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Synthesis, Characterization and Biological Studies of Novel Biodegradable Aconitic Acid Based Copolyester for Application in Skin Tissue Engineering

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Biodegradable polyester elastomers have gained a greater attention in the field of skin tissue engineering. A series of novel biodegradable polyesters are synthesized, based on non toxic monomers *e.g.*, aconitic acid, citric acid and 1,12-dodoecanediol, which are usually extracted from natural components. In the present work, a co-polyester poly (1,12-dodecanediol acotinate-co-1,12-dodecanediol citrate) (PACDDL) is synthesized by melt poly-condensation without any toxic catalyst. The chemical structure of the elastomers are then characterized by FT-IR, ^{1}H NMR and ^{13}C NMR. TGA, DSC techniques. The biological studies such as, *in vitro* cyto-compatibility, anticancer activity and CAM assay (angiogenesis) are examined. The physical properties exhibit that the elastomer is suitable for application in tissue engineering. The biological studies reveal that the polymer has excellent cell compatibility, making it suitable as potent biomaterial in skin tissue engineering.

Keywords: Aconitic acid, Elastomers, Biomedical application, Angiogenesis, Anticancer activity.

INTRODUCTION

Elastomers are long and highly flexible polymer network with cross-linking between them. The high mobility of these polymer chains, which can reconfigure on stress is attributed to the flexible chain length, whereas the elasticity and mechanical strength is attributed to the cross linking. The combinations of these properties make them suitable as biomaterials in medical fields [1]. Aliphatic polyester elastomers can mimic the tissues in our body, as they can be tailored to give a wide range of physiochemical, mechanical and degradative properties [2,3]. So the focus on synthesis, characterization and application of biodegradable polyester elastomers are drastically increasing and they have emerged as a vital class of biomaterials. These elastomers are relatively beneficial as they can be produced at a lower cost with controlled properties.

Many such elastomers have been synthesized in the past, such as poly(glycerol sebacate) [4,5], poly(1,8-octanediol citrate) [6], poly(ethylene glycol-co-citric acid) [7], poly-(polyol sebacate) [8], (citric acid-co-polycaprolactone-triol) [9], poly(aconitic-glycerol-cinnamicacid) [10], poly(diethylene glycol acotinate) [11], *etc.* Even though they are used widely as biomaterials, most of them require complex synthetic procedure, high temperature long post polymerizing time.

The monomers chosen play an important role for determining and controlling the functionality, degradability and biocompatibility of the biomaterials to be produced [12]. Many such elastomers based on multifunctional monomers such as citric acid have been reported [6,11-13]. Investigations on aconitic acid based monomers are comparative less and Kanitkar *et al.* [10] have synthesized and characterized aconitic acid based polyester. Yet, no study has been reported on aconitic acid based co-polyesters in combination with citric acid and aliphatic diol as co-monomers by catalyst free synthesis. Moreover, natural monomers such as, aconitic acid and citric acid, usually extracted from natural products such as baggase are endogenous to human metabolism [14,15] as they are intermediates in the Kreb's cycle and attempts a route to minimize the side effects, which owes to their biocompatibility and biodegradability. The aim of the present investigation is to synthesize a crosslinked polyester elastomer from non-toxic monomers, by a simple and cost effective method with good physiochemical and biological properties for biomedical applications.

EXPERIMENTAL

Synthesis of elastomers: Many synthesis reported before are complex and expensive, but the polyester poly(1,12-dodecanediolacotinate-co-1,12-dodecanediolcitrate) (PACDDL) is synthesized by a simple two step method by preparing multifunctional pre-polymer and then post-polymerizing it by thermal crosslinking. Equimolar amounts of aconitic acid, citric acid and 1,12-dodecanediol (0.01 mol:0.01 mol:0.03 mol) were placed in three-necked round-bottom flask and the monomer mixture was first heated upto 170-175 °C for 15 min till the monomer melts followed by heating at 140-145 °C for 2 h and 15 min under a constant stream of nitrogen without any catalyst [6,16]. The pre-polymers obtained were then dissolved in 1,4-dioxane (20 % w/w solution) to remove any unreacted monomer. The pre-polymer solution was then purified by dropwise precipitation in deionized water. The pre-polymers was again dissolved in 1,4-dioxane and then casted into a silicon petri dish and left at room temperature for 24 h. This petri dish is then placed in an air oven at 80 °C for post polymerization of the pre-polymers. The polymer was obtained in the form of a thin film.

The pre-polymers were studied for characterization by FT-IR, ^{1}H NMR, ^{13}C NMR and molecular weight was determined by MALDI-MS. The post polymers used thermal and biological studies.

FTIR spectra analysis: A Fourier transform infrared spectrum of the purified pre-polymer was recorded using FT-IR 4700 type A spectrometer. Pre-polymer samples were prepared by a solution casting technique over KBr crystals.

Nuclear magnetic resonance spectroscopy: ^{1}H NMR and ^{13}C NMR were recorded for the purified pre-polymer, dissolved in DMSO using 400 MHz solution Bruker Flex-PC microflex NMR spectrometer.

Thermal analysis: Thermogravimetric analysis (TGA) of the post polymer was performed in a range of room temperature to 500 °C in nitrogen atmosphere at a heating rate of 10 °C/min with a TGA instrument TGA Q50 V20.13 Build79. Differential scanning calorimetry was performed in a range of -70 °C to 350 °C at a heating rate of 10 °C/min using DSC Q200 V23 Build 79 instrument under nitrogen atmosphere.

Mass spectroscopy: The pre-polymer was dissolved in DMSO and the ionizing agent NaI was added and then placed on MALDI plate. The molecular weight of the pre-polymer was determined by BRUKER 1825 MALDI mass spectrometer.

***in vitro* Assay for cytotoxicity activity and anticancer activity: (MTT assay) [17]:** Vero cell lines were used to determine the cytotoxicity and A549 cell line (lung cancer cells) were used to determine the anticancer effect of the polymers.

Cells (1×10^5/well) were plated in 24-well plates and incubated in 37 °C with 5 % CO_2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24 h. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100 µL/well (5 mg/mL) of 0.5 % 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) was added and incubated for 4 h. After incubation, 1 mL of DMSO was added in all the wells. The absorbance at 570 nm was measured with UV-spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50 % inhibition (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{A_{570} \text{ of treated cells}}{A_{570} \text{ of control cells}} \times 100$$

Graphs are plotted using the % of cell viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments.

Chorion allaontoic membrane (CAM) assay: Synthesized compounds were dissolved in sterile phosphate buffer saline (PBS). For ease application, pellets of these solutions 100 µL/pellet (corresponds to 1000 µg/pellet) were prepared and applied drop wise on gelatine sponge and then on to chorioallantoic membrane. Seven day embryonated white leghorn chicken eggs were collected from a local hatchery and the eggs were cleaned with 70 % ethanol. A small window of 1 cm^{-2} was made in the shell of each egg. Then, air was sucked out from the eggs to bring their membrane down [18]. Through the window of each egg, sterile disc of gelatine sponge containing concentration (1000 µg/pellet) of methanol extracts were implanted inside the egg at the junction of two blood vessel of the chorioallontoic membrane. Subsequently, the opening was resealed with parafilm and the eggs were re-incubated at 37 °C for 72 h [19]. The windows were then reopened and formation/inhibition of vessels were observed in terms of number and caliber and finally compared with PBS used as control.

RESULTS AND DISCUSSION

Polymer characterization: The FT-IR spectra of the pre-polymer (Fig. 1) shows a strong absorption band at 1725 cm^{-1}, which attributes to the presence of carbonyl stretching of ester group. The absorption bands at 2926 and 2857 cm^{-1} were assigned to the methylene groups in the diol and acids [6,16]. The broad band at 3491 cm^{-1} attributes to the stretching vibrations of hydrogen bonded carboxyl and hydroxyl groups. The observed ^{1}H NMR chemical shift (Fig. 2) values shows a δ 3.71 ppm and δ 6.76 ppm attributes to the $–CH_2$ and –CH of the aconitic acid. The multiple peaks from δ 1.2-1.4 attributes to $–CH_2$ groups of diol. The peak at δ 3.7 ppm could be due to the $–OCH_2CH_2$- group of diol. The ^{13}C NMR confirms the formation of ester group by strong peaks at δ 167 ppm and δ 175 ppm. The absorption bands of FT-IR shows the formation of the ester linkage and the chemical shift values correlates with the proposed structure. The MALDI-MS spectra confirms the formation of the oligomer and the molecular weight of the pre-polymer at *m/e* 1825 was assignable to (TA_4-DDL_5-CA_2-Na). The MALDI-MS proves the incorporation of all the monomers used in the pre-polymer.

Thermal properties: The DSC and TGA analysis revealed that the elastomer is thermally stable as its T_m and T_d are 127.98 and 296 °C, respectively as shown in Figs. 3 and 4. The highly elastic nature of the synthesized elastomer is revealed from its lower T_g value of -40.67 °C. Thus it is evident that the cross-linking due to multifunctional groups decreases the rigidity

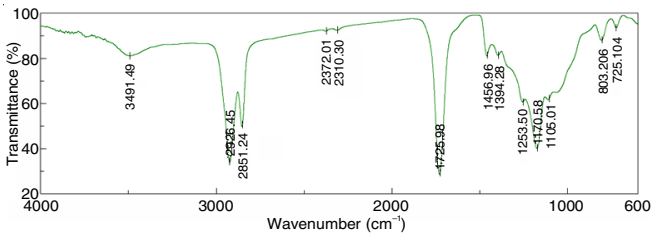

Fig. 1. FT-IR spectra of PACDDL

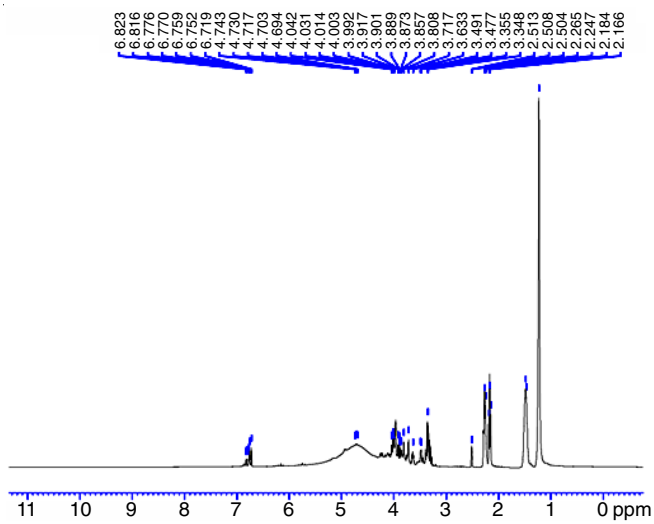

Fig. 2. ^{1}H NMR spectra of PACDDL

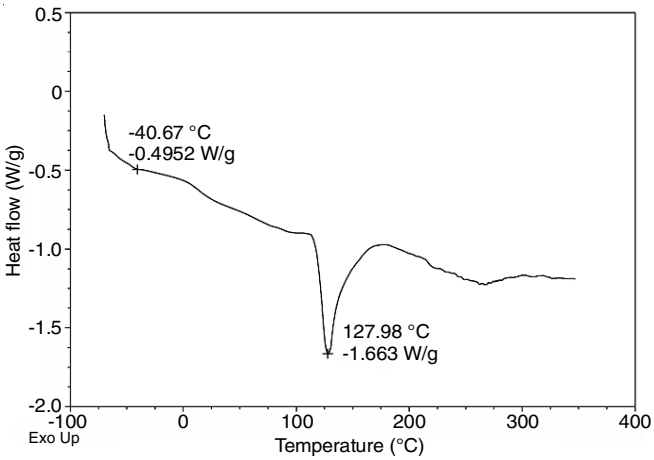

Fig. 3. DSC thermogram of PACDDL

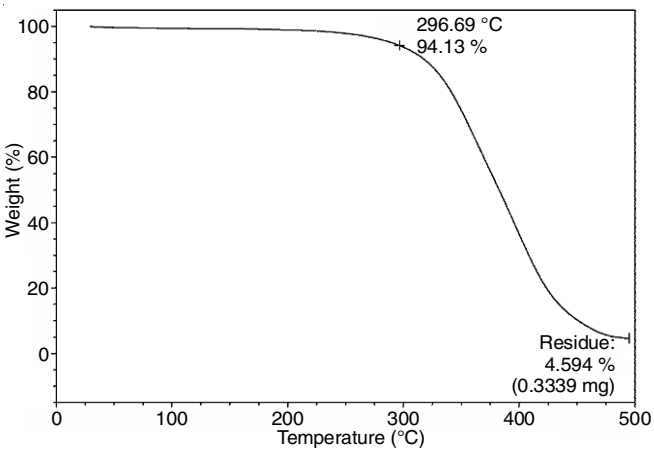

Fig. 4. TGA curve of PACDDL

of the polymer, which ultimately leads to low glass transition temperature. The elasticty of the polymer is suggested due to monomers such as aconitic acid and citric acid. The elastic nature and decomposition temperature much above the body temperature proves its suitability as an implant material.

Chorion allaontoic membrane (CAM) assay: The chorion allaontoic membrane assay shows the formation of the new blood vessels, which supports the wound healing process after the implantation of the biomaterial. After 72 h, endothelial cells became adherent and showed confluent cell layers formed on the synthesized polymer and also on the window of the egg. The number of branch points of the blood vessels formed on the PACDDL was found to be 200 % than the control of 100 % (increased by 2 folds) as shown in Fig. 5(A) & 5(B). This result shows the biocompatibility of polymer to well adhere to the surface of implanted site and also to mimic the extra-cellular matrix (ECM). This unique property is attributed to the non-toxic and natural monomers aconitic acid and citric acid.

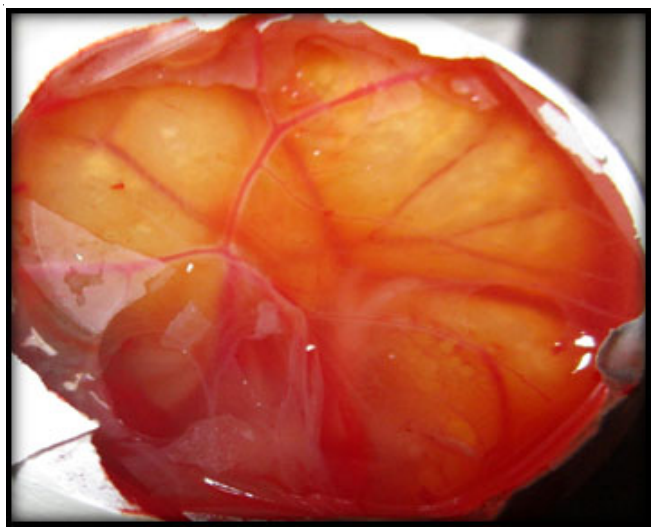

Fig. 5(A) CAM assay of the control showing the formation of blood vessels

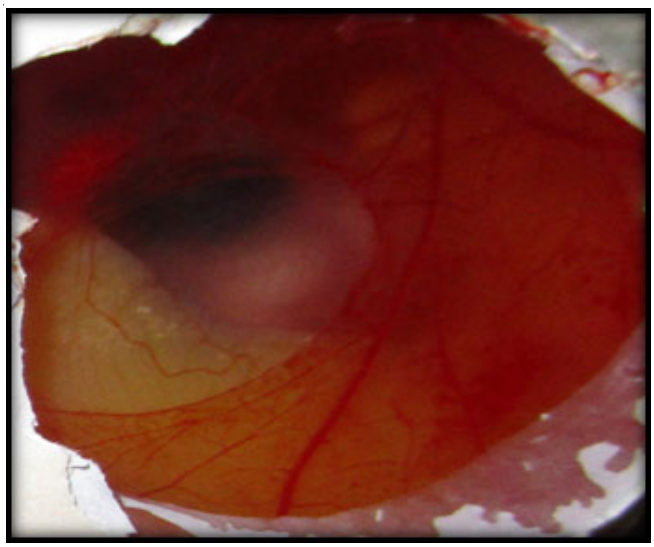

Fig. 5(B) CAM assay of the PACDDL showing the formation of blood vessels

Cytotoxicity and anitcancer activity of the co-polyester: Viable cells were determined by the absorbance. Concentration

required for a 50 % inhibition (IC_{50}) was determined graphically from the Fig. 6 for VERO cell line and Fig. 7 for cancer cell line. The effect of the PACDDL on the cell proliferation of VERO cell line and A549 cell line was expressed as cell viability. The affected cell lines at different concentration are shown in Figs. 6 and 7.

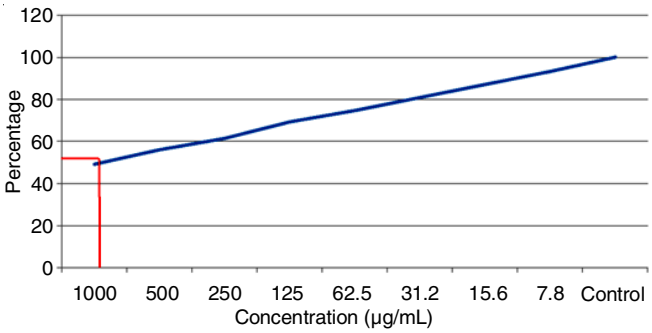

Fig. 6. Graphical representation of PACDDL on normal cell line

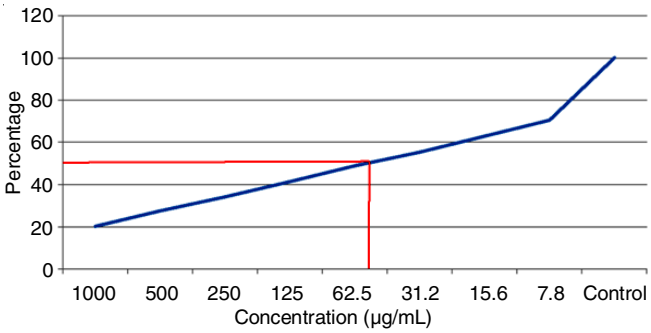

Fig. 7. Graphical representation of PACDDL on cancer cell line

It was found that the synthesized polyester against normal cell line shows the IC_{50} value at a concentration of 1000 µg/mL, which reveals that the PACDDL is toxic to normal cells only at a very high concentration (Table-1). On the other hand the polymer is toxic to the cancerous cell like A549 (lung cancer cell line) even at a low concentration of 62.5 µg/mL as in Table-2. This implies that the polyester is toxic to cancer cells even at a low concentration than the normal cells.

TABLE-1
CELL TOXICITY STUDIES ON NORMAL CELL LINE

S. No.	Conc. (µg/mL)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.405	49.15
2	500	1:1	0.462	56.06
3	250	1:2	0.506	61.40
4	125	1:4	0.571	69.29
5	62.5	1:8	0.615	74.63
6	31.2	1:16	0.666	80.82
7	15.6	1:32	0.717	87.01
8	7.8	1:64	0.768	93.20
9	Control	–	0.824	100

Conclusion

The polyester poly(1,12-dodoecanediol acotinate-co-1,12-dodoecanediol citrate) (PACDDL) was synthesized using melt poly-condensation without the use of toxic catalyst, followed by thermal curing. The structures were confirmed by FT-IR and NMR spectroscopy. The thermal properties reveal the thermal stability and the cross-linking in the elastomers. The CAM assay reveals the wound healing property by the formation of blood vessels without any haemorrhage. It is explicit from the cell selectivity assay and anticancer assay that the co-polyester is toxic to cancer cell lines at a low concentration, but is well tolerated by normal cell line even at a high concentration. These evidences show that the polyester PACDDL holds promising potential as a biomaterial in skin tissue engineering.

TABLE-2
ANTICANCER STUDIES ON CANCER CELL LINE

S. No.	Conc. (µg/mL)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.299	20.18
2	500	1:1	0.407	27.48
3	250	1:2	0.505	34.09
4	125	1:4	0.613	41.39
5	62.5	1:8	0.724	48.88
6	31.2	1:16	0.819	55.30
7	15.6	1:32	0.931	62.86
8	7.8	1:64	1.043	70.42
9	Control	–	1.481	100

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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