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STATISTICAL OPTIMIZATION OF GELATIN IMMOBILISATION ON MODIFIED SURFACE PCL MICROCARRIER TO IMPROVE PCL MICROCARRIER COMPATIBILITY

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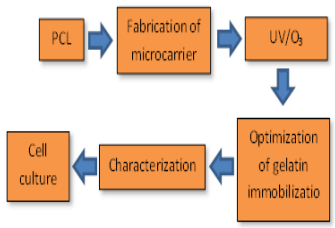
Graphical abstract



Abstract

Growing cells on microcarriers may have overcome the limitation of conventional cell culture system. However, the main challenge remains at ensuring the surface biocompatibility with cells. Polycaprolactone (PCL), a biodegradable polymer, has received considerable attention because of its excellent mechanical properties and degradation kinetics that suit various applications, but its non-polar hydrocarbon moiety renders it sub-optimal for cell attachment. In this present study, the aim was to improve biocompatibility of PCL microcarrier by introducing oxygen functional group via ultraviolet irradiation and ozone aeration (UV/O_3 system) to allow covalent immobilization of gelatin on the PCL microcarrier surface. Respond surface methodology was used as a statistical approach to optimized parameters that effect the immobilization of gelatin. The parameters used to maximized amount of gelatin immobilize were the mol ratio of COOH:EDAC, NHS concentration and gelatin concentration. The optimum conditions for maximum amount of gelatin (1797.33 µg/g) on the surface of PCL were as follows: 1.5 of COOH:EDAC ratio, 10 mM NHS concentration and, 80 mg/ml gelatin. The result shows that gelatin coated PCL microcarrier promote more and rapid cell adhesion with density of 16.5×10^5 cells/ml as compared to raw PCL microcarrier (2.4×10^5cells/ml) and UV/O_3 treated PCL microcarrier (4.25×10^5cells/ml). Therefore, immobilization of gelatin with optimized parameters onto PCL microcarrier improved biocompatibility of PCL microcarrier.

Keywords: Microcarrier, gelatin immobilization, polycaprolactone, statistical optimization

Abstrak

Pengkulturan sel menggunakan "microcarriers" mungkin telah mengatasi kekangan sistem kultur sel konvensional. Walau bagaimanapun, cabaran utama untuk memastikan biokompatibiliti (keserasian permukaan) "microcarrier" dengan sel masih belum dapat diatasi sepenuhnya. Polycaprolactone (PCL), sejenis polimer terbiodegradasikan semakin mendapat perhatian berdasarkan ciri-ciri mekanikal yang sangat baik dan kinetik degradasi yang sesuai untuk pelbagai kegunaan. Namun begitu, moieti hidrokarbon tanpa kutub pada PCL menjadikan ia sub-optimum untuk pelekatan sel. Di dalam kajian ini, kumpulan berfungsi oksigen diperkenalkan melalui sistem penyinaran ultraungu dan pengudaraan ozon (system UV/O_3) untuk membolehkan imobilisasi (pemegunan) gelatin secara kovalen pada permukaan "microcarrier" PCL dengan menggunakan 1-etil-3-(3-dimetilaminopropil) hidroklorida karbodiimid dan N-hidroksisuksinimid (EDAC/NHS). Keadaan optimum untuk memaksimumkan jumlah gelatin (1797.33 µg/g) pada permukaan PCL adalah seperti berikut: Nisbah1.5 COOH:EDAC, 10 mM NHS dan, 80 mg/ml gelatin.

Kata kunci: Microcarrier, imobilisasi (pemegunan) gelatin, polycaprolactone, pengoptimuman statistik

1.0 INTRODUCTION

Microcarrier is a term used when referring to the microspheres that support cells in mammalian cell culture in which cells grow as monolayer on the surface of the particles [1, 2]. Microcarriers are spherical particles with a size range between 100 to 200 µm. Due to its small size, microcarrier have a wide variety of applications, one of which is cell culture and tissue engineering as mentioned above. Other applications include drug delivery system, protein immobilisation, and gene delivery. The microspheres used in drug delivery system are termed as microparticles [3]. Meanwhile, the microspheres that may entrap cells in the inner compartment are called microcapsule [2]. In this article, the term microcarrier is used since microspheres were evaluated for cell and tissue culture applications. Polycaprolactone (PCL) is a synthetics polyester that has found many uses in medical and tissue engineering field as biomaterials due to its good mechanical properties, biocompatibility, biodegradability and flexible structure.

Recently, it is of interest to immobilize protein or oligopeptides onto polymer surface as it significantly improves the biocompatibility of the polymer [4]. Immobilisation of biologically active molecules can generate a specific, predictable and controlled response from the cell seeded on the materials [5]. Carbodiimide coupling reagents are the most popular type of zero-length crosslinker. Water-soluble carbodiimides are being the common choice for biochemical conjugations because most macromolecules of biological origin are soluble in aqueous buffer solutions. Not only the carbodiimide itself is able to dissolve in the reaction medium, but the by-product of the reaction, an isourea, is also water-soluble, facilitating easy purification. Among the carbodiimide, EDAC {1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride} and EDAC/NHS (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/N-hydroxysuccinimide) are frequently used to immobilize biomolecules such as gelatin on functionalized carrier [7, 8]. EDAC will react with carboxylic group on the modified polymer surface to produce intermediate compound namely *O*-acylisourea (by-product). *O*-acylisourea is not stable in solution and it will undergo cyclic electronic displacement thus producing *N*-acylurea which is unreactive towards primary amine [9]. The addition of *N*-hydroxysuccinimide (NHS) in the reaction resulted in the intermediate product (succinimidyl ester) that is more stable towards hydroysis, more reactive towards primary amine and formation of *N*-acylurea is hindered since the succinimidyl ester cannot undergo cyclic electronic displacement [9].

EDAC/NHS-coupled reactions are highly efficient and usually increase the yield of conjugation compared to EDAC alone. Khan *et al*., (2007) claimed that EDAC/NHS strategy provide a good platform to improve the biocompatibility for various biomedical purposes. This present study aims to optimized gelatin immobilized on the surface of polycaprolactone (PCL) microcarrier using this strategy.

In the present study, the surface modification process was conducted to i) introduce functional groups on the microcarrier surface and ii) statistically optimized the gelatin immobilisation process condition, with the purpose to improve cell attachment on the microcarrier. Topographical and chemical changes induced upon optimization of PCL microcarrier surfaces were characterized by scanning electron microscopy (SEM), attenuated total reflection–Fourier transform infra-red spectroscopy (ATR-FTIR) and surface energy. Rat amniotic fluid stem cell (AFSC) growth and expansion on the untreated, UV/O_3 treated and UV/O_3-gelatin

coated PCL microcarriers were examined and compared.

2.0 METHODOLOGY

2.1 Microcarrier Preparation

Polycaprolactone (PCL) microcarriers were prepared by emulsion solvent evaporation process according to Maia and Santana (2004) with slight modifications [16]. The prototype system of UV/O_3 to introduce oxygen functional group on the microcarrier surface was set following Murakami *et al*. (2005) with optimized parameters.

2.2 OFAT Optimisation

Optimization of gelatin immobilisation was conducted in two phases. The first phase was to perform a one-factor-at-a-time experiment (OFAT) to get the initial framework of response variation according to factor level and determine where the optimum could be located [9]. The factors are varied until the best setting was found by changing one factor and keeping other factors constant. Gelatin immobilisation protocol using EDAC/NHS crosslinker couple relative to the concentration of carboxyl functional group (COOH) deposited on the microcarrier surface lead to the selection of the variables (data not shown).

2.3 Experimental Design of Gelatin Immobilisation Process Conditions

Experimental design was generated using statistical software, Design Expert® 7.0 (Stat Ease Inc.). The influence and interaction between COOH:EDAC mol ratio (X_1), NHS concentration (X_2), and gelatin concentration (X_3) on the amount of immobilized gelatin (Y) on the surface of microcarriers were evaluated using faced centred composite design requiring 20 formulations which include six replicates at the centre point to represent process variations. This design was used to explore the quadratic response based on the second order polynomial model. Table 1 shows three variables namely (COOH:EDAC) molar ratio (X_1), NHS concentration (X_2), and gelatin concentration (X_3) with the range of factors used in the optimization directed from the OFAT result.

Table 1 Factors and levels used in 2^3 faced centered composite design (FCCD) for optimization of process conditions of gelatin immobilisation on PCL microcarrier surface

	Central composite design			
	Variables	***(-1)***	***(0)***	***(+1)***
X_1	(COOH:EDAC)	1	1.5	2
X_2	NHS conc. (mM)	5	10	15
X_3	Gelatin (mg/ml)	60	80	100

The influence and interaction between COOH:EDAC molar ratio (X_1), NHS concentration (X_2), and gelatin concentration (X_3) on the amount of gelatin immobilized (Y) on the surface of microcarriers were evaluated using faced centered composite design requiring 20 formulations which include six replicates at the center point to represent process variations. This design was used to explore the quadratic response based on the second order polynomial model. A significant difference between the mean of three independent conditions was based on statistical analysis of variance (ANOVA).

2.4 Characterisation

Characterisation of the physically optimized surface of PCL microcarrierswas carried out to elucidate the improvement of wettability and biocompatibility of the microcarriers. The water contact angle was measured using Phoenix 300 Contact Angle analyzer (S.E.O., Korea) by means of sessile drop method. The drops image was captured by a video camera and Surfaceware 8 software was used to calculate the contact angle from the shape of the water drop on the UV/O_3 treated and gelatin coated PCL film. Surface chemistry of UV/O_3 treated PCL was analyzed by attenuated total reflectance (ATR) mode using Nicolet™iS™ 50 FT-IR spectrometer (Thermo Scientific, USA) to observe the addition of oxygen functional group after UV/O_3 treatment and the presence of amide bond on the gelation coated PCL microcarrier.

2.5 Microcarrier Culture Cultivation

Inoculum for spinner flasks was obtained from static monolayer growth in 175 cm^2 tissue culture flasks. Spent medium was discarded and the flask was washed twice with PBS and then incubated for 15 min in accutase until complete cell removal from the flask's surface. The accutase was inactivated by the addition of fresh culture medium to the cell suspension. The suspension was then centrifuged at 100×g for 5 min. The supernatant was discarded and the remaining pellet was resuspended in 30ml fresh complete media. Cell concentration was determined using Neubauer hemocytometer under an inverted phase microscope (Olympus CK40,

Japan). Five hundred milliliter spinner vessel (BellCo, USA) with 200 ml working volume was coated with 5% silicon oil in ethyl acetate (to prevent microcarrier from attaching to the inner surface). PCL microcarriers (3 g/ml) suspended in 50 ml culture medium and cells at a concentration of 1.5×10^5 cell/ml were inoculated in the spinner flask and culture medium was added to a final volume of 200 ml. Samples (2 ml) of cell suspension were taken every 12 hours and analyzed for cell viability and morphology.

2.6 Sampling and Cell Counting

One millilitre of microcarriers culture was aseptically pipetted out from the spinner flask culture and placed into 15 ml tube. The microcarriers were allowed to settle. Supernatant was discarded. Microcarriers were washed twice with PBS before treatment with Accutase and the tube was incubated in CO_2 incubator for 15 min at 37 °C. After 15 min the mixture was gently flushed to detach the immobilised cells. The concentration of cells in the suspension was determined using a haemacytometer with the aid of trypan blue. Twenty microlitres of cells suspension was mixed with an equal volume of trypan blue dye. Ten microlitres of the mixture was placed on the haemacytometer and allowed to spread by capillary action. Cells were counted under an inverted microscope and concentration of cells (cells/ml) was calculated using Eqn 1.

$$c = {}^{n}/_{v} \quad (1)$$

c = cell concentration (cells/ml)
n = number of cells
v = volume counted (ml)

Standard Heamacytometer were used have the depth of chamber of 1 mm and the area of the central grid is 1 mm^2. Therefore v = 0.1 mm^3. The formula then become

$$c = n \times 10^4 \quad (2)$$

If the cells were too concentrated, the cell suspension can be diluted and the dilution factor was added in the calculation as follows:

$$c = n \times dilution\ factor \times 10^4 \quad (3)$$

3.0 RESULTS AND DISCUSSION

3.1 Optimization of Process Conditions Of Gelatin Immobilisation

The traditional one-factor-at-a-time (OFAT) method was conducted in order to determine the possible optimum level of variables that would result in the highest amount of gelatin immobilized on the surface (data not shown). The deisign of experiment is shown in Table 2. Based on the OFAT results, the independent variables were then investigated at three levels according to the central composite design (CCD) [10]. Effect of linear, interactive and quadratic terms on the amount of gelatin was developed using second order polynomial model. The regression equation to predict the optimal condition was given by the following equation:

Amount of gelatin (Y) = 1775.89 - 12.20*A + 2.00*B - 0.74*C - 10.88*A*B - 5.79*A*C - 1.12*B*C -86.80*A^2 - 32.47*B^2 - 20.47*C^2 (4)

Table 2 Measured and predicted carboxyl concentration in the experiments obtained by central composite design

Run	Experimental factors			Y (Amount of gelatin) (µg/g)	
	A (COOH:EDAC)	*B (mM)*	*C (mg/ml)*	*measured*	*predicted*
1	2.0	10.0	80.0	1658.67	1676.89
2	1.0	15.0	100.0	1666.33	1665.16
3	1.0	10.0	80.0	1709	1701.28
4	2.0	15.0	60.0	1633	1622.72
5	1.5	10.0	60.0	1745.67	1756.16
6	1.0	15.0	60.0	1659.67	1657.29
7	2.0	5.0	100.0	1627.67	1627.43
8	1.5	10.0	80.0	1781	1775.89
9	1.0	5.0	60.0	1625.67	1629.29
10	2.0	5.0	60.0	1639.7	1638.25
11	2.0	15.0	100.0	1613.67	1607.42
12	1.0	5.0	100.0	1634	1641.65
13	1.5	10.0	80.0	1766.33	1775.89
14	1.5	10.0	80.0	1777.67	1775.89
15	1.5	10.0	80.0	1797.33	1775.89
16	1.5	10.0	100.0	1754.67	1754.68
17	1.5	10.0	80.0	1775.33	1775.89
18	1.5	15.0	80.0	1725.33	1745.41
19	1.5	5.0	80.0	1751	1741.42
20	1.5	10.0	80.0	1778.67	1775.89

Note: ***A***: molar ratio of (COOH:EDAC); ***B***: NHS concentration; ***C***: concentration of gelatin

Table 3 Analysis of variance ANOVA of the model generated for optimization of process conditions of gelatin immobilisation on PCL microcarrier surface

Source	Sum of Squares	*F*-value	*p*-value
Model	79351.79	48.69	< 0.0001
A-COOH:EDAC	1487.42	8.21	0.0168
B-NHS concentration	39.84	0.22	0.6491
C-Gelatin concentration	5.43	0.03	0.8660
AB	946.77	5.23	0.0453
AC	268.54	1.48	0.2512
BC	10.057	0.06	0.8184
A^2	20719.37	114.43	< 0.0001
B^2	2899.40	16.01	0.0025
C^2	1151.79	6.36	0.0303
Lack of fit	1295.73	2.52	0.1670
R^2 = 0.9777; Adj R^2 = 0.9576; Pred R^2 = 0.8783			

According to the design experimental response model (Table 2) maximum experimental values was 1797.33 µg/g with high reproducibility with 0.79% relative standard deviation (RSD) value. *F*-value of the model was 48.69 (Table 3) which indicates that the model was highly significant and that the effect of factors was real [10]. The probability value (*p*-value) is also a measure of significance of a regression model and parameters that contribute to the model equation [10]. The very low probability value ($p < 0.0001$) of the model confirms the valid measures of variation in the data. Linear terms of model coefficients of COOH:EDAC ratio, the interaction term between COOH:EDAC ratio and NHS concentration, and all quadratic terms were found to be significant with $0.0001 < p\text{-value} < 0.05$. The coefficient of determination, $R^2 = 0.9777$ estimated for the above equation implies that 97.77% of the variability in the response (amount of gelatin) could be explained by the model and that only 3% of total variation was not explained by the model. The adjusted $R^2 = 0.9576$ was also found to be very high which is particularly useful when comparing models with a different number of terms, therefore indicating a high degree of significance of the model [10]. From the validation experiments (Table 4), the optimum cross-linking conditions were determined to be EDAC to COOH ratio of 1.5:1, NHS concentration of 10 mM and gelatin concentration of 80 mg/mL. Under these conditions, the maximum amount of immobilized gelatin of 1797.33 µg/g was obtained. This optimization study has successfully increased by 6 fold from amount of gelatin before optimization.

Table 4 Validation experiments designed by Design Expert 7.0

Run	EDAC to COOH ratio	NHS conc. (mM)	Gelatin conc. (mg/mL)	Amaount of immobilized gelatin (µg/g)		Residuals
				Experimental	Predicted	
1	1.3917	5.635	71.92	1713.33	1734.16	20.83
2	1.549	10.317	77.96	1830	1743.66	-86.34
3	1.598	9.152	85.996	1526.67	1719.18	192.51

Figure 1 shows the regression model (two-dimensional contour plots (a) and three-dimensional response surface (b)) of the interaction between COOH:EDAC ratio and NHS concentration. The plot shows that the amount of immobilized gelatin is affected by varying the COOH:EDAC ratio and NHS concentration. The maximum immobilisation was at the point of intersection of variables X_1 and X_2 at given range (center of the elliptical plot). The interaction of COOH:EDAC ratio and NHS concentration was significant ($p<0.05$). The response was affected by function of these factors. The linear effect of COOH:EDAC ratio was significant at ($p<0.05$) whereas the effect of NHS concentration was insignificant ($p>0.6491$).

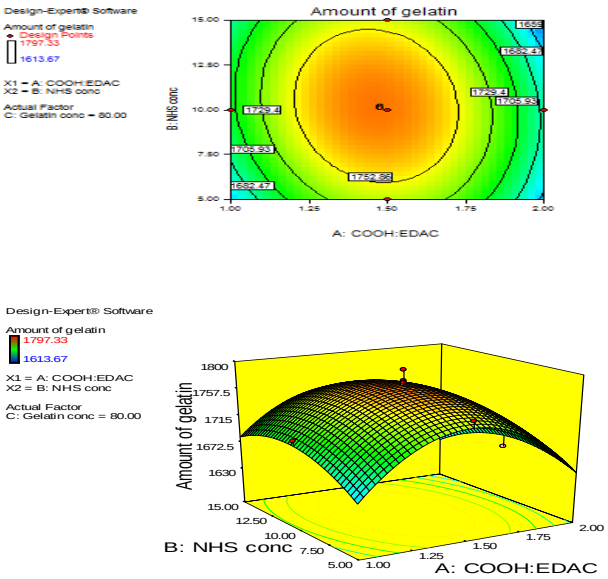


Figure 1 2-D contour plot and 3-D response surface interaction between COOH:EDAC ratio and NHS concentration on the amount of gelatin immobilized

3.2 Amount of Gelatin Immobilized on Microcarrier Surface

Table 5 shows the amount of gelatin being immobilized onto the microcarrier before and after UV/O_3 treatment. The measurement was based on the detection of hydroxyproline from the hydrolyzed gelatin that is immobilized on the microcarrier surface [11]. There was an increased in concentration of gelatin immobilized (82.2% of the UV/O_3 treated PCL microcarrier) on the microcarrier surface after the optimization of the process condition.

Table 5 Concentration of immobilized gelatin on UV/O_3 treated PCL microcarrier before optimization and after optimization of process condition of gelatin immobilization

Before optimization			After optimization		
Parameters		Amount of gelatin	Parameters		Amount of gelatin
EDAC:COOH (ratio)	0.5:1	320±0.9	EDAC:COOH (ratio)	1.5:1	1797.33±21
NHS conc. (mM)	5		NHS conc. (mM)	10	
Gelatin conc. (mg/ml)	10		Gelatin conc. (mg/ml)	80	

The introduction of oxygen functional group onto PCL microcarrier not only improved the hydrophilicity, indeed it provides a favorable interaction site for biomacromolecules such as protein, peptide, polysaccharide or growth factor. The immobilisation of gelatin onto the modified surface was achieved through covalent bonding using zero-length crosslinker [12].

3.3 Characterisation

In order to further clarify the effects of introduction of gelatin on the surface of PCL microcarrier, the hydrophilicity and composition of the treated surface were investigated. Surface energy measures the hydrophilicity of the treated microcarrier, Meanwhile, Attenuated Total Reflection–Fourier Transform Infra Red (ATR-FTIR) confirmed the addition of functional groups on the microcarrier surface. The morphology of microspheres surface was studied by scanning electron microscopy (SEM).

3.3.1 Surface Energy

The wettability of UV/O_3 treated film was compared to the gelatin coated film. Table 6 shows the surface energy of UV/O_3 treated PCL film and gelatin coated PCL film. Treatment was made by relative measurement under similar condition as the microcarrier.

Table 6 Surface energy of untreated, UV/O_3 treated and gelatin immobilized PCL microcarrier

Microcarrier	Surface energy (mJ/m²)
Untreated PCL	26.98
UV/O_3 treated PCL	41.12
Gelatin coated PCL	61.28

The introduction of the functional and 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 [7]. The increased in surface energy is due to the incorporation of the polar components on the surface by the presence of the polar groups, electric charges and free radicals [13]. Drastic increase in surface energy from 41.12 mJ/m^2 to 61.28 mJ/m^2 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 [5].

3.3.1 ATR-FTIR

Figure 2 shows the successful immobilisation of gelatin onto the UV/O_3 treated PCL surface which 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. Increase in the relative intensity of amide I band (at 1654 cm^{-1}) and amide II at (1544 cm^{-1})[5] also attributed to the successful of gelatin immobilisation. The peaks at 1723, 1175 and 1230 cm^{-1}(Figure 2) are the signature peaks of polyesters which correspond to C=O, C-O-C and C-C respectively in the IR spectra [14].

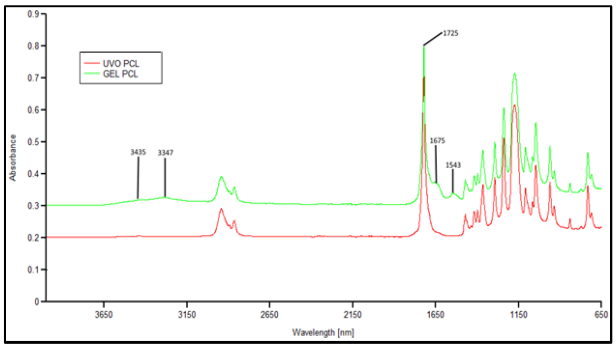


Figure 2 ATR- FTIR spectra of UV/O_3 treated PCL (UVO PCL) and gelatin immobilized PCL (GEL PCL)

3.4 AFSC Growth on Microcarrier Culture

This part of the study demonstrates that rat amniotic stem cells (AFSC) adherence and proliferate during expansion on microcarrier on the basis of light microscopy, SEM and growth kinetic. Figure 3 shows growth performance of AFSC on gelatin coated PCL microcarrier, UV/O_3 and raw PCL microcarrier as a control. All experiments were seeded with 1.5×10^5cells/ml and with microcarrier concentration of 3 g/l. The number of cells adhered to microcarrier was calculated every 12 hours for 5 days. The result shows that gelatin coated PCL microcarrier promote better cell adhesion and proliferation as compared to UV/O_3 PCL and raw PCL microarrier, with maximum cell concentration of 1.65×10^6 cell/ml on the second day of cultivation. The number of cells starts to decrease beyond 70 hours due to nutrient depletion and accumulation of waste in the culture media. Maximum cell concentration for UV/O_3 PCL microcarrier and raw PCL microcarrier were 4.25×10^5cells/ml and 2.4×10^5cells/ml respectively (Table 7).

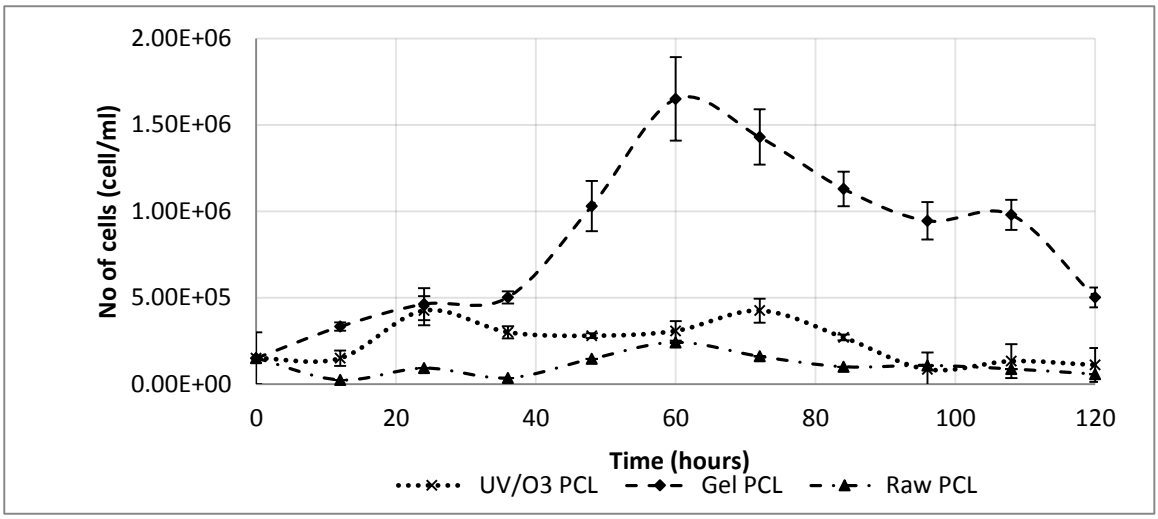


Figure 3 Growth kinetics of rat amniotic stem cell (AFSC) on different microcarriers in stirred spinner flasks: (x) UV/O_3 PCL, (●) gelatin immobilized, (♦) untreated PCL

Table 7 Values of maximum cell concentration, growth kinetics and doubling time of AFSC cell on different types of microcarrier

Microcarrier	Maximum cell concentration ($\times 10^5$ cells/ml)	Growth rate, μ (h^{-1})	Doubling time, t_d (h)
Raw PCL	2.4 ± 5.9	0.0056	123.88
UV/O_3 PCL	4.25 ± 7.0	0.0124	55.91
Gelatin PCL	16.5 ± 24.1	0.0250	27.75

Faster cell growth rate was also observed in gelatin coated PCL microcarrier followed by UV/O_3 PCL microcarrier and eaw PCL microcarrier (Table 5). Gelatin coated microcarrier was found to be suitable for culturing primary and sensitive cells with low plating efficiency [15]. In the case of stem cell, immobilisation of gelatin on the microcarrier substrate promotes excellent cell attachment, spreading and expansion of stem cell while preserving their differentiation potential along multiple lineages [16]. Figure 4 shows the morphology of AFSC cells on gelatin coated PCL observed using phase contrast microscope and SEM at 96 hours of cultivation. No aggregates were observed for all cultures for the first 3 days of cultivation but cell-bridges start to form.

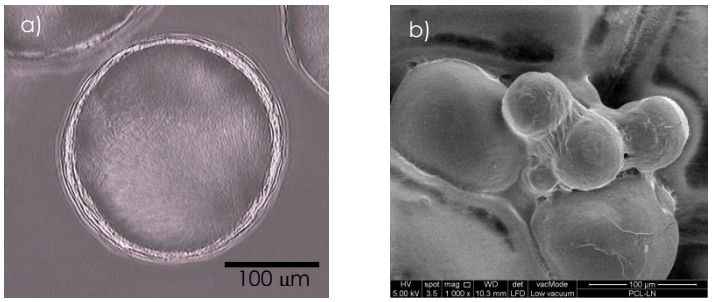


Figure 4 Micrograph of AFSC at 96 hours on gelatin coated PCL visualized using a) an inverted phase contrast microscope (100xamplification) Scale bar:100 μm. b) SEM image of AFSC on gelatin-coated PCL (1000xamplification) Scale bar: 100 μm

4.0 CONCLUSION

Based on the statistical optimization, the optimum conditions for maximizing the amount of gelatin immobilized (1797.33 µg/g) on the surface of PCL were as follows: ratio of COOH to EDAC of 1:1.5, NHS concentration of 10 mM, and gelatin concentration of 80 mg/ml. Amount of gelatin was increased by 82% on the optimized microcarrier as compared to the non-optimized microcarrier. Gelatin immobilisation on PCL microcarrier surface enhanced surface wettability and promotes higher cell adhesion and proliferation. The gelatin-coated microcarrier is particularly suitable for stem cells as compared to UV/O_3 PCL microcarrier and Raw PCL microcarrier.

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