



Regular article

Investigation of cellular response to covalent immobilization of peptide and hydrophobic attachment of peptide amphiphiles on substrates

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ABSTRACT

An overall investigation was made of the cell morphology, adhesion, viability, and proliferation of human fibroblast cells on surfaces functionalized with peptide and peptide amphiphiles. We compared surfaces in which the RGD peptide was immobilized covalently onto silicon with those in which lipidated versions of the RGD peptide were hydrophobically attached to the alkylated silicon surfaces. The hydrophobically attached peptide amphiphile on alkylated silicon surfaces produce structures that are somewhat akin to the structure of a cell membrane. Scanning electron microscopy (SEM) and Laser scanning confocal microscopy (LSCM) were used to characterize the seeding human fibroblast cells on all prepared surfaces. Surfaces were also evaluated with a methyl tetrazole sulfate (MTS) assay to compare the proliferation ability. Cell-substrate interactions were examined through cell adhesion assay. Peptide-amphiphile modified surfaces exhibited substantially superior cellular responses compared to those on the covalently immobilized peptide. It was also shown that the length of alkyl tail in lipidated peptides may influence cellular response. Hydrophobically attached peptide amphiphiles on alkylated silicon surfaces may suggest new biomimetic platforms for further studies of the interaction between cells and extracellular matrix.

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1. Introduction

There is a significant interest in the development of biomimetic materials that can mimic extracellular matrix (ECM). Cell to cell and cell to ECM interactions are important for the formation of in vivo tissues and organs [1–7]. Therefore, the production of a stable, functional, and bioactive film is of interest for applications ranging from biosensors, biochip development, and tissue engineering [8–10]. In early research, long chain of ECM components such as fibronectin and vitronectin was used for surface modifications of inorganic substrates for this purpose [11,12]. However, it is now known that interaction between ECM and cell membrane receptors occurs via only short segments of ECM [13,14]. One of these sequences is the RGD sequence and is now known to promote cell adhesion on inorganic or polymeric surfaces [11,15–20]. Cell adhesion is mediated by integrins through a cascade of four different events including cell attachment, spreading, organization of actin cytoskeleton, and for-

mation of focal adhesions [16,21]. During the adhesion step of cells, integrins undergo several conformational alterations throughout ligand binding and transmembrane signaling [22,23].

Different methods including bioconjugation, chemoselective ligation, and click chemistry have been used to covalently immobilize an RGD peptide on different substrates [24–33]. The techniques used for peptide immobilization must ensure that the attachment site does not interfere with the accessibility of the active site and that active sites remain active during attachment with a high surface density of the peptide. Molecular self-assembly is a powerful approach to design such a model that satisfies some of these requirements. The best known of such self-assembled structures is a lipidated peptide which is achieved by attaching a hydrophobic moiety to a peptide to create an amphiphilic molecule [8,34,35]. The hydrophobic tail serves to orient the molecules while the peptide head group imparts the specific biological activity [6,36]. Previous studies have shown that the lipidated peptide amphiphiles (PAs) have potential applications in tissue engineering and drug delivery [37–39]. Stroumpoulis et al. [40] reported an effective method to screen biological probes for cell adhesion and growth through the incorporation of PAs gradients in a membrane environment. Unique biomimetic materials have been

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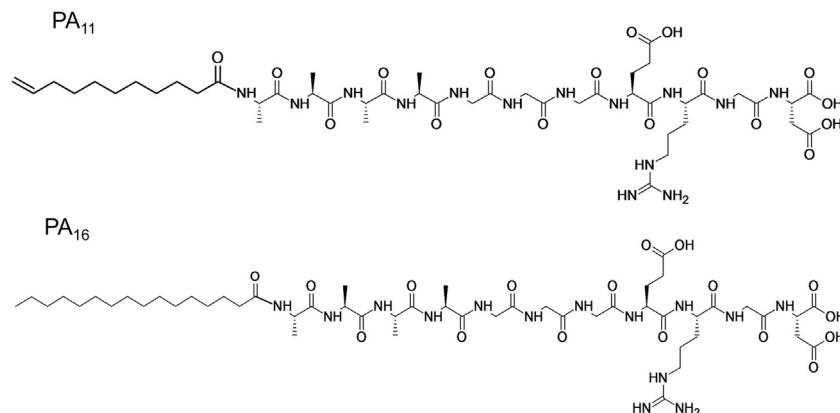


Fig. 1. The chemical structure of undecanoyl and palmitoyl peptide.

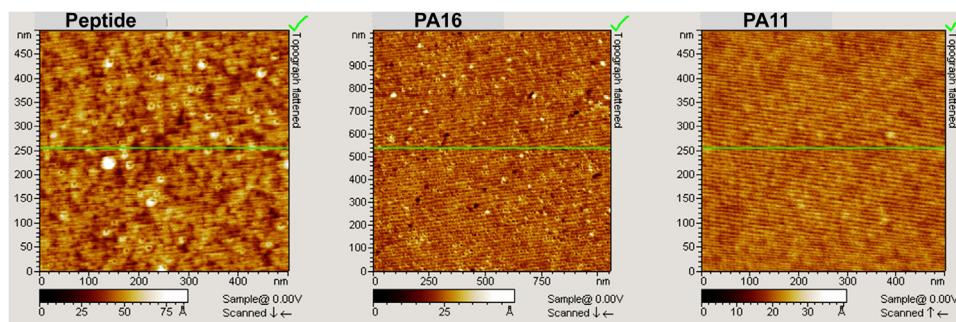


Fig. 2. AFM images of peptide, PA₁₆ and PA₁₁ on alkylated silicon surfaces.

developed through the incorporation of lipidated peptides into self-assembled structures such as films [8]. In a previous research [41] we synthesized and characterized two derivatives of PAs including undecanoyl lipidated peptide (PA11) and palmitoyl lipidated peptide (PA16) (Fig. 1). TEM observations revealed [41] the presence of a network of nanofibers for PA16 and PA11. We developed bilayer surfaces with restricted fluidity via covalent attachment of tetradecane monolayers on hydrolyzed surfaces in a self-assembly method. Hydrophobic attachment of PA on these surfaces then produced bilayer structures composed of a hydrophobic interior and a hydrophilic exterior.

In this paper, we compare cellular responses of the hydrophobically attached PA on the alkylated surfaces with the covalently immobilized peptide on silicon surfaces. It should be noted that the same sequence of amino acids (A-A-A-A-G-G-G-E-R-G-D) were used in both the peptide and the PAs.

2. Experimental methods

2.1. Materials

Human dermal fibroblasts (HDF; line GM3348) were obtained from Sigma-Aldrich. All chemicals were purchased from Sigma-Aldrich (Sydney, NSW, Australia) and used as received without further purification. Milli-Q water ($18\text{ M}\Omega\text{ cm}$) was used for the rinsing and preparation of solutions.

2.2. Synthesis of peptide and peptide amphiphiles

Peptide synthesis was performed on a PS3 automated peptide synthesizer which is described in the previous paper [25]. To produce the lipidated form of the peptide, it was acylated with a mixture of either 0.5 mmol palmitic acid or unde-

canoic acid, 0.5 mmol N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-N-methyl-methanaminium hexafluorophosphates N-oxide, 1 mmol diisopropyl methylamine and 5 ml Dimethylformamide which has been described in detail in a previous paper [41]. Mass spectra for peptide, PA11 and PA16 were 931.53 m/z [M + H]⁺, 1086 m/z [M + H]⁺ and 1012 m/z [M + H]⁺, respectively. The crude peptide and peptide amphiphiles were analyzed by HPLC and it was found that all of them had a purity of >95%.

2.3. Immobilization of RGD peptide on the surface of silicon

The chemical attachment of the peptides onto the silicon surface was performed as follows. An N hydroxyl succinimide (NHS) ester terminated organic monolayer film was first produced using a self-assembly method [42]. 100 mg of the peptide was attached to this NHS terminated self-assembled monolayer by incubating it in 10 ml phosphate buffered saline (PBS) at pH 7 for 3 h with 2 min sonication every 15 min. The samples were then rinsed consecutively with copious volumes of chloroform, ethyl acetate, ethanol, and water. Details of this procedure are given in reference [25].

2.4. Hydrophobic attachment of peptide amphiphiles on alkylated silicon surfaces

The process begins by removal of the oxide layer from the silicon surface using 40% ammonium fluoride which yields a hydroxylated surface. The wafer is then immersed in tetradecane and exposed to the ultraviolet light. The terminal double bond then links to the hydroxylated surface to yield tetradecane covalently bonded to the silicon. Detail of this process has been given elsewhere [42]. Formation of the hydrophobically attached films of the lipidated peptide amphiphiles onto alkylated silicon surface involved incubation of the alkane functionalized silicon surface in 100 mg lipidated pep-

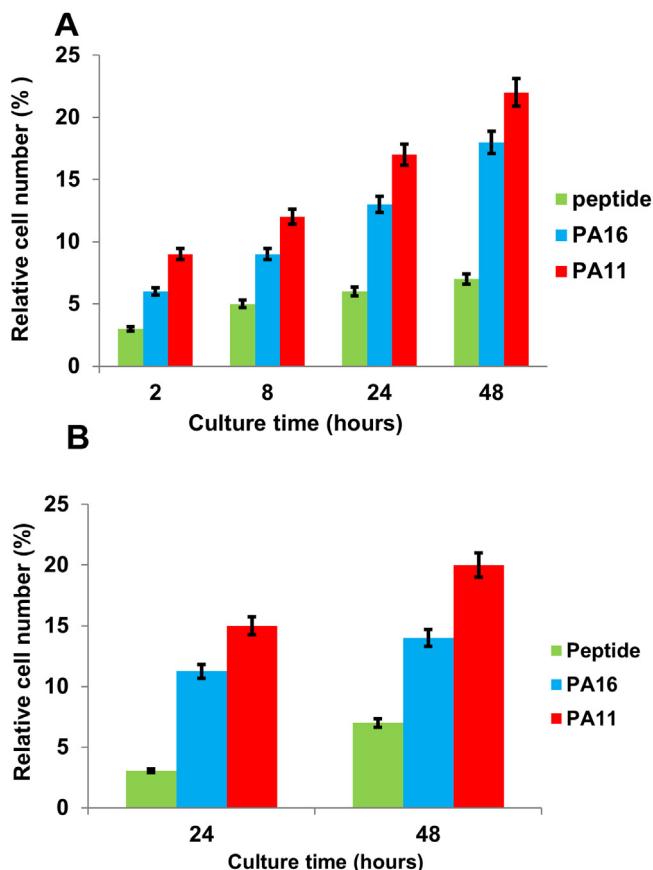


Fig. 3. Cell adhesion on covalently immobilized peptide, PA11 and PA16 at the interval time of (2, 8, 24, and 48 h). Number of viable seeded cells on covalently immobilized peptide, PA11, and PA16 after 24 and 48 h. (The results are normalized to bare surface and each value and error bar represents the mean of triplicate samples and its standard deviation).

tide dissolved in 10 ml of PBS. More detail of this has been described previously [41].

2.5. X-ray photoelectron spectroscopy (XPS) measurements

The XPS measurements were made using an ESCALAB 220iXL spectrometer with a monochromatic Al KR source (1486.6 eV), hemispherical analyzer, and a multichannel detector. All spectra were accumulated at a take-off angle of 90° with a 0.79 mm² spot size and were recorded at a pressure of less than 10⁻⁶ Pa.

2.6. Cell culture

Human skin fibroblast cell lines (GM3348) were grown in Dulbecco's modified eagle medium supplemented with 10% (by volume) fetal bovine serum (FBS), 50 IU/ml streptomycin in an incubator with 5% CO₂ (by volume). The fibroblasts were detached from the culture flasks by incubating in 2 ml of 0.25% trypsin for 5 min. Aliquots of the cells were added to each well of 6-well plates containing silicon wafers with a SiO₂ surface, functionalized silicon surfaces with the covalently immobilized peptide, the hydrophobically attached PA11 and PA16 to the alkylated surfaces. Prior to use, the surfaces were rinsed with 70% ethanol, and dried under sterile conditions.

2.7. Cell adhesion assay

Aliquots containing approximately 4 × 10⁴ fibroblast cells were added into each well of 6-well plates on all samples for various adhesion time intervals (i.e., 2, 8, 24, and 48 h). After an interval of cell culture, the substrates were washed two times with PBS. The adhered cells were harvested with trypsin-Ethylenediamine tetraacetic acid (EDTA), and the cell number was counted using a hemocytometer. The cell adhesion experiments were run three separate times.

2.8. Ellipsometry

A Woollam M2000V spectroscopic ellipsometer with Tungsten-Halogen light source was used to carry out ellipsometry measurements. The ellipsometric spectra were obtained at 65, 70 and 75° incident angle and 400 nm wavelength. A model with a Bruggeman effective medium approximation layer which contains a Cauchy layer with 17% voids on top of a silicon substrate was applied to fit the data. Thickness and optical constants were acquired from the best fit to the model. Experimental uncertainty in the thickness was estimated at approximately ±2 × 10⁻¹⁰.

2.9. Atomic force microscopy

Surface roughness was measured using a Pico SPM atomic force microscope (Keysight technologies, 5500 SPM/AFM, USA) which enables accurate imaging in nanoscale. Silicon nitride probes mounted on cantilevers and were performed in tapping mode. The data is represented in gray scale, with light pixels correspond to the highest points on the topography and dark pixels characterize the lowest points. AFM images were acquired by scanning the sample in air under ambient laboratory conditions.

2.10. Scanning electron microscopy (SEM)

A Philips XL30 scanning electron microscope was used to obtain the SEM images of samples. After 48 h culturing, the silicon samples with the attached fibroblast cells were fixed with 2.5% (v/v) glutaraldehyde for 2 h at 37 °C. Samples were dehydrated for 10 min each in 70%, 80%, and 90% ethanol solution and then in 100% ethanol. After critical point drying, the samples were coated with gold for the SEM imaging.

2.11. Laser scanning confocal microscopy

A Nikon C1 confocal microscope was used to determine the viability of cells. A pulsed diode laser was used for performing fluorescence lifetime microscopy in Limbo mode. The system was equipped with an inverted Nikon Eclipse TE200 fluorescence microscope which had a number of filters available for visualizing samples. Propidium iodide and fluorescein diacetate in PBS were used to assess cell viability after 48 h. Cells fluorescing red were counted as dead, since red fluorescent can penetrate and intercalate into the nucleic acids only in cells with damaged cytoplasmic membranes. Cells fluorescing green were counted as viable since fluorescein diacetate enters normal cells and emits a green fluorescence when it is cleaved by esterases [43].

2.12. MTS assay

MTS assay was used as a colorimetric method to measure metabolic activity of live cells and the absorbance at 490 which is linearly correlated with the number of viable cells attached to the surfaces. Approximately 4 × 10⁴ cells were seeded on the

various substrate chips ($10\text{ mm} \times 10\text{ mm}$) in each well of a 24-well culture plate. The cells were cultured with growth medium containing 10% FBS at 37°C in an incubator with 5% CO_2 . At the indicated time (24 and 48 h), the chips were washed with PBS three times and were immersed in culture medium with 20% MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Promega, Madison WI). The reaction was completed for 1 h, and the absorbance at 490 nm with an ELISA reader was recorded.

3. Results

3.1. Surface characterization

FTIR spectra collected from samples in the previous papers confirmed the presence of peptide and peptide amphiphiles on substrates (25, 41). Ellipsometry measurements yielded a thickness of $6 \pm 1 \times 10^{-9}$, $7.5 \pm 1 \times 10^{-9}$, and $8.9 \pm 1 \times 10^{-9}$ for peptide, PA11 and PA16 respectively. AFM analysis (Fig. 2) revealed a uniform coating for PA11 modified surfaces, with a “peak-to-valley” distance of $\sim 2\text{ nm}$. Analysis of PA16 modified surfaces revealed the presence of $\sim 2.5\text{ nm}$ high energy dispersed groups and larger clumps with a “peak-to-valley” distance of $\sim 5\text{ nm}$ were acquired for peptide modified surfaces. X-ray photoelectron spectroscopy was used to measure the concentration of the peptide on the surfaces. The density of the peptide on the substrate is one of the determining factors mediating cell adhesion. The N:C ratio is a useful indicator of the concentration of the peptide on a substrate [44]. There are large differences in the peptide surface density between the hydrophobically attached PAs and the covalently immobilized peptide. While the N:C ratio of the covalently immobilized RGD peptide was 0.24, this ratio significantly increased to 0.75 and 0.58 in PA11 and PA16 modified surfaces respectively [41]. Davis et al. [28] reported a ratio of 0.20 for the covalent bonding of RGDC peptides to aminosilane silicon (111) surfaces via a maleimide hetero bifunctional crosslinker. Immobilization of RGD peptides on polymer nanofibers [45] yielded an N:C ratio of 0.36. A value of 0.39 was reported for immobilization of the RADA16-I peptide on the functionalized silicon surfaces [46]. Previous research showed a close relationship between the peptide density on a substrate and cell adhesion, spreading, cell survival, and proliferation [40,47–49].

3.2. Cellular behavior of human fibroblast cells on different substrates

Human fibroblast cells were seeded onto 1) bare silicon wafer, 2) the covalently immobilized peptide, 3) PA11 and PA16 modified surfaces under serum-free conditions. The addition of serum may decrease the level of short-term cell adhesion to the RGD peptide due to the adsorption of factors which mask the adhesive peptide. The number of adherent fibroblast cells was evaluated and normalized to bare surface after 2, 8, 24, and 48 h on the aforementioned substrates. Cell adhesion increased in all samples as a function of time, up to 48 h. The results shown in Fig. 3a indicate that after 2 h cell seeding, cell attachment to the PAs modified surfaces was 2-fold greater than the covalently immobilized peptide. Within these substrates, the highest level of cell adhesion was obtained on PA11 modified surfaces. The MTS assay was performed to measure the number of live cells attached to the aforementioned surfaces after 24 and 48 h. The results derived from MTS measurements are normalized to bare surface and shown in Fig. 3b. It can be seen that the rate of proliferation of fibroblast cells on peptide amphiphiles modified surfaces was more than twice of those on the covalently immobilized peptide surfaces. SEM was used for qualitative assessment of cell spreading and morphology of the fibroblast cells on

all substrates after 48 h. Results show that the cells on the silicon substrates failed to attach and retained a rounded shape (Fig. 4a). While the cells grown on the covalently immobilized peptide were in various advanced stages of spreading and proliferation and displayed more typical dendrite morphology (Fig. 5a). The morphology of human fibroblast cells on the surfaces modified with PA was extensively spread and had both enlarged nuclei and long dendritic extensions (Figs. 6 and 7a). The morphology of these cells appeared to be similar to the fibroblast cells in the native tissues [50]. SEM images show that spreading of cells on PA11 modified surfaces (Fig. 7a) was slightly higher than those on PA16 surfaces. Confocal microscopy images further confirmed the superior cell attachment and spreading on the alkylated surfaces bearing the PAs. As can be seen in (Fig. 6b &7b), after 48 h the cells on the surfaces modified with PAs were homogenously distributed across the substrates and had a uniform appearance. Fibroblast cells also aligned in parallel clusters on these surfaces. Although the PAs modified surfaces supported a large number of viable cells, a few dead cells could be observed on PA16 modified surfaces (Fig. 6b). In contrast, relatively fewer live cells were attached to the covalently attached RGD peptide and some of them were generally round in appearance (Fig. 5b).

4. Discussion

The research was motivated to establish the most effective surfaces for promoting cellular responses. To this end, surfaces including covalent immobilization of the peptide and hydrophobic attachment of the PA on the alkylated surfaces were prepared. Then adhesion, morphology, viability, and viable cell numbers on all surfaces were investigated. Our data show that the alkylated silicon surfaces bearing the PAs support extremely superior cellular response in term of cell adhesion, spreading and viability compared to those on the covalently immobilized peptide. The overall strength of cell adhesion is managed via three factors: (i) the intrinsic affinity of the individual receptor-ligand bonds, (ii) the density of available ligands on the substrate, and (iii) the ability of both the receptor and the ligand to move [51]. The superior adhesion and spreading abilities of the PA modified surfaces might be explained by the high peptide surface density as well as high flexibility and accessibility of the RGD peptide. The PAs modified surfaces are arranged in a way that the hydrophilic RGD sequence is oriented outside the surface and hydrophobic alkyl tails participate in hydrophobic interaction with the tetradecane monolayers on the silicon surfaces. In fact, these structural features appear to effectively increase the presentation of the uniformly oriented RGD ligands to cells. Limited cell attachment onto surfaces modified with the covalently immobilized peptide may be attributed to either the low peptide surface density or the limited number of effective binding domains. It was found that the surface peptide density is the major factor that controls cell attachment and spreading [52]. A research on PAs containing residues 1263–1277 (peptide IV-H1, Gly-Val-Lys-Gly-Asp-Lys-Gly-Asn-Pro-Gly- Trp-Pro-Gly-Ala-Pro) from human type IV collagen also reported that such amphiphilic peptides promoted melanoma cell adhesion and spreading in a dose-dependent manner [4,53]. Previous studies [54,55] have shown that random orientation of the molecules to the surface in the covalently immobilized peptide may bury the active sites of the peptide which result in a decrease in the number of effective binding domains. Limited binding domains also hinder the spreading of adhered cells on the substrate. In most immobilization techniques, integrins undergo a remarkable conformational change that this significantly reduces receptor-ligand affinity [51]. Therefore, only small fraction of the adsorbed peptide is conformationally available to the cell adhesion receptors.

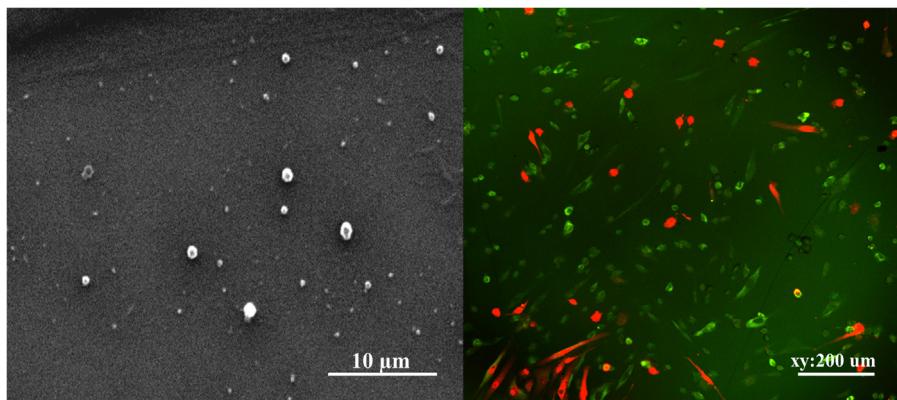


Fig. 4. SEM and fluorescent confocal microscopy images of fibroblast cells on bare silicon surfaces after 48 h.

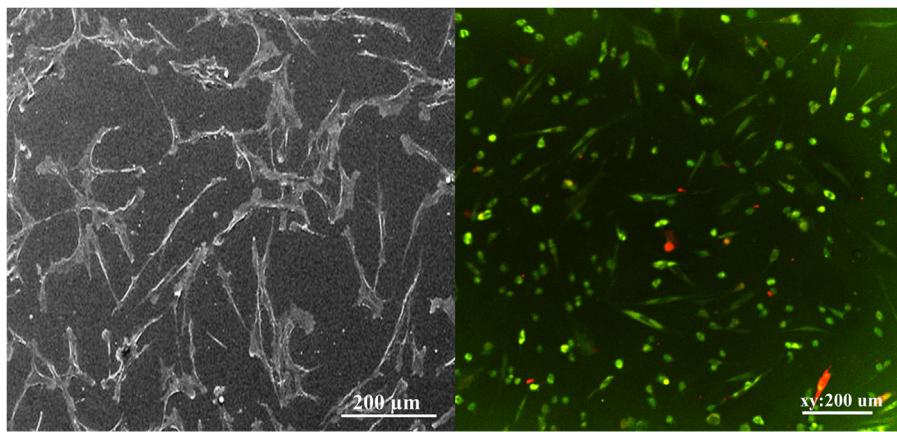


Fig. 5. SEM and fluorescent confocal microscopy images of fibroblast cells on the covalently immobilized peptide after 48 h.

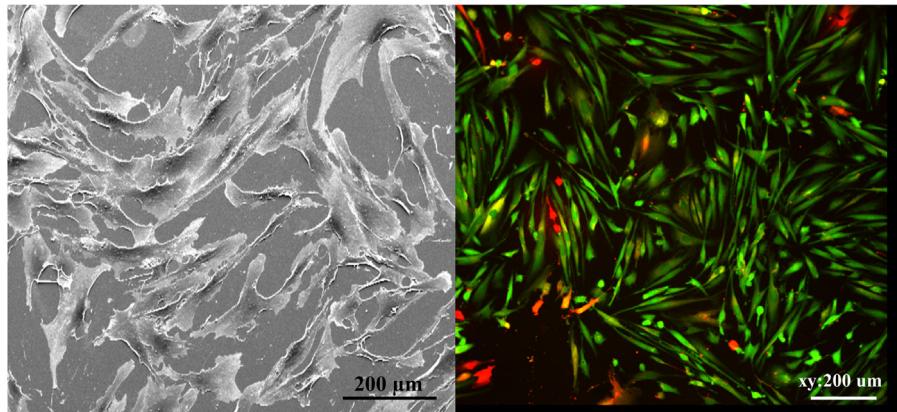


Fig. 6. SEM and fluorescent confocal microscopy images of fibroblast cells on the hydrophobically attached PA16 modified surfaces after 48 h.

The dramatic difference in the number of initially adherent cells on the PA modified surfaces and the covalently attached peptide may suggest that the integrin-binding events that enable cell adhesion may occur more quickly on the PAs modified surfaces. The extent of initial cell adhesion regulates further cell behavior such as migration, proliferation, and differentiation [56]. It is well known that the shape of a cell is correlated with the number, availability, and distribution of the binding sites [54]. The fully spread cell morphology on the PAs modified surfaces may suggest that most of the available binding domains have participated in the cell binding providing essential traction force and mechanical support for

cell spreading which is in good agreement with previous results [40,54,57].

An additional potential factor that may contribute to the superior cellular response on the PA modified surfaces is that the cells may respond to the higher-order assembly structures of RGD peptide formed by the PAs on alkylated silicon surfaces which are in agreement with a previous report [58]. It was previously reported that incorporation of lipidated PAs into self-assembled structures such as films promote the formation of higher order structures that effectively enhance cell adhesion, spreading and growth [8,39].

Results from Atomic force microscopic images revealed that surface roughness of all surfaces (peptide and peptide amphiphiles) are

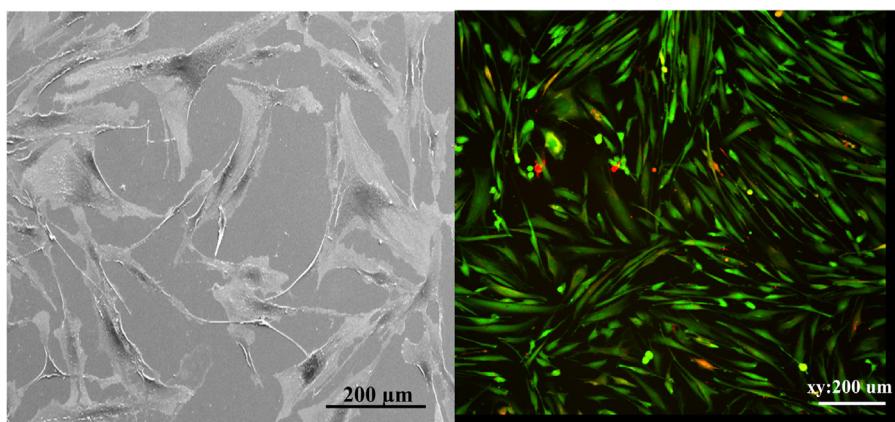


Fig. 7. SEM and fluorescent confocal microscopy images of fibroblast cells on hydrophobically attached PA11 modified surfaces after 48 h.

in the nanoscale range. The observed roughness may be attributed to the organic materials either from modification process or during the cleaning process. It is well known that surface roughness has an effect on cellular responses. However, because of the slight difference in AFM results, it cannot be stated with certainty that the superior cell response to the PAs are attributed to the more flattened surfaces of the PAs compared to those of the peptide.

Our findings also indicate that the PAs with the shorter alkyl chain (PA11) possess slightly better cellular response compared to those with the longer alkyl chain (PA16). The differences observed in the cellular response between PA11 and PA16 modified surfaces may be attributed to the more uniform binding domains on PA11 modified surfaces. In a previous study [41], we have shown that self-assembly of PA11 on alkylated silicon surfaces produces more homogeneous and well-organized layers compared to those on PA16 modified substrates. The other possible explanation for the higher cellular response on PA11 modified surfaces may be ascribed to the length of undecanoyl moiety which may improve the accessibility of the RGD ligands to the specific receptors. Previous research has shown that the length of the spacer group influences the peptide ability to specifically engage integrins [8,55,59]. Although a certain amount of spacing between the RGD sequence and the anchoring site is vital for the cell attachment activity, the use of a longer spacer group may reduce cell attachment via lower binding affinity [55]. These findings are not comprehensive and further experiments are required to investigate the effect of the alkyl length in PAs on cell response.

5. Conclusion

It was established that the well-organized PAs on the alkylated silicon surfaces support cell adhesion, spreading and proliferation more effectively than those on the covalently immobilized peptide. Cell membrane-like structures of the PAs on silicon surfaces seem to significantly enhance availability and accessibility of the RGD ligands at high density to the integrin receptors. Significant cell response was obtained on surfaces modified with undecanoyl lipidated peptide (PA11). While the PAs modified surfaces appear to be biocompatible surfaces, further investigations are needed to ensure that they are suitable for new biomimetic surfaces.

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