a surface in proportion to a number of cells attached to tissue culture polystyrene (TCPS) in the same culture media. Having CAR value of 92%, virgin poly(DTE-*co*-20%DT carbonate) can be considered as a good substrate for cell adhesion as compared to 100% of TCPS. Evidently, the alteration of surface functionality has a significant impact on fibroblast adhesion and proliferation. Hydrophobic *N*-succinimide groups deteriorated the ability of the substrate to adhere cells. Adding soluble RGD (RGD-s) slightly increased the cell adhesion ratio of the virgin polymer film. The CAR values of RGD- and RGDS-immobilized surfaces were lower than expected considering that at least 75% of carboxyl groups on the polymer surfaces were immobilized with RGD or RGDS. The CAR value of poly(DTE-*co*-20%DT carbonate) film was raised from 92.3% to 117.0% after GRGDS immobilization even though its surface substitution was only 30%. The extra glycine

spacer obviously introduces flexibility to the polymer surface and thus allows the RGD part to effectively mediate its specific response to the cells.

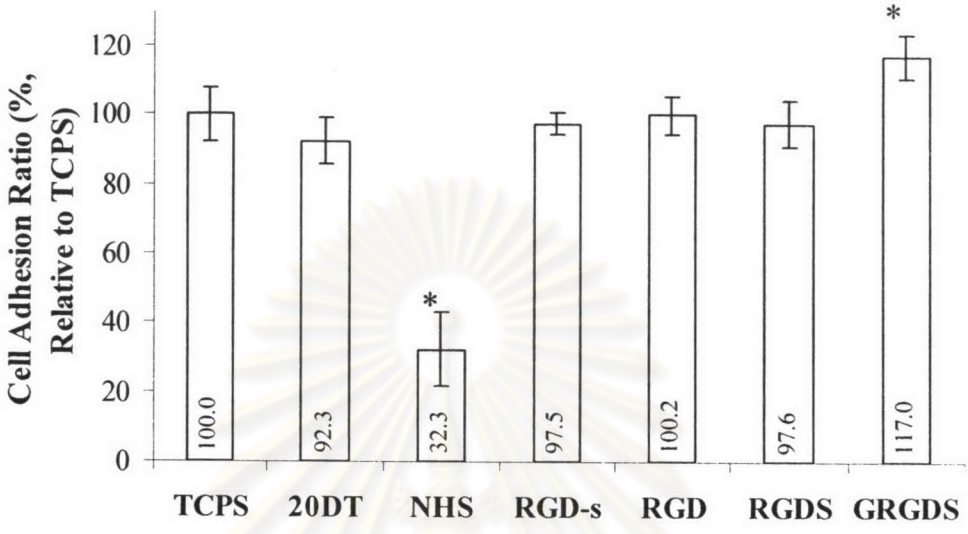


Figure 4.14 *In vitro* cell adhesion ratio (CAR) of fibroblasts on polymer substrates.
* This data are significantly different from other substrates ($p<0.01$).

According to cell proliferation ratio (CPR) shown in Figure 4.15, the RGD and RGDS-modified substrates became relatively poor substrates for cell proliferation unlike GRGDS-modified substrates. This set of data really confirmed that GRGDS can best enhance cytocompatibility of the polymer surface.

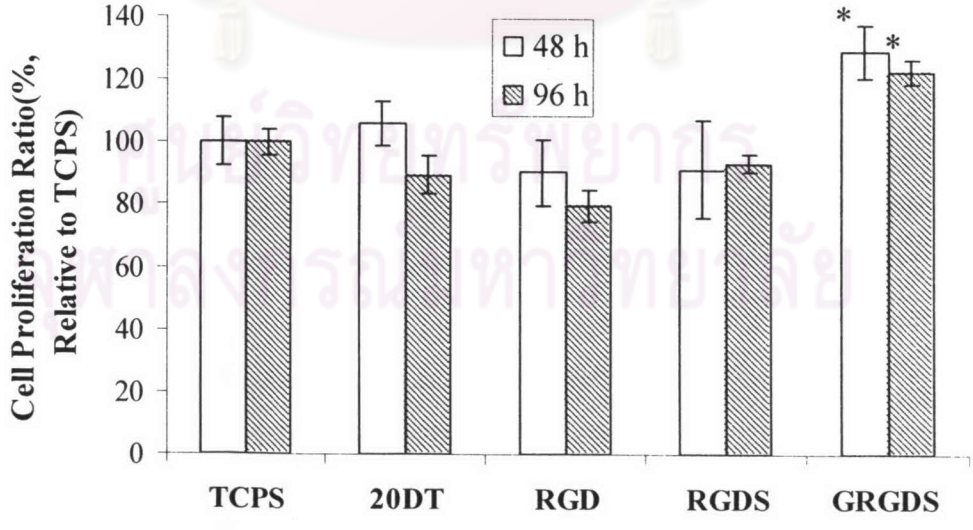


Figure 4.15 *In vitro* cell proliferation ratio (CPR) of fibroblasts on polymer substrates.
* This data are significantly different from other substrates ($p<0.01$).