Collagen Immobilization onto Acrylic Acid Laser-grafted Silicone for Using as Artificial Skin: In Vitro

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ABSTRACT

The skin has an important role in human health and its defect can produce many problems in normal physiology. Because of its anatomy and physiology, most likely the skin is exposed to pathologic agents more than other organs. In this study, grafting of poly(acrylic acid) onto silicone rubber was carried out using CO₂-pulsed laser as an excitation source in order to create functional groups to immobilize collagen. Collagen was immobilized onto poly(acrylic acid) grafted silicone through covalent bonding. Modified and unmodified surfaces were characterized by water contact angle; scanning electron microscopy (SEM) and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). Fibroblast cells (L929) were cultured onto collagen immobilized poly(acrylic acid)-grafted silicone and poly(acrylic acid)-grafted silicone films. It was observed that collagen immobilized surfaces showed significant cell adhesion and growth in comparison with poly(acrylic acid)-grafted silicone samples. It seems that collagen immobilized surfaces may have an excellent potential to be used as a derm-like matrix.

INTRODUCTION

The skin loss is one of the oldest and still not totally resolved complications in surgical field. Due to the non-spontaneous healing of the dermal defects, the scar formation for the full thickness skin loss would be inevitable unless some skin substitutes are used. In the past decades, many skin substitutes such as engrafts, allograft and autografts have been employed for wound healing. However, because of the antigenicity or the limitation of donor sites, the skin substitutes mentioned above cannot accomplish the purpose of the skin recovery and yet they are not widely used [1-5]. Skin tissue engineering
provides a prospective source of advanced therapy for treatment of skin defects because it has comparable results with traditional methods while can avoid those limitations [6]. Therefore, many studies are turning towards the tissue engineering approach, which utilizes both engineering and life science disciplines to promote organ or tissue regeneration and sustain and recover their functions [7].

Polymers have generated considerable interest as biomaterials in the field of tissue engineering, such as tissue replacement, the polymeric materials provide support surfaces for the immobilization of biologically active molecules and living cells. Scaffolds have also been made from extracellular protein matrix components such as collagen. These materials show excellent cell adhesion, biodegradability and biocompatibility but suffer from the disadvantage that they cannot be freely or reproducibly processed into stable objects with three-dimensional shapes of good mechanical strength. Scaffolds made out of polymers, on the other hand, have excellent strength and ductility and can readily be made into various shapes, but their surfaces do not favourably interact with cells. It is the physicochemical and morphological nature of polymers that governs the cell interaction at the interface [8].

It is known that the surface properties of a biomaterial dominate the interactions between the material and the biological environment. In the field of tissue engineering, polymeric materials provide surfaces for the immobilization of biologically active molecules and living cells [9].

Since most conventional polymers except for polysaccharides have no such functional groups on the surfaces, they should be modified so as to have reactive groups for covalent immobilization of biomacromolecules [10]. Synthetic polymers therefore often require selective modification to introduce specific functional groups to the surface for the binding of biomolecules [7]. In order to covalently immobilize biomacromolecules onto polymer surfaces, they are required to have functional groups such as amine, imine, carboxyl, hydroxyl, isocyanate, expoxy, etc [10]. Graft polymerization is an attractive way in which a desired monomer can be grafted onto the surface with covalent bond, giving it good stability [11]. Surface graft polymerization is one of the most versatile means for the molecular design of polymer surface to enhance their physical and chemical properties for specific applications [12].

Surface modification is a widely adopted method because it can enhance the biocompatibility of a material surface while keeping the bulk properties intact [13]. Radiation-induced graft copolymerization of monomers onto the surface of polymer materials by the use of energy sources such as UV, gamma ray and electron beam has provided convenient methods to impart desired surface properties. However, these techniques still have some shortcomings, such as long gamma irradiation time, possible damage to the substrate, and the bulk mechanical properties of the substrate may change as well.

Laser surface modification of polymeric materials is a unique and powerful method without altering their bulk properties. CO2-pulsed lasers have large beam pattern areas, large pulse energy, use non-toxic gases and are cheap and easy to operate. This technique offers possibilities to improve the performance of biomaterials. The resulting surface is also particle free and sterile, due to the low amount of chemicals formed [14].

PMDS has been used in a wide range of biomedical applications during the past four decades [14,15]. Because of its special properties of silicone elastomer, such as biocompatibility, it is generally applied as soft tissue replacement. A silastic layer with proper thickness and pores provides the barrier needed while maintaining the proper water flux. In addition, the silastic layer also provides the mechanical rigidity needed to suture the graft in place, preventing movement of the material during healing process [16].

Although silicone has been used mostly as soft tissue substitutes because of its excellent softness, stability, and bioinertness, serious problems have occurred when the silicone devices were implanted for a long time. They include damages to the tissues in direct contact through mechanical friction and dense fibrous tissue formation around the silicone surface without bulk deterioration. Therefore, a series of works was initiated in an attempt to make the silicone surface more biocompatible by grafting water-soluble, synthetic polymers and biological macromolecules [17]. Poly(acrylic acid) is a kind of hydrogel that exhibits the ability to swell in water and retains a
significant fraction of water within its structure without being dissolved. Hydrogels have physical properties similar to those of human tissues and possess excellent tissue compatibility. They may interact less strongly with the immobilized species than more hydrophobic materials. Thus, molecules and cells immobilized on (or within) hydrogel may be more likely to retain their biological activity for longer periods of time. The main disadvantage of hydrogels is their poor mechanical properties after swelling. To eliminate this disadvantage, a hydrogel was grafted onto some base polymers with good mechanical properties [18].

For grafting, carboxyl group is one of the best functional groups. Chemical bonds are produced between amino-groups from proteins and carboxyl functional groups. Acrylic acid (AAc) is commonly used in such grafting process. AAc acts as a spacer to bind proteins and the substrate. The carbon-carbon double bond in the structure of AAc binds easily with polymers. In addition, the base materials can retain their own chemical and physical properties after graft polymerization. All the given advantages make AAc a popular chemical agent in graft polymerization [8,19,20]. Many natural materials, such as collagen, are used as biomaterials in tissue replacement and wound healing. These materials show excellent biocompatibility, biodegradability and cell adhesion [21-22]. Collagen, usually exists in skin, vessel, bone, tendon, and basement membrane, and it is used as a biocompatible material. It is fabricated by three \(\alpha\)-chains, which are formed by a [glycine (Gly)-amino acid (X)-amino acid (Y)]\(_n\) series of polypeptide [23]. Collagen binds to the wound bed and is invaded by neovascular-ture and fibroblasts and with time the collagen is resorbed by collagenase and is completely replaced by remodelled dermis [16].

The cell culture on a defined surface has rarely addressed the challenges of differentiated cells. Fibroblasts, for example, which are commonly used in such studies, maintain considerable pleiotropy in culture and are relatively easy to culture [24]. Fibroblast has been specifically chosen for specific animal bioassays because it is one of the early populates in a healing wound and the most common cell in derm and other connective tissues, and produces collagen and other connective tissue proteins [16,25].

In this work, we performed immobilization of collagen onto poly(AAc) grafted silicone to study cell behaviour on different surfaces via in vitro test. A pulsed tunable \(\text{CO}_2\) laser to introduce peroxide groups onto the surface of polydimethylsiloxane, which normally possess low free surface energy and then the grafting of poly(AAc) onto silicone was carried out. Immobilization of collagen was achieved through covalent bonds between the amino groups in the collagen molecules and the carboxyl groups in poly(AAc) chains grafted onto the silicone films as shown in Scheme I. L929 mouse fibroblast cells were cultured onto grafted and unmodified different samples surfaces.

**EXPERIMENTAL**

**Materials**
The substrate polymer used for peroxidation process was polydimethylsiloxane (PDMS), M3090 medical
grade which was purchased from Wacker. Acrylic acid (AAc>99%) and water soluble carboxidiimide (WSC) from Merck were used in this study for activating COOH groups on the AAc grafted silicone. Collagen type I was obtained from Sigma Aldrich.

Methods

**PDMS Vulcanization**

PDMS was milled with 0.5 phr dicumylperoxide (90%) as curing agent at 80°C. The rubber was formed into 2 mm thick sheets and compression cured at 165°C for 5 min in hot press. Vulcanized films of 0.3 mm thickness were Soxhlet extracted with toluene: methanol (60:40 v/v) for 48 h and then dried in a vacuum oven at ambient temperature to constant weight.

**Irradiation Procedure**

Laser irradiation was carried out by a line-tunable pulsed CO₂ laser [Lumonics TEA-840/1]. The laser provides beams of wavelengths 9.1-10.6 μm (1098-943 cm⁻¹). The samples were exposed to laser pulses under the selected conditions of the wavelengths 9.58 μm, repetition frequency 1 Hz and various fluency and number of pulses. After exposure, the samples were thoroughly washed with distilled water many times, and after drying in a vacuum oven at ambient temperature, weight decreases were determined.

**Graft Polymerization**

After laser treatment, peroxides on the surface were formed [11]. Then a reactor was used. The treated PDMS films were placed in the reactor and then 30% (by wt) of AAc aqueous solution was poured into the reactor and degassing was carried out by purging the solution with N₂ for 20 min. Afterwards, without further exposure to air, the reactor valves were closed. Graft polymerization of acrylic acid onto treated silicone was performed to proceed at different temperatures (40, 50, 60, 65, 70, 80, and 90°C) for 4 h. After removal of homopolymer and monomer by many repeated washings with distilled water, the samples were dried under standard condition and then weight increases were determined.

**Wettability**

Hydrophilicity was evaluated by measuring the contact angle formed between water drops and the surface of the modified samples using contact angle measuring system. For this purpose, the drops of water were mounted on 5 different areas of the surface with a microsyringe. Results are mean values of five measurements on different parts of the film.

**Graft Density and Surface Water Uptake**

The samples are weighed before and after graft polymerization to obtain graft density as follows:

$$\text{Graft density} = \frac{W_g - W_0}{A}$$

where $W_g$ is weight after graft polymerization, $W_0$ is weight before graft polymerization, and $A$ is surface area. For determination of surface water uptake, the samples were dried in a vacuum oven at ambient temperature and weighed. Then they were immersed in distilled water for 30 s and weight increase was determined, to obtain the surface water uptake as follows:

$$\text{Surface water uptake} = \frac{W_a - W_b}{A}$$

where $W_a$ is weight after immersing in water, $W_b$ is weight before immersing in water, and $A$ is surface area. The data obtained are mean values of five readings.

**ATR-FTIR Measurement**

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) with Zn-Ce crystal and horizontal ATR was used.

A Brucker (Equinox 55) FTIR spectrometer employed used spectra generated from 32 scans.

**Scanning Electron Microscopy**

Scanning electron microscopy (SEM) was performed on gold-coated samples using a polaron sputter coater. A Cambridge S-360 SEM operating typically at 10 kV was employed for morphology measurements.

**Immobilization of Collagen**

Poly(acrylic acid)-grafted silicone films were placed in 10 mg.mL⁻¹ of an aqueous solution of N-(3-dimethyl aminopropyl) N’-ethyl carboodiimide...
hydrochloride (WSC) at 4°C for 30 min to activate carboxyl groups in the grafted poly(AAc) chains. The activated poly(AAc)-grafted silicone films were immersed into collagen solution at 4°C for 24 h to allow the activated carbonyl groups in the grafted poly(AAc) chains and amino groups in the protein to form covalent bonds. The concentration and pH of collagen solution were 1 mg.mL⁻¹ and 3.0, respectively. Collagen-immobilized silicone films were rinsed with distilled water many times and then the samples were stored at 4°C before use.

Cell Culture Test
The collagen-immobilized silicone films as well as poly(AAc)-grafted silicone films were immersed into sterilized saline for 12 h just before cell culture. The mouse fibroblast cell (L929) was obtained from National Cell Bank, Pasteur Institute of Iran. The cells were maintained in RPMI-1640 growth medium supplemented with 10 fetal bovine serums (F.B.S), 100 IU mL⁻¹ streptomycin. A routine subculture was used to maintain the cell line. The cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. After one week incubation the monolayer was then harvested by trypsinization. The cell suspension of 4×10⁵ cell.mL⁻¹ was prepared before seeding. The samples were placed into each well using a multi-well plate with 5 mL of cell suspension and seeded in each well, keeping one well as a control without any sample, and then maintained in incubator for 96 h. The samples were removed from the well, washed with phosphate buffer saline (PBS) solution twice, placed on a glass slide and fixed with ethanol and then stained with hematoxylin-eosin (H&E). All the samples were air-dried and then cover slips were mounted on them. The samples were examined by a light microscope.

RESULTS AND DISCUSSION
Reduction of Sample Weight after Irradiation
As mentioned in previous report, the oxidation reaction is a result of the laser irradiation at 9.58 μm wavelength [14]. In spite of this fact, a reduction in sample weight after irradiation due to surface ablation is observed. Figure 1 shows weight decreases after irradiation in different pulses and fluencies.

Figure 1. Weight decreases of PDMS films (μg/cm²) after irradiation by CO₂-pulsed laser with different number of pulses and fluencies; wavelength = 9.58 μm, frequency = 1 Hz. (a) 0.6 J/cm²; (b) 1.25 J/cm²; and (c) 3 J/cm².

As it is noticed, increases in fluency and number of pulses result in further weight decreases. It is supposed that increases in fluency and number of pulses would increase the laser ablation. As it is reported
earlier [14], continuous and homogeneous porosity on the treated surface of silicone has been shown after irradiation in comparison with smooth surface of unmodified sample. This implies that the exposing surface to laser beam is increased as the laser irradiation proceeds by further pulses.

Surface Graft Polymerization
As earlier reported [14], the maximum concentration of peroxides formed in the surface layer of silicone by CO2-pulsed laser treatment at 9.58 μm is obtained only by one pulse and then decreases by increasing the laser pulse number due to formation of other oxygen containing groups, such as carbonyl, hydroxyl and hydrocarboxyl groups instead of peroxides. According to this mechanism the radicals are formed and then hydrogen abstraction is carried out and then hydroperoxides groups are formed.

Figure 2 shows the effect of number of laser pulses on the water drop contact angle and surface water uptake of the poly(AAc)-grafted silicone films. As it is observed the minimum water drop contact angle and maximum graft density and surface water uptake were obtained by one pulse of laser and then increasing of water drop contact angle and decreasing of graft density and surface water uptake were obtained by increasing the laser pulse number. Figure 1a shows significant decrease of the surface water uptake from 1 pulse to 20 pulses laser poly(AAc) grafted silicone surfaces [P value < 0.0001]. Poly(AAc) is hydrophilic and silicone is hydrophobic. Thus it seems that density increases of grafted poly(AAc) results in decreasing trend of water drop contact angle and increasing of surface water uptake.
The effect of graft temperature on: (a) graft density and surface water uptake, and (b) contact angle. Concentration of monomer: 30%, fluence of laser beam: 1.25 J/cm², number of pulse: 1 pulse.

This is consistent with concentrations of peroxide generated on PDMS films by CO₂-pulsed laser with different number of pulses, as mentioned earlier [14].

The effect of the number of laser pulses on the density of grafted poly(AAc) on the laser treated silicone is seen in Figure 3 as well. Attenuated total reflectance Fourier transform (ATR-FTIR) spectra of silicone surface grafted with poly (AAc) are presented in Figures 3a and 3b. The characteristic absorption bond of AAc appearing at 1710 cm⁻¹, corresponds to the grafted (C=O) group of acrylic acid. Comparison of these spectra with that of the unmodified silicone (Figure 3c) is an evidence for the presence of the grafted poly(AAc) onto the modified surface of silicone. Comparison of Figure 3a with Figure 3b also shows some reductions of density of grafted poly(AAc) samples on the laser treated silicone from 1 pulse to 10 pulses.

Graft copolymerization of poly(AAc) onto the laser treated silicone films takes place in the presence of monomer when the system is heated to temperatures higher than 40°C, whereas no graft polymerization was observed with untreated silicone in the same conditions. Figure 4 shows the effect of graft temperature on the graft density and surface water uptake (Figure 4a) and water drop contact angle (Figure 4b) of poly(AAc) grafted silicone films.

As it is observed the maximum graft density and surface water uptake and minimum water drop contact angle were obtained at about 65°C. The data
imply that temperature of copolymerization system is one of the important factors in density increases of grafted poly(AAc) onto the laser-treated silicone. The figure shows that the grafting degree increases with the reaction temperature. Significant increase of the surface water uptake was found from 40ºC to 60ºC grafting temperature \([P \text{ value } < 0.0001]\). The result assumes that an elevated temperature can accelerate the monomer diffusion followed by decomposition of the peroxides into radicals produced by laser treatment of the silicone film surface. As a consequence, a higher grafting degree is achieved, because the AAc homopolymerization occurs easily as the reaction temperature is increased. The homopolymerization will cause the viscosity of AAc solution to increase. The high viscosity further inhibits AAc monomer moving to the surface of silicone. For this reason, the grafting degree of AAc is reduced [26].

Thus, the temperature below or above 65ºC reduces the density of grafted poly(AAc). It seems that lower temperature reduces the copolymerization of acrylic acid and higher temperature increases the rate of homopolymerization.

The effect of temperature on the density of grafted poly(AAc) on laser treated silicone is shown in Figure 5. Comparison of Figure 5a with 5b is an evidence for the density of the grafted poly(AAc) onto the treated surface of silicone films in the system at 65ºC is higher than the system of 50ºC. Figure 6 shows the scanning electron micrograph (SEM) of poly(AAc)-10 pulses laser grafted silicone with granular surface.

### Collagen Immobilization

The effect of surface modification on the hydrophilicity was illustrated by the water drop contact angle and surface water uptake is shown in Table 1.

The contact angle of polymer surface can be decreased by introducing hydrophilic groups. Because collagen immobilized poly(AAc)-grafted

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<td>PDMS-AAc</td>
<td>71.5 ± 3/5</td>
<td>450 ± 45</td>
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<td>PDMS-AAc-COL</td>
<td>71.1 ± 4/3</td>
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silicone surface, has carboxyl and amino groups, its contact angle is nearly equal in comparison with poly(AAc)-grafted silicone surface which has carboxyl group.

ATR-FTIR Spectra of collagen immobilized poly AAc-grafted silicone is presented in Figure 7. The characteristic absorption band of collagen appearing at 1651 cm⁻¹ corresponds to the grafted amide groups of collagen. Comparison of these spectra (Figure 7b) with those of poly(AAc)-grafted one (Figure 7a) gives evidence for the presence of immobilization of collagen onto poly(AAc)-grafted silicone surface.

**Cell Culture**

Effect of the two different surfaces, poly(AAc)-grafted silicone and collagen immobilized poly(AAc)-grafted silicone, on cell adhesion, spreading and growth is shown in Figure 8.

We quantified the mean number and area of cells cultured on different surfaces by Image ProPlus soft-

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<th>Table 2. The mean area and number of cells after 48 h cultured on surfaces ±SD, n=3.</th>
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<tr>
<td><strong>Poly</strong>(AAc) <strong>grafted surfaces</strong></td>
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<tr>
<td>----------------------------------</td>
</tr>
<tr>
<td>Area (μm²)</td>
</tr>
<tr>
<td>a = 110 ± 17</td>
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<td>a = 577 ± 81</td>
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ware (version 3). The results are shown in Table 2.

As it is observed in Figure 8 and Table 2, cell adhesion, spreading and growth onto the collagen immobilized onto poly(AAc)-grafted silicone films were better than poly(AAc)-grafted silicone films. This is attributed to the presence of collagen which provides a suitable substrate for cell growth [25].

Although wettability is an important factor for cell adhesion, spreading and growth onto polymer surfaces as reported by earlier work, surface chemistry or charge character are also very important factors. It seems that the carboxylic acid groups grafted on the surface have a negative effect on cell adhesion, spreading and growth [27].

In addition, the number of pulses was important factor in the density of grafted poly(AAc) and consequently the density of immobilized collagen onto silicone films.

Figure 8 and Table 2 show density effectiveness of grafted poly(AAc) and immobilized collagen onto silicone films as well. Figures 8a and 8b in comparison with Figures 8c and 8d indicate the negative effect of grafted poly(AAc) and positive effect of immobilized collagen on cell adhesion, spreading and growth in 1 pulse laser treated silicone films that were greater than 10 pulses laser treated silicone films.

Data in the literature suggest that cells specifically recognize collagen substrates. Fibroblasts grown on collagen matrices appear to differentiate morphologically and biochemically. Cells grown on collagen adopt an elongated morphology and are observed to have large pseudopodia-like processes extending into a collagen matrix. In addition, the spatial deposition of newly synthesized collagen follows the orientation of collagen fibres in a reconstituted collagen matrix. Fibroblast cell bodies have been observed to exert tension that leads to reorientation of a collagen matrix, to reduce the regulation of collagen synthesis. Also, it was shown that skin cells could be grown onto collagen substrates yielding a tissue-like product [25].

Because of relatively non-antigenic property, atelocollagen and allogenic fibroblasts were applied as part of many different artificial skins or skin replacements. Besides, there are different methods to decrease their antigenicity, so that they do not show early rejection [25].

Thus, cultured allogenic fibroblast on collagen immobilized onto poly(AAc)-grafted silicone films may be produced in large quantities and cryopreserved until the time they are required for treating full thickness wound defects.

The above developed system has the potential to be used as artificial skin in tissue engineering.

CONCLUSION

The carboxylic acid groups of poly(AAc)-grafted silicone had a negative effect on the adhesion, spreading and growth of fibroblast cells, while the amino groups of collagen immobilized on the surfaces, showed better adhesion, spreading and growth. Since a large portion of cell protein surface and some proteins, are well known to play an important role in cell attachment onto substrates are negatively charged, therefore they are adsorbed less on the negatively charged surfaces. But collagen is cell compatible and has positive effect on fibroblasts' adhesion, spreading and growth.

Besides, the number of pulses has its own effect on the density of grafted poly(AAc) and consequently immobilized collagen onto silicone films.

Thus, fibroblast culture onto the collagen immobilized samples, while testing cell compatibility, would be applied as a method for production of artificial derm.

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