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In vitro biocompatibility evaluation of biscoumarin based random copolyesters

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ABSTRACT

Polymeric materials finds versatile applications in various biomedical disciplines but lacks compatibility issue and this may be attributed to the nature of polymeric material. Of more particular, the choice of suitable bioactive monomer incorporated into the polymer chain decides the compatibility of polymeric material. This motivated the generation of biscoumarin based copolyesters. In the present investigation a series of biscoumarin copolyesters (**CP1-CP7**) were evaluated for their *in vitro* haemocompatibility, cytotoxicity and antioxidant activities. The aim of this study was to review the results and assess the suitability of series of structurally divergent biscoumarin copolyesters as an operable biocompatible polymeric material in various medical disciplines. Haemolysis studies have been performed to check the haemocompatible of the copolyesters. 2,2,-diphenyl-1-picrylhydrazine (DPPH) radical scavenging assay was performed to evaluate the antioxidant activity of copolyester. (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliuum bromide-based (MTT) cytotoxicity assay was performed over normal Vero cell line to assess the cytocompatibility of the copolyesters. All the copolyesters exhibited low cytotoxicity even at higher concentration over Vero cell lines in an IC_{50} range of 290-463 µg/mL. Cancerous Hep-2 cell line was more sensitive to the cytotoxic activity of copolyester **CP5** compared to the normal Vero cells. The haemolysis results of the copolyesters were in the range of 4.47-8.95%. Curcumin coupled copolyester exhibited very good antioxidant activity, while the remaining exhibited low antioxidant activity. This may be attributed to the considerable structural-dependent DPPH radical scavenging activity. The outcomes of these assessments manifest the importance of biscoumarin copolyesters as suitable biocompatible material.

Keywords: Biscoumarin copolyester, Antioxidant, Haemolysis, Cytotoxicity, Vero cell line.

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1. Introduction

A probe through the literature reveals that recently most of the researchers focused more on the polymeric materials by tuning its properties either by synthetic or by processing approach based on its applications in various disciplines. Of more particular, it exhibit wide range of application in biomedical disciplines. Polymeric materials are being investigated in developing various surgical devices such as synthetic skin, cartilage, vascular nerve, heart valve and synthetic blood vessel. It can also be used extensively in the field of orthopaedic medicine[1] as screws, wires, plates, rods and pins to provide mechanical support for the bone treatments. The development and application of polymeric material in the field of drug delivery[2,3] play a vital role in the treatment of many diseases. In a system of drug delivery, polymeric materials are designed in such a way, that it has to release the bioactive agents in erosion or diffusion controlled process or the combination of both. By modulated the matrix parameter of polymeric material the release characteristics of bioactive agents can be achieved. Nowadays metallic implants have been replaced by polymeric surgical implants. Issues associated with metallic implants include incomplete healing due to stress shielding and need for further surgery to remove the implant material.

Surgical implantation of biomaterials often leads to detrimental side effect due to host response that reflects the first step of tissue repair. In general host reactions after implantation of biomaterial materials include foreign body reaction, blood material interaction, fibrosis [4], acute inflammation and chronic inflammation. Major responses of host tissue to biomaterial deserve attention such as carcinogenicity, cytotoxicity, oxidative stress and coagulation. On considering the compatibility of biomaterials the first and best requirement was cytocompatible and haemocompatible. Along with it should also exhibit antioxidant property because oxidative stress may arise due to the incompatibility of biomaterials.

The biocompatibility of implant material in the human body is associated with interaction with living cells and biomaterial. The haemocompatibility [5-12] of biomaterial refers to the degree of mutual adaptation between materials and blood components. The major i*n vitro* haemocompatibility experiment should include effect on haemolysis caused by biomaterials. Since RBCs (Red blood cells) are the major component of blood system, osmotic fragility of RBC membrane has to be investigated by haemolysis method. The cytotoxicity [13-17] of implants over normal cells is another important criterion to fulfil the requirement of biomaterials.

Sometimes incompatibility of polymeric biomaterial may leads to oxidative stress [18]. The antioxidant defence mechanism with excess reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced during oxidative stress may lead to imbalanced redox state. Surgical injury during the implantation of biomaterial brings the biomaterial in contact with blood and activates wound healing process. Oxidative stress plays a vital role in wound healing process. The excess generation of free radicals and other reactive species in the vicinity of implantation leads to imbalanced redox state and ultimately results to prolonged inflammation phase. The heterogeneous media of RBCs are specifically exposed to endogenous oxidative damage because of their vital role as oxygen carrier. RBCs enriched with polyunsaturated fatty acid are susceptible to free radical mediated peroxidation.

Of particular interest synthetic copolyester exhibits an attractive avenue for biocompatible biomaterials because of their modifiable properties. [19, 20] Biocompatible polymers [21, 22] can be made into implantable material of desired shape or it can be incorporated in to the body as coated medical devices. Often polymer implants leads to biofilm formation due to the microbial adhesion on the surface of implants. Sometime long-term inflammation leads to dysplasia.

In this area of research most of the reported polymeric materials are more specific in enhancing either one of the biocompatible property and that may be either cytocompatible or haemocompatible. By considering the above facts it is essential to develop a polymeric biomaterial possessing haemocompatible, cytocompatible and antioxidant [23-29] properties along with antimicrobial and anticancer properties. Degradative properties can be achieved by the incorporation of aliphatic carboxyl flexible spacers. Selection of suitable aromatic monomer is an important criterion in deciding the biocompatible properties of polymer. The choice of suitable bioactive monomer is important in deciding the properties of polymers. In this work dicoumarol was preferred as suitable monomer, since it is a very good anticoagulant; it was expected to exhibit haemocompatible properties once it gets incorporated into the polymer chain. In general materials with high hydrophobicity exhibit good anticoagulation due to low surface energy, resulting in minimal interaction with blood components[30]. As per occupational safety health administration (OSHA) coumarin was considered as non-carcinogen for humans. In the earlier work it was found to exhibit anticancer activity. Hence it was expected to exhibit cytocompatible property over normal cells. Coumarin and its analogues are well known for its wide spectrum of biological activities including anticoagulant, antimicrobial, analgesic, antibacterial, antifungal, anti-

inflammatory and antitumor. Of particular interest 3,3'-methylene-bis(4-hydroxycoumarin) exhibits anticoagulant, anti-proliferative activity with less toxicity. Considering the importance of 3,3'-methylene-bis(4-hydroxycoumarin) in polymer synthesis. The objective of our present work was to investigate haemocompatible, cytocompatible and antioxidant properties of biscoumarin copolyesters.

Copolyester derived from the random synthesis of 3,3'-methylene-bis(4-hydroxycoumarin), aliphatic diacid chloride (spacer) such as sebacoyl chloride and aromatic diols such as hydroquinone, resorcinol, ethyl resorcinol, bisphenol and curcumin respectively, were prepared and their physical characterisation, *in vitro* antimicrobial and anticancer activity over Hep-2 cell line were reported in the earlier work. [31]

2. Experimental

2.1 General synthetic details

The experimental details regarding the preparation of copolyester have been reported earlier.[31] All the reagents and chemicals were purchased from Sigma-Aldrich and used without further purification. Synthesis of copolyester were achieved *via* phase-transfer-catalysed interfacial polycondensation of 3,3'-methylene-bis(4-hydroxycoumarin) with aromatic diols such as hydroquinone, resorcinol, ethyl resorcinol, bisphenol-A and curcumin through acid dichloride with varying methylene spacer as illustrated in Table 1 and the synthetic scheme is depicted in Figure 1

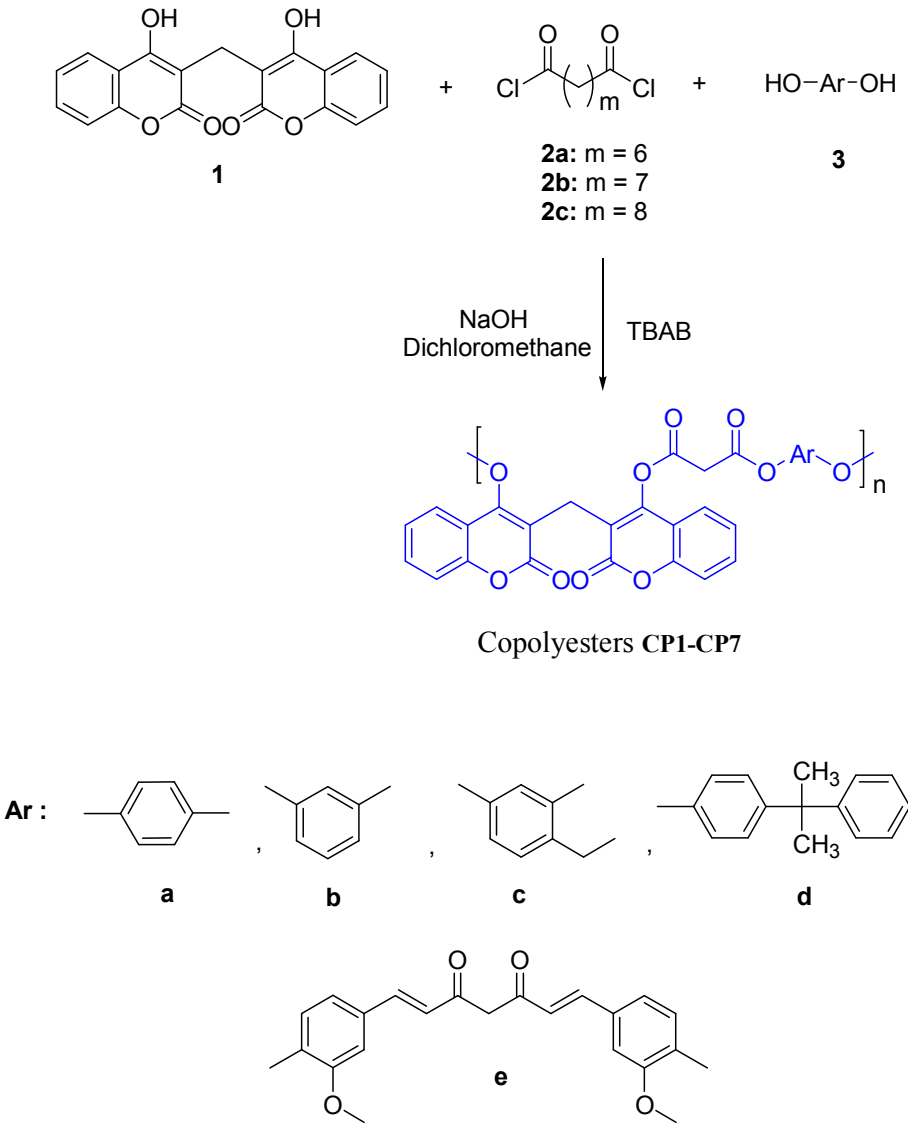
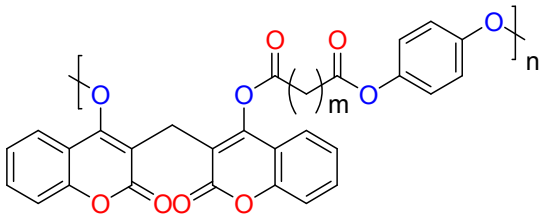
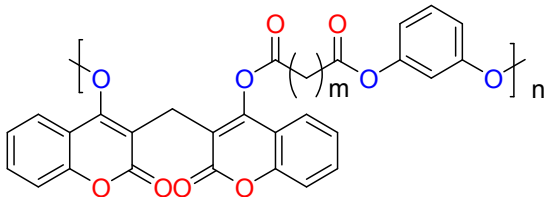
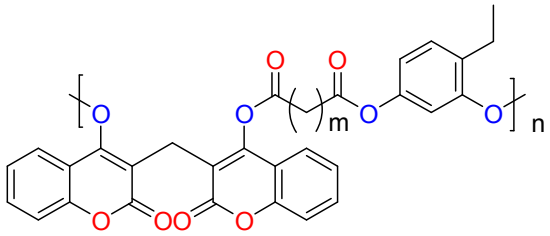
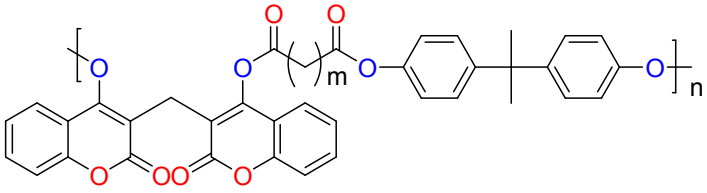
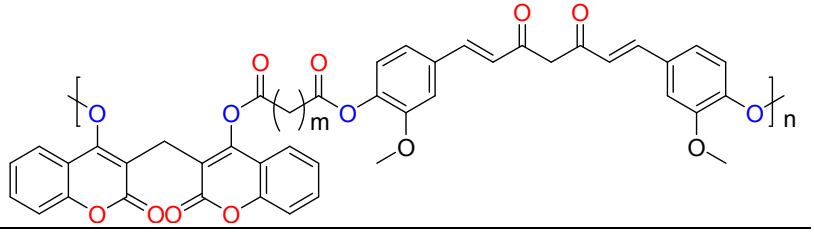


Figure 1 Schematic of the synthetic procedure for biscoumarin based copolyester

Table 1 Structure of biscoumarin copolyesters synthesised with data of comonomers used

Polymer code [a]	Aromatic diol	Acid dichloride	Polymer structure
CP1	3a	2c	
CP2	3b	2c	
CP3	3c	2c	
CP4	3d	2c	
CP5	3e	2c	
CP6	3e	2b	
CP7	3e	2a	

[a] Copolyester CP5-CP7 have same aromatic unit (3e) with difference in chain length of methylene spacer.

2.2 Outline of Copolyester characterisation techniques

Structures of the copolyesters were confirmed using NMR spectroscopy (Bruker 400 MHz spectrometer) with tetramethylsilane as internal standard. About 10 and 50 mg of sample were dissolved in 1mL of $CDCl_3$ were used for ^{1}H and ^{13}C NMR respectively. FTIR measurements were carried out using Perkin Elmer 883 spectrophotometer. The solubility behaviour of all the random copolyesters was tested qualitatively in chlorinated and polar aprotic solvents. The inherent viscosities of the copolyesters were determined in NMP at 30 ^{o}C at the concentration of 0.5 g/dL using an Ubbelohde suspended level viscometer. From GPC analysis the number average molecular weight (M_n) and weight average molecular weight (M_w) and polydispersity index (PDI) of polymers were determined by a WATERS 501 gel permeation chromatograph equipped with differential refractive index detector and 3 ultra styra gel column. The thermal properties of copolyesters were investigated by thermogravimetric analysis (TGA) at a heating rate of 10 $^{o}C\ min^{-1}$ in nitrogen atmosphere. Glass transition temperature (T_g) of random copolyesters was determined by differential scanning calorimetry (DSC) at a heating rate of 10 $^{o}C\ min^{-1}$ under nitrogen atmosphere. Wide angle X-ray diffractogram of polymer samples were obtained using Bruker XRD D8 FOCUS instrument operating with CuKα radiation source (λ=1.5405980 Å) at 30 kV and 15 mA. Samples were scanned at the bragg angle (θ) range of 2θ = 5-80^{o}.

2.3 Haemocompatibility

The tests here are mainly aimed in finding the extent of haemolysis. The haemolysis percentage is defined as

$$haemolysis(\%) = \frac{\mathbf{OD(test) - OD(negative)}}{\mathbf{OD(positive) - OD(negative)}} * 100$$

Where optical density (OD) is the light absorbency and is given by log ($I0/I$), where $I0$ and I are the original and transmitted light intensities.

For this test, blood samples were collected in a beaker containing sodium citrate in the proportion of 3.8 g of sodium citrate per 100 ml of blood to avoid coagulation. The anti-coagulated blood was then diluted with N-saline in the proportion of 8:10. For checking the haemolysis 0.2 ml of diluted blood was added to 10 ml of 1% sodium carbonate solution and incubated for 60 min at 37 °C. The OD of the incubated solution was measured in an UV spectrometer at 545 nm wavelengths.

Since sodium carbonate was known to cause large-scale rupture of RBC the OD count of this is taken as positive control referred to as OD (positive). Similarly, for negative control 0.2 ml of diluted blood was added to 10 ml of N-saline and again this was incubated for 60 min at 37 °C. The OD of this solution was found again in an UV spectrometer at 545 nm wavelength and the OD is referred to as OD (negative). The reason for adding N-saline solution for negative control test is that this is known to cause the least RBC rupture.

10 mg of sample was taken in a standard test tube containing N-saline and incubated at 37 °C for 30 min for providing temperature equilibrium. 0.2 ml of diluted blood was then added to the test tube, mixed gently and incubated for 60 min. OD of the sample is then obtained. This process was referred to as OD (test). The blood samples used above were of a goat and were obtained from tamilnadu veterinary and animal science university by life teck research centre in which the haemocompatibility studies were carried out. If the haemolysis percentage is less than 10, the test material is taken as haemocompatible and if it is less than 5, the material is highly haemocompatible. Three replicates were performed for each copolyesters and the data are expressed in mean ± standard deviation.

2.4 Cytocompatibility

2.4.1 Cell line and culture:

VERO cell lines were obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in Minimal Essential Medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

2.4.2 Reagents:

DMEM was purchased from Hi Media Laboratories Fetal Bovine Serum (FBS) was purchased from Cistron laboratories Trypsin, methylthiazolyl diphenyl- tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals Mumbai). All of other chemicals and reagents were obtained from Sigma Aldrich Mumbai.

2.4.3 In Vitro assay for Cytotoxicity activity (MTT assay)

Cells (1×10^5/well) were plated in 24-well plates and incubated in 37 ^oC 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 MEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT)[32,33]

was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570nm 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:

% Cell viability = A570 of treated Cells / A570 of control Cells × 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.

2.5 Antioxidant

The effect of given samples on DPPH radical was estimated according to the procedure described by Von Gadow et al.[34] Two mL of 6×10^{-5} M methonolic solution of DPPH was added to 50 µl of a methonolic solution (10 mg ml-1) of the sample. Absorbance measurements commenced immediately. The decrease of absorbance at 515 nm was continuously recorded in a spectrophotometer for 16 min at room temperature. A methanolic solution of pure compound [quercetin] was tested at 1 mg/ml concentration. The scavenging effect (decrease of absorbance at 515 nm) was plotted against the time and the percentage of DPPH radical scavenging ability of the sample was calculated from the absorbance value at the end of 16 min duration as follows: All determinations were performed in triplicate and the data are expressed in mean ± standard deviation. The percentage inhibition of the DPPH radical by the samples was calculated according to the formula of Yen and Duh.[35]

$$IP = [(AC(0)- AA(t)/ AC(0))] \times 100$$

Where AC(0) is the absorbance of the control at t = 0 min; and AA(t) is the absorbance of the antioxidants at t = 16 min.

3. Results and discussion

3.1 Synthesis and Characterisation

Biscoumarin based copolyester synthetic scheme are depicted in Figure 1. A series of aliphatic-aromatic random copolyesters (**CP1-CP7**) bearing biscoumarin group were

synthesized by condensation of bioactive biscoumarin unit with aromatic diol and aliphatic acid dichloride in the respective mole ratio (1:1:2) by using DCM-H_2O as interphase, alkali as an acid acceptor and TBAB as phase transfer catalyst with 90-95% yield along with spectral data are depicted in our previously reported study[31]. The formation of ester group and the incorporation of 3,3'-methylene-bis(4-hydroxycoumarin) were confirmed by their characteristic absorption band at 1722-1725 cm^{-1} and 1648cm^{-1} respectively. The peak at 3.85ppm corresponds to the bridged carbon bearing two protons from the coumarin units. Thus confirming, the incorporation of biscoumarin moiety, effectively get involved in polymerisation. The signal characterising the methylene groups of aliphatic unit appears at 2.39-1.27ppm. 1.15 and 1.07 are the polydispersity index value for the copolyester **3a** and **3d** respectively. The initial decomposition temperature of all the copolyesters ranged from 221-263 ^oC and the T_{10} values were in the range of 259-292 ^oC and the glass transition temperature was in the range of -11 to 54 ^oC. The incorporation of bulky or the use of bent monomers such as 3,3'-methylene-bis(4-hydroxycoumarin) **1** containing polar carbonyl groups along with the insertion of of meta oriented aromatic units **3b** and **3c** through the flexible spacer in the polymer chain, tends to reduce the interaction between polymer chains and eventually leads to increase in free volume and solubility with enhance processability due to low glass transition temperature and also retaining its thermal stability. X-ray diffraction studies revealed the amorphous character of the copolyester with some degree of crystallinity at the region of $2\theta \sim 17.5^0$ and 20.1^0 and this may be attributed to the flexible methylene spacer.

3.2 Haemolysis

Haemolysis is regarded as significant screening test in deciding the compatibility of copolyesters. Higher the degree of haemolysis leads to extensive rupture of red blood cells and the compatibility towards blood decreases. It is known from the literature that the haemolysis value for biomaterials used in various medical disciplines should be less than 5%.[36] The results obtained from the haemolytic assay for the copolyesters **CP1-CP7** are highlighted in Figure 2, showing the measured extend of haemolytic index verses the respective copolyesters and also the percentage values of haemolysis are displayed in Table 2. Almost all the biscoumarin copolyesters exhibit extensive anti-haemolytic activity (< 10% haemolysis). In general coumarin and its analogues exhibited very good anti-haemolytic

activity. Of all, curcumin coupled random biscoumarin copolyesters exhibited good anti-haemolytic activity.

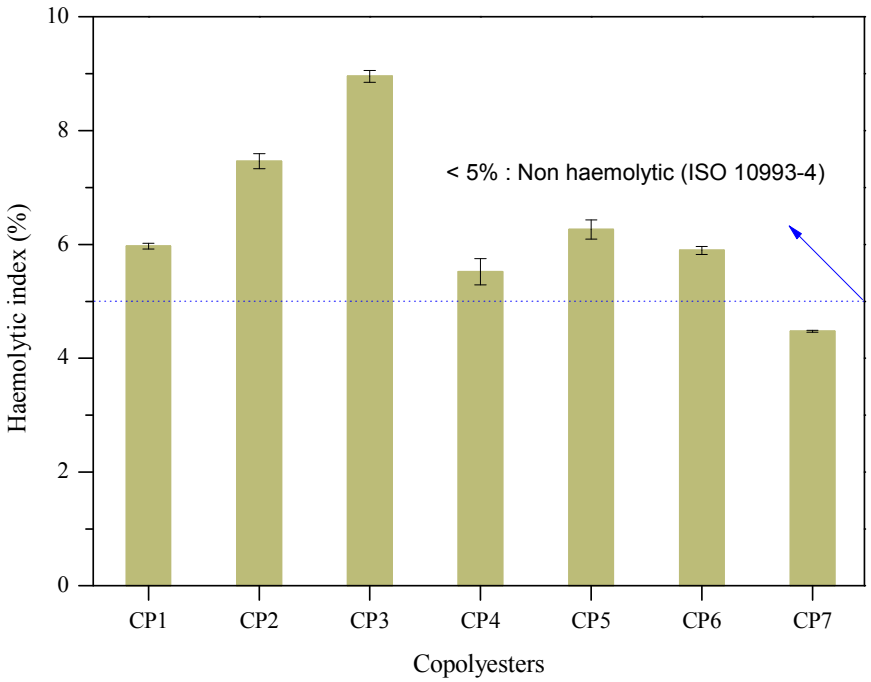


Figure 2. Haemolytic index vs biscoumarin copolyesters (**CP1-CP7**)

The heterogeneous mediums of red blood cells (RBCs) are unmasked to endogenous oxidative damage because of their distinct role as oxygen carriers.[9] RBCs are enriched with polyunsaturated fatty acids which are sensible to free radical-mediated peroxidation. Since the lipid peroxidation involves free radical chain reactions, the RBC membrane immediately get rupture and ultimately leads to haemolysis. On the other hand the incorporation of curcumin, a very good antioxidant would react in the free radical chain propagating stage and terminate the peroxidation process, consequently inhibit haemolysis.

The hydrophobicity of methylene repeating units in the copolyesters was found to exhibit pronounced effect on the haemolytic activity. And it is explicit from the copolyesters **CP5**, **CP6** and **CP7**. Thus the anti-haemolytic activity of these copolyesters on the basis of hydrophobicity (flexible spacer chain length) could be arranged as follows: **CP7** > **CP6** > **CP5**. The incorporation of hydrophobic group on the repeating unit also contributes considerable effects on the haemolytic activity of the copolyester. Copolyester **CP2** found to exhibit lesser haemolytic effect in comparison with copolyester **CP3** as this may be attributed to the presence of hydrophobic ethyl unit. Thus overall increase in hydrophobicity was in

accordance with the reported literature[37] that appraise, increase in hydrophobicity exhibit strong interaction with the cell membrane leading to loss of selectivity. The regulation of protein adsorption on the polymeric surface plays a vital role in deciding the long term blood compatibility of polymeric material. From the X-ray diffraction studies it is evident that the methylene spacer influences the hydrophobicity of the polymer which in turn influences the haemolysis of RBCs and it is clear from the degree of crystallinity observed in the region of $2\theta \sim 17.5^0$ and 20.1^0 respectively.

Table 2 Data of haemolysis and antioxidant activity[a]

Polymer code	Haemolysis[b] (%)	DPPH scavenging activity (%)[c]
CP1	5.97±0.052	28.93 ± 0.35
CP2	7.46±0.132	8.01 ± 0.37
CP3	8.95±0.100	26.01 ± 0.23
CP4	5.52±0.228	5.99 ± 0.46
CP5	6.26±0.170	87.02 ± 0.66
CP6	5.89±0.070	-
CP7	4.47±0.020	-

[a] Data are presented as mean ± SD
[b] Less than 5% was consider to be non haemolytic as per ISO 10993-4
[c] 99.92% is the DPPH scavenging activity of Quercetin standard

3.3 Cytotoxicity:

In vitro cytotoxicity studies were performed on copolyester samples by the use of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT)[32, 33]. Since MTT is a vital dye for the assessment of cell viability. Vero cells were employed for cytotoxicity evaluation. The results of cytotoxic activity were summarised as IC_{50} (μg/mL) of compounds in Table 3. The IC_{50} values of the copolyesters reveal low cytotoxic activity (IC_{50} = 290-463 μg/mL) against normal Vero cell line. It is evident from Table 3 that all biscoumarin copolyesters have an

effect on the viability of the vero cells at higher concentrations with IC_{50} values greater than 290μg/mL. Of all the copolyesters, the copolyesters **CP5** and **CP7** exhibited low cytotoxic even at higher concentrations. **CP6** was not investigated for cytotoxicity since it contain six carbon methylene units and it was propose to exhibit cytotoxicity value in between **CP5** and **CP7**. Generally curcumin and coumarin pharmacore selectively induces apoptosis in highly proliferating cells and more death is pronounced in tumor cells than normal cells.[38, 39] From the IC_{50} values it is explicit that adding hydrocarbon side chain such as ethyl group from ethyl resorcinol increases cytotoxicity at higher polymer concentration.

The effect of copolyester **CP5** on the viability of Vero and Hep-2 cell were investigated by MTT assay. It is evident from Figure 3 that the copolyester **CP5** exhibited differential effect on both normal and cancerous cell line. The IC_{50} value of Hep-2 cell and vero cell line was found to be 34 and 463 μg/mL. These data indicates that the copolyester **CP5** had higher cytotoxic activity towards cancerous Hep-2 cell at lower concentration and had relatively lower cytotoxic activity with normal cell even at higher concentration. This may be due to the sensitivity of tumor cells over curcumin unit coupled in the copolyester than normal cells. This sensitivity towards tumor cell may be attributed to the difference in membrane structure, protein composition and oversized against normal cells. A probe through the literature have shown that α,β-unsaturated carbonyl compounds such as conjugated dienones enable a Michael addition of intercellular thiol compounds such as glutathione to the olefinic double bond.[40-43] Since the concentration of glutathione is very low in blood, but sufficiently high in cancer tissue the copolyesters can persist in blood but quickly inhibit glutathione-S-transferase, which intensify the cytotoxicity of these copolyesters. The pH of extracellular fluid of tumor has been found to be 6.81±0.09 on average with lowest value of 5.55,[44,45] at this condition the copolyester **CP5** would be expected to be stable in intercellular fluid of normal tissue and in blood but hydrolysed in acidic extracellular fluid of cancer tissue and in this way copolyester **CP5** improve its efficacy over cancer cell and thus lower its cytotoxicity over normal Vero cells. In addition to the aforementioned structural aspect responsible for the cell viability towards both Hep-2 cell and vero cell line, polymer used as stent coatings must also be cytocompatible and generally to fulfil the requirement of cytocompatibility the polymer used as stent coating must be amorphous and have glass transition points below body temperature. Copolyesters CP1-CP5 exhibit low glass transition temperature[31] and amorphous behaviour exhibited by Xray diffraction studies. The amorphous nature of copolyester CP5 exhibited by XRD was

responsible for cytotoxicity towards Hep-2 cell lines and this may be attributed to the fact, that amorphous material are more sensible towards acidic condition.

Table 3 *In vitro* cytotoxic activity of synthesised copolyesters by MTT assay

Compound	Viability % Sample concentration (µg/mL)									IC_{50}(µg/mL)[a] VERO[b]
	1000	500	250	125	62.5	31.2	15.6	7.8	Cell control	
CP1	37.28	45.76	50.84	54.23	59.32	61.02	64.40	69.49	100	290
CP3	35.50	45.76	52.54	55.93	59.32	62.71	66.10	71.18	100	339
CP4	38.98	45.76	52.54	55.93	62.71	64.40	67.79	71.18	100	349
CP5	45.76	49.15	54.24	57.62	62.71	66.10	67.79	69.49	100	463
CP7	40.68	47.46	54.23	55.93	59.32	62.71	66.10	69.49	100	408

[a] "IC_{50}, The concentration that causes a 50% reduction of cell growth"
[b] Normal cell line (VERO)

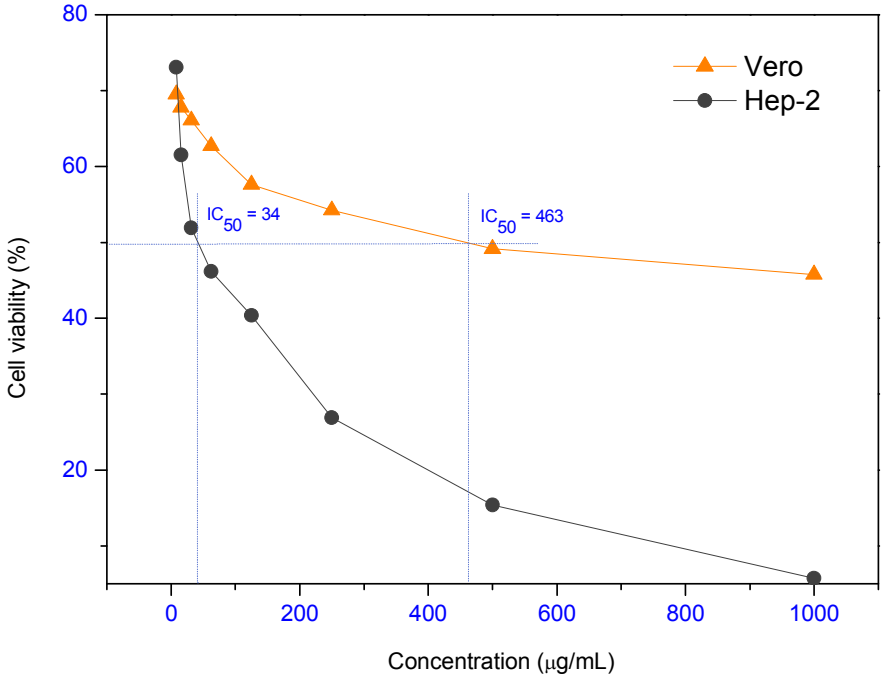


Figure 3. The inhibitory effect of copolyester CP5 concentration on Vero cell and Hep-2 cell lines

The low cytotoxicity exhibited by the copolyesters (**CP1**, **CP3-CP5** and **CP7**) when treated with normal cells supports that the prepared copolyesters are biocompatible and can be employed for various biomedical disciplines. The comparative graph of haemocomptible in percentage verses cytocompatible over normal vero cell line on the basis of IC_{50} value was shown in Figure 4. It clearly shows the significance of curcumin unit in the biscoumarin copolyesters. Of particular interest copolyester **CP5** and **CP7** with curcumin unit can be preferred more for further investigation due to its extensive biocompatible property.

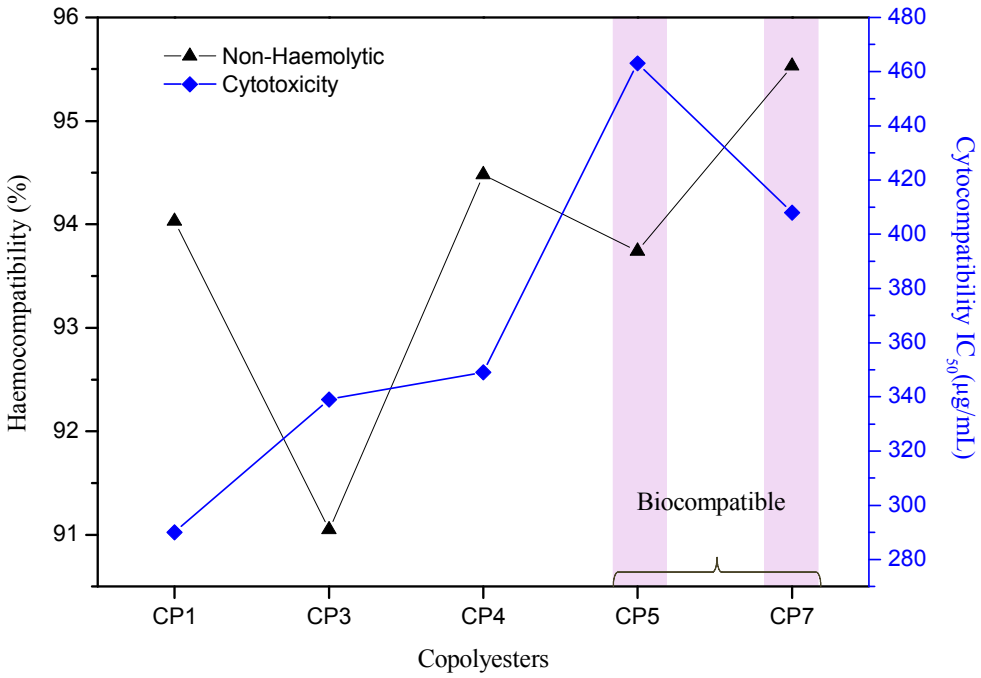


Figure 4. Comparative graph of Haemocompatible and Cytocompatible copolyesters (**CP1, CP3-CP5, CP7**). Copolyesters that exhibt moderate to high biocompatibility are highlighted in purple stripes (**CP5**, **CP7**).

3.4 Antioxidant activity

Oxidative stress plays a vital role in limiting the biocompatibility of many biomaterials due to inflammation, chronic diseases and also contributes to other complications that may cause the failure of biomaterial implant. Designing a polymeric material bearing antioxidant properties is therefore a potential avenue to improve the biocompatibility of polymers. Inclusion of antioxidant in to the polymer backbone structure has high relative antioxidant content and

may provide prolonged, continuous attenuation of oxidative stress while the polymer or its degradation products are present.

DPPH assay has been widely used to assess radical-scavenging ability of polymeric material under investigation. DPPH is relatively stable nitrogen radical and it is reduced to its corresponding hydrazine when reacts with polymeric material bearing any hydrogen donor. Copolyesters from **CP1-CP5** have been discussed for antioxidant study. From the results it is explicit that the biscoumarin copolyester **CP5** coupled with curcumin through flexible spacer exhibit intense antioxidant activity equivalent with standard quercetin and it is shown in Figure 5. The results of DPPH radical-scavenging ability along with haemolysis percentage of copolyester are summarised in Table 2.

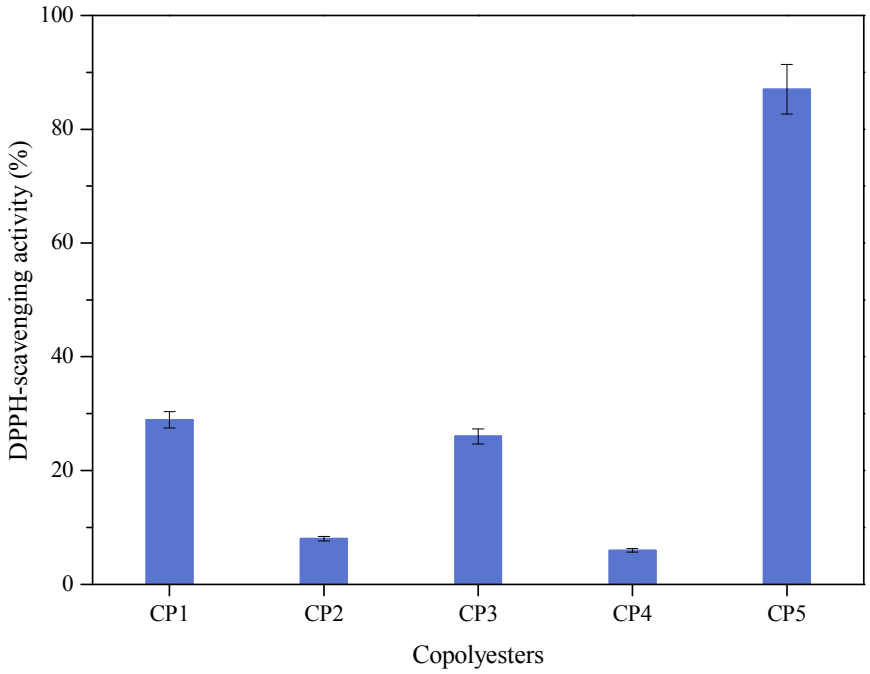


Figure 5. DPPH radical scavenging profile of copolyester (**CP1-CP5**)

In general the 1,3-diketone and the substituents on the phenolic side chain of curcumin are the important structural features that contributes to its antioxidant properties. As per the computational result enol form of curcumin was found to be more stable than keto form. Its unique enol form of diketone may have a typical radical trapping ability and a chain breaking antioxidant activity. The enol form of curcumin incorporated in the polymer was also reflected in the ^{1}H-NMR spectrum.[31] Another possible observable reaction is oxidation of methylene proton (CH_2) which may lose proton subsequently and produce carbon centred radical.[46] The enol and methylene group are the two possible sites of radical generation which

leads to enhanced antioxidant activity of curcumin coupled copolyester **CP5**. Other than **CP5**, all the copolyesters exhibited low antioxidant activity. Copolyester **CP6** and **CP7** are not investigated for antioxidant study. Since copolyester **CP5** was found to be cytocompatible and moreover the curcumin moiety in the polymer chain plays a vital role in deciding the antioxidant activity. Variations in methylene unit by one or two carbon atom also propose to exhibit similar antioxidant activity.

4. Conclusions

The present study clearly explains the importance of biscoumarin based copolyesters possessing dual haemocompatible and cytocompatible activites. This was further corroborated by the haemolysis studies which clearly highlighted that the presence of flexible spacer chain length plays a vital role in haemolysis process and further the presence of curcumin manifest its importance in deciding the haemolytic activity of copolyesters. Among the tested polymers, copolyester **CP7** displayed very low haemolytic activity as per ISO 10993-4. While the remaining copolyesters such as **CP1**, **CP4** and **CP6** exhibits marginal haemolytic activity. The cytocompatibility studies also reveal biscoumarin coupled curcumin copolyester such as **CP5** are found to exhibit low cytotoxic even at higher concentration. Further the cytotoxicity of copolyester **CP5** was investigated over cancerous cell (Hep-2 cell line) and normal cell (Vero cell line) and it was conferred that 13 times higher concentration of copolyester **CP5** was required to exhibit IC_{50} (μg/mL) value of 463 over normal Vero cell line in comparison with Hep-2 cell line. Overall copolyesters **CP5** and **CP7** are proposed to be biocompatible polymers and it was subjected for further investigations. The antioxidant study (DPPH radical scavenging) also signifies the importance of biscoumarin copolyesters **CP5** as lead scaffold to sketch the development of novel antioxidant polymers.

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Graphical abstract:

Copolyester **CP5** exhibits cytocompatible property over normal cell line (Vero cells) and it required 13 times higher concentration in comparison with Hep-2 cells.