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Surface modification of Polycaprolactone (PCL) microcarrier for performance improvement of human skin fibroblast cell culture

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Surface modification of Polycaprolactone (PCL) microcarrier for performance improvement of human skin fibroblast cell culture

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Abstract. Polycaprolactone (PCL) has many advantages for use in biomedical engineering field. In the present work PCL microcarriers of 150-200 μm were fabricated using oil-in-water (o/w) emulsification coupled with solvent evaporation method. The surface charge of PCL microcarrier was then been improved by using ultraviolet/ozone treatment to introduce oxygen functional group. Immobilisation of gelatin onto PCL microspheres using zero-length crosslinker provides a stable protein-support complex, with no diffusional barrier which is ideal for mass processing. The optimum concentration of carboxyl group (COOH) absorbed on the surface was 1495.9 nmol/g and the amount of gelatin immobilized was 1797.3 μg/g on UV/O_3 treated microcarriers as compared to the untreated (320 μg/g) microcarriers. The absorption of functional oxygen groups on the surface and the immobilized gelatin was confirmed with Fourier Transformed Infrared spectroscopy and the enhancement of hydrophilicity of the surface was confirmed using water contact angle measurement which decreased (86.93^o – 49.34^o) after UV/O_3 treatment and subsequently after immobilisation of gelatin. The attachment and growth kinetics for human skin fibroblast cell (HSFC) showed that adhesion occurred much more rapidly for gelatin immobilised surface as compared to untreated PCL and UV/O_3 PCL microcarrier.

1. Introduction

Microcarrier is a term used in reference to microspheres that support cells in mammalian cell culture in which cells grow as monolayer on the surface of microspheres' surface [1],[2]. In a laboratory scale

cell culture anchorage-dependent cells are commonly cultivated on the walls of roller bottles or non-agitated vessels such as tissue culture flasks [3].

Moving upscale, particularly to produce large amounts of bioproducts, animal cells, are routinely carried out in a bioreactor. In this condition, microcarriers are required as substrate to the anchorage-dependent cells. Different types of bioreactors such as stirred tank and fluidised bed bioreactor have utilised microcarrier to grow anchorage-dependent cells. Microcarriers are among the most established technological platforms for industrial production to increase productivity [4]. Biomaterials such as biodegradable polymer are particularly suited for the development of microcarrier. The surface can be further improved as desired by introducing functional groups such as hydroxyl and carbonyl onto the surface of polymer microparticle. For instance, a combination of ozone aeration and UV irradiation have been reported to improve immobilisation of gelatin onto the microcarrier, therefore, enhancing the anchorage dependent cell proliferation [5].

This study was set to fabricate biodegradable microcarrier beads using solvent evaporation method and identify the controlling parameters that affect particle size. Ultraviolet ozone (UV/O_3) process conditions were then optimised to improve the surface of the microcarrier beads by introducing functional groups prior to optimisation of gelatin immobilisation on the microcarrier surface. Finally, the fabricated microcarrier beads were evaluated for their cytocompatibility by culturing mammalian cell lines on the microcarrier in spinner vessel culture system.

2. Experimental procedures

2.1Materials

Polycaprolactone (PCL) (MW=45,000) in pellet form, dichloromethane used to dissolve PCL, and poly(vinyl alcohol) (PVA) were supplied by Sigma Aldrich (USA). Pure oxygen (>99%; Linde Malaysia Sdn. Bhd), toluidine blue O (Bendosen Laboratory Chemical, Germany), phosphate buffer (PBS; EMD Chemical Inc, USA), sodium dodecyl sulfate (SDS; Merck, Germany), and absolute ethanol (HmbG Chemicals, Germany) were used. Bovine gelatin was purchased from Halagel (Malaysia). Absolute ethanol from HmbG Chemicals, (Germany) and olive oil was obtained from a local hypermarket. Dulbecco's modification of eagle's medium (DMEM) in powder form, fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin, 0.1 g/l streptomycin) were supplied by Gibco (USA). Sodium bicarbonate, hydrochloric acid, sodium hydroxide and trypan blue were supplied by Sigma-Aldrich. Silicone oil and ethyl acetate were purchased from Merck Millipore (Germany).

2.2 Microcarrier preparation

The preparation of microcarrier was achieved by a solvent evaporation method [6,7] with slight modifications [8**]**. PCL was dissolved in organic solvent. The dissolved mixture was then added dropwise into a polyvinyl alcohol (PVA). The resulting emulsion was stirred at appropriate speed for 6 hours at room temperature. The microcarrier was collected and washed with distilled water prior drying overnight at 30 ^{o}C. Parameters tested to study the effect of operation condition on the size of PCL microcarrier was stirring speed, PVA concentration and polymer matrix ratio. The UV/O_3 system equipped with ozone generator that supply with pure oxygen at flowrate of 0.5 lpm at constant standard working pressure of 20 psig. The generated ozone was supply to the Dresher bottle containing microcarrier that was placed in the UV box for 60 minutes to introduce oxygen functional group [9]. The UV/O_3 surface of PCL microcarrier were activated using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC) and *N*-hydroxysuccinimide (NHS). The microcarrier was then washed with MES buffer prior to gelatin immobilized in 80 mg/ml gelatin solution. Lastly the microcarrier was washed and dried in an oven overnight.

2.3 Characterization

For characterization, analytical techniques were used to magnify the microcarrier, visualized the structure and investigate the distribution elements on the surface of microcarrier by means of scaning

electron microscope (SEM) (Hitachi, S3400N), contact angle (Phoenix, 300), attenuated total reflectant Fourier transform infrared spectroscopy (ATR-FTIR) (Thermo scientific, Nilolet iS50), and gel permeation chromatography (GPC) (Waters, 2690/DAD 996/RID V2410/LSD CD 432). Lastly, in the biological testing component, the microcarriers were tested in mammalian suspension cell culture (human fibroblast cell, HSFC) to determine the biocompatibility of the microarrier.

3. Results and Discussion

3.1 Effect of preparation condition on particle size of PCL microcarrier

The effects of the preparation conditions on the size and morphology of the microspheres were investigated. The result showed that high stirring speed (300 rpm), the emulsion is easily dispersed therefore producing smaller droplets. The size of microspheres was independent of surfactant at low concentrations (0.05-0.1%) as the surfactant is not sufficient to stabilize the droplet formation. However, the size of microspheres decreased from 384.50-190.77 μm as the surfactant concentration is increased. The microspheres size was not only affected by those two parameters, but also by the amount of (PCL) in dichloromethane (DCM) solvent. At high ratio (1:30) of the polymer matrix to the organic solvent, small microspheres size (94.64 μm) was observed and as the ratio decrease (1:10), the microspheres size increase (293.26). Convincingly, the size of microspheres can be control by controlling the parameter's variables by mean of solvent emulsion method.

3.2 Optimised microcarrier

Polycaprolactone (PCL) microcarrier was successfully produced using solvent evaporation method. The microcarrier was then treated with UV/O_3 treatment, which intendedly to introduce the oxygen functional group on the surface of the microcarrier to make it susceptible for gelatin immobilisation. The amount of oxygen functional group increased from 283.4 nmo/g to 1495.9 nmol/g [10]. Subsequently, gelatin was immobilised on the surface of treated also shows increment (Table 1) in amount of gelatin immobilised on the microcarrier surface with optimised condition.

Table 1. Amount of absorbed gelatin on untreated PCL microcarrier and UV/O_3 treated PCL microcarrier**.**

Microcarrier	**Concentration of immobilised gelatin (μg/g)**	**% increased**
Non-optimised PCL microcarrier	320.00±0.9	82.2%
Optimised PCL microcarrier	1797.33±21	

3.3 Contact angle measurement

The wettability of UV/O_3 treated film was compared to the untreated and gelatin coated film. Table 2 shows the water contact angle values and surface energy of untreated PCL film, UV/O_3 treated PCL film and gelatin coated PCL film. Treatment was made by relative measurement under similar condition as the microcarrier.

Table 2. Contact angle values and surface energy of untreated, UV/O_3 treated and UV/O_3 treated and gelatin immobilised PCL microspheres.

Microcarrier	**Contact angle (^{o})**	**Surface energy (mJ/m^2)**
Untreated PCL	86.93	24.94
UV/O_3 treated PCL	69.34	41.12
UV/O_3 treated + gelatin immobilisation (i.e. gelatin coated PCL)	49.34	61.28

The value of contact angle of untreated microcarrier (86.93°) imposed a relatively hydrophobic behaviour. The angle was consequently decreased to 69.34° after UV/O_3 treatment, thus leading to the increased surface energy from 24.94 mJ/m^2 to 41.12 mJ/m^2. This increase in surface energy could be due to the incorporation of oxygen-containing functional groups like O–C=O, C=O, C–O and OH [11] on the surface of the microcarrier. According to Gomathi and Neogi [12], an increase in surface energy is due to the incorporation of the polar components on the surface by the presence of polar groups, electric charges and free radicals.

The introduction of the functional polar components on the PCL microcarrier surface not only improved its hydrophilicity but may also accommodate biomolecules components such as protein and cell growth factors to make the surface more biocompatible for cell growth and proliferation [13]. A drastic decrease in contact angle from 69.34° to 49.34° for gelatin coated PCL microcarrier was observed. This indicates further improvement in hydrophilicity as compared to UV/O_3 treated PCL microcarrier which could be due to the presence of large amount of amino terminal and carboxyl groups [14]. Figure 1 shows the decrement of contact angle of water drops on the raw PCL surface, UV/O_3 treated and gelatin coated PCL surface, respectively.

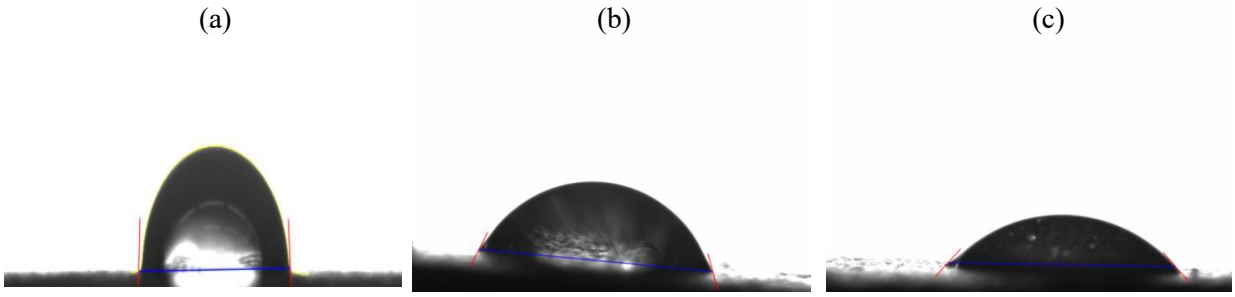

Figure 1. Water drop images of contact angle analysis on the surface of: (a) raw PCL, (b) UV/O_3 treated PCL and (c) gelatin coated PCL.

3.4 ATR-FTIR analysis

Figure 2 shows the ATR-FTIR spectra of PCL, UV/O_3 treated PCL and gelatin coated PCL, respectively. The peaks at 1723, 1175 and 1230 cm^{-1} are the signature peaks of polyesters which correspond to C=O, C-O-C and C-C respectively in the IR spectra [15].

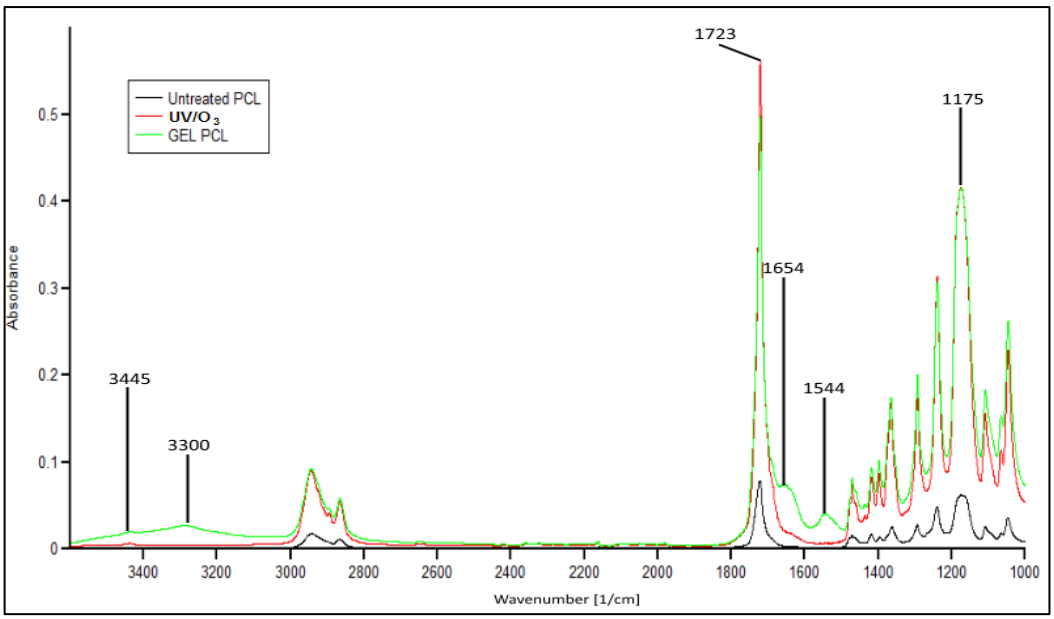

Figure 2. ATR- FTIR spectra of untreated PCL, UV/O_3 treated PCL (UV/O_3 PCL) and gelatin coated PCL (GEL PCL).

Interestingly, figure 2 shows the presence of a new peak in UV/O_3 PCL spectra at 3445 cm^{-1}. This suggests that the oxidation process by UV/O_3 could introduce O-H group in the main chain of PCL [16]. Meanwhile, marked broadening in the region of 1600-1750 cm^{-1} could be attributed to the free acids, modification of chemical environment around the carbonyl group or the formation of vinyl groups [17]. Subsequently, the successful immobilisation of gelatin onto the UV/O_3 treated PCL surface could be deduced by the presence of a broadband at 3300 cm^{-1}, possibly due to the overlapping of a hydroxyl group (O–H) and an amine group (N–H) stretching vibrations. An increase in the relative intensity of amide I band (at 1654 cm^{-1}) and amide II (at 1544 cm^{-1}) [14] also attributed to the successful of gelatin immobilisation.

3.5 SEM analysis

The morphology of the PCL microcarrier was examined by SEM, as displayed in figure 3. The microcarriers were observed to be spherical with a uniform particle size in the range of 100 to 150 µm. The surface of the untreated PCL (figure 3 (a)) appears to be smoother than the surface of the UV/O_3 treated PCL microcarrier (figure 3 (b)). Upon UV/O_3 treatment the surface appears to crease and has plenty of holes which can be clearly seen in figure 3 (e). According to Teare and Bradley [18], the roughness of the PCL surface were due to distortion caused by a series of photo-cleavage reaction involving UV rays and ozone on the polymer surface.

Further, microcarrier coated with gelatin exhibit rather smooth surface as compared to UV/O_3 treated PCL (figure 3 (c) & (f)). The creases were covered by gelatins that were immobilised onto the surface through covalent bonding with intermediate crosslink. The immobilisation was uniform over the microcarrier surface and this may help improve the biocompatibility of the microcarrier [19].

3.6 Gel Permeation Chromatography (GPC)

The effect of PCL microcarrier surface modification on weight-average molecular weight (M_w) were examined by using GPC. HPLC-grade tetrahydrofuran (THF) was used as mobile phase at room temperature with a solvent flow rate of 1 ml/min with 35 bar pressure. From the analysis, the average molecular weight of untreated PCL microcarrier was 23×10^4. After the treatment with UV/O_3, M_w of PCL microcarrier slightly decreased to 22×10^4. The change in M_w of PCL microcarrier was due to the

oxidation and chain scissions of polymer through the formation of many free radicals which consequently lowers the molecular weight of the treated sample [20,21].

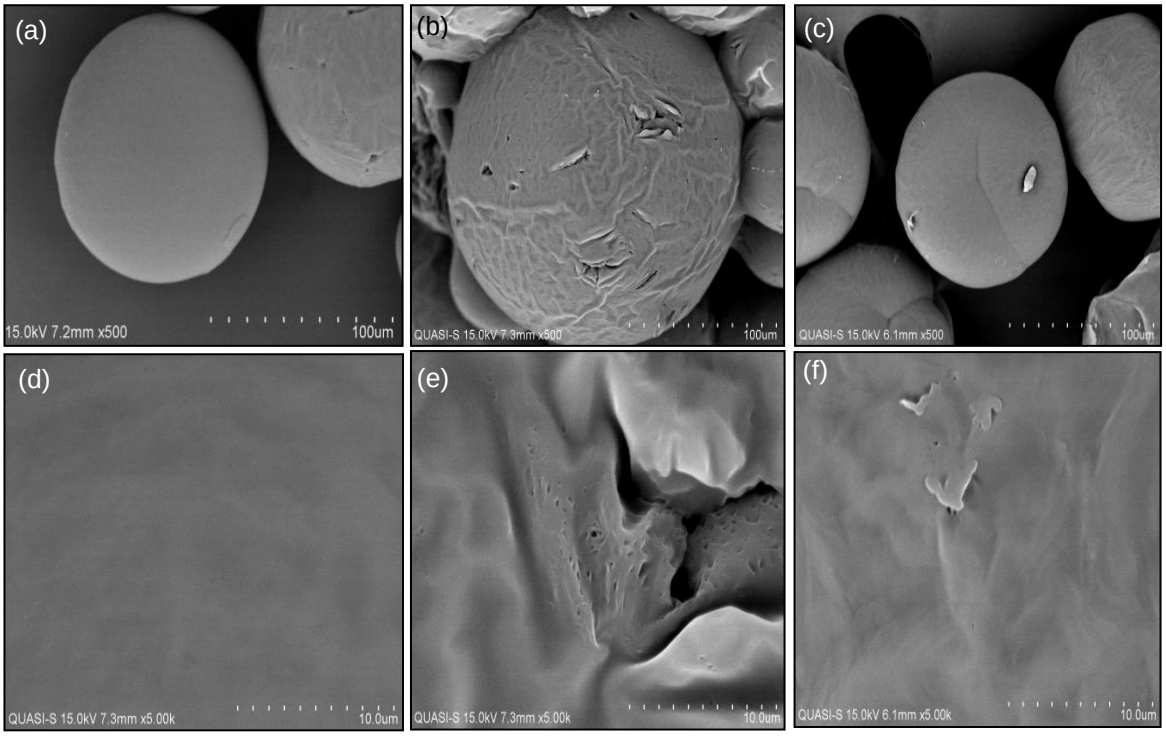

Figure 3. SEM images of the untreated PCL microcarrier (a), UV/O_3 treated PCL microcarrier (b), and gelatin coated PCL microcarrier (c). (d), (e) and (f) are higher magnification of (a), (b) and (c) respectively.

3.7 Cell attachment on gelatin coated PCL microcarrier

The growth of human skin fibroblast cells (HSFC) (1184 (ECACC 90011883) on different PCL microcarrier surfaces was investigated by suspension culture in stirred spinner flasks. Figure 4 shows the growth kinetics of HSFC on gelatin coated PCL microcarrier, UV/O_3 treated PCL microcarrier and untreated PCL microcarrier as a control. Table 3 shows the number of maximum cells attached on microcarrier, growth rate and doubling time of the three different cultures. From the graph, HSFCs were observed to attach and proliferate well on the gelatin coated microcarrier (Gel PCL) within two days after inoculation. The attached cells continued to grow until it reached maximum number of cells of 2.06×10^6 cells/ml (14 fold) as compared to the seeding concentration (1.5×10^5 cell/ml) at 72 hours after cultivation, with the fastest doubling time of 25.4 hours as compared to the cell growth on UV/O_3 PCL microcarrier (maximum cell number of 6.1×10^5 cells/ml, 84 hours after inoculation).

Meanwhile, in the untreated PCL microcarrier culture, slow cell growth with doubling time of 193.37 h and a low maximum number of cells (1.2×10^5 cells/ml) was observed. This low yield can be attributed to the difficulties of cells to attach on the surface of the untreated microcarrier which is less biocompatible as compared to gelatin coated PCL microcarrier.

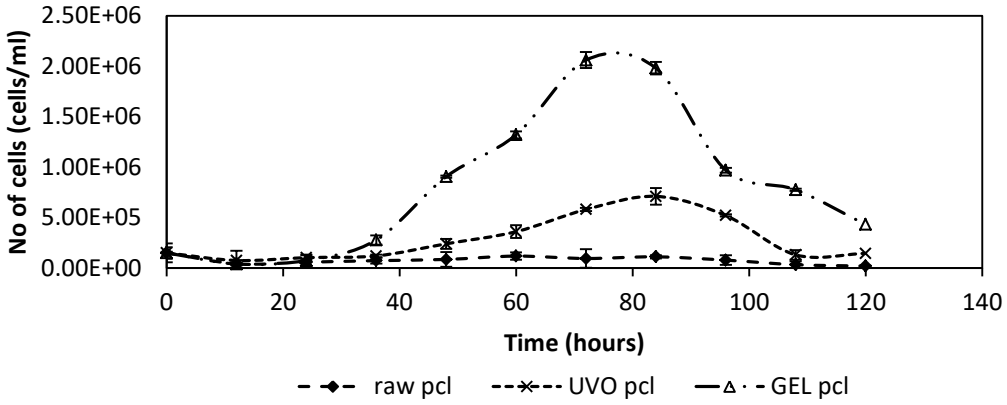

Figure 4. Growth kinetics of human skin fibroblast cell (HSFC) on different microcarriers in stirred spinner flasks: (x) UV/O_3 treated PCL, (▲) gelatin coated PCL, (●) untreated PCL.

Table 3. Values of maximum cell concentration, growth kinetics and doubling time of HSFC on different types of microcarrier.

Microcarrier	**Maximum cell concentration ($x10^5$ cell/ml)**	**Growth rate, μ (h^{-1})**	**Doubling time, t_d (h)**
Untreated PCL	1.2 ± 14.1	0.004	193.37
UV/O_3 treatedPCL	6.1 ± 8.4	0.017	41.51
Gelatin coated PCL	20.6 ± 4.7	0.027	25.40

In the light of the selection of dermal related cells (skin fibroblast) as models in this study, the aim was to demonstrate the usability of the biodegradable PCL microcarrier in tissue engineering applications. Microcarriers offer the advantage of mass production of cells, large scale production of bioproduct, as well as more specific application such as transplantation tools to carry cells in wound healing therapies. Skin fibroblasts have been reported to have great potential for treating skin diseases such as genetic abnormalities, infections and skin cancer [22]. They are also commonly utilized as an *in vitro* cell model for toxicity testing and the discernment of process of chemically induced skin carcinogenesis [23].

4. Conclusion

The characterization of PCL microcarrier indicates the presence of new functional groups on the surface as were evident by ATR-IR spectroscopy. The oxidation process by UV/O_3 treatment introduced O-H group in the main chain of PCL. The successful immobilisation of gelatin onto the UV/O_3 treated PCL surface was also revealed by FTIR measurement. A drastic decrease in contact angle from "69.34°" to "49.34°" for gelatin coated PCL microcarrier was observed, indicating further improvement in hydrophilicity as compared to UV/O_3 treated PCL microcarrier. The ability of gelatin coated PCL microcarriers to support growth and proliferation of cells were assessed using primary cell (human skin fibroblast cell, HSFC) shows that gelatin coated PCL microcarriers were able to support the growth of HSFC which is the primary type of cell that have low plating efficiency. The oxidation

of PCL microcarrier surface by UV/O_3 treatment enhances surface wettability and promotes higher gelatin immobilisation and in turn improved cell adhesion and proliferation on the microcarrier.

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