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Hyperbranched Poly(citric acid) and Its Application as Anticancer Drug Delivery System

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ABSTRACT: As citric acid decomposes in its melting point, polycondensation of this monomer to prepare its polymers and copolymers is not possible. This study describes a feasible way to synthesize hyperbranched polyesters consisting of citric acid and glycerol monomers. In the first strategy, different ratios of citric acid and glycerol as AB_3 and A_3 monomers were mixed and heated step by step to avoid decomposition of citric acid. The produced water, as byproduct of polycondensation, was removed from the mixture by vacuum pump. Synthesized hyperbranched copolymers were characterized by nuclear magnetic resonance, gel permeation chromatography, thermogravimetric analysis, and dynamic light scattering. Ability of the synthesized hyperbranched polyesters to load and transfer cisplatin as an anticancer drug was investigated and it was found that their loading capacity is high and the prepared drug delivery systems are stable in saline buffer for several months. The toxicity of anticancer drug delivery systems against C26 cancer cell line was evaluated. Cisplatin loaded in polyesters showed lower IC_{50} value than the free cisplatin, confirming the efficacy of the synthesized citric acid–glycerol hyperbranched polyesters as biocompatible cargos to transport anticancer drugs. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2013

KEYWORDS: biocompatibility; dendrimers; hyperbranched polymers and macrocycles; drug delivery systems

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INTRODUCTION

A wonderful and well-known case study in the growth of science is found in the birth of polymer chemistry in the early 20th Century.[1,2] A new class of polymers is dendritic polymers which include three subgroups namely (a) random hyperbranched polymers, (b) dendrigraft polymers, and (c) dendrimers.

Dendritic polymers have excellent chemical and physical properties compared to other types of polymers. They are a relatively young class of polymers but well-established body of interdisciplinary research, exploring a remarkable variety of potential applications.[3] Dendrimers and hyperbranched polymers are characterized by highly branched structures, large numbers of end functional groups, low intrinsic viscosities, and very high solubility.[4–9] The low intrinsic viscosity is attributed to their packed structure, whereas the high solubility is assigned to the large number of end functional groups available per macromolecule.[5]

Dendrimers have a wide range of applications such as drug transport,[10] gene transport,[11] high-loading supports for organic synthesis,[12] water purification systems,[13] and molecular nanocarriers.[14] However large-scale use of dendrimers is restricted because of their labor-intensive synthesis and the resulting limited availability in bulk quantities.[15] Hyperbranched polymers, unlike dendrimers, are relatively inexpensive to produce and are easy to synthesize in large quantities through simple methods that do not need the tedious isolation and purification procedures.[5,6]

Therefore, hyperbranched polymers as a unique type of dendritic polymers can be an attractive alternative to dendrimers, because they possess both interesting properties of the dendritic structures and also feasibility for large-scale manufactures.[6]

This study explains the synthesis of the hyperbranched polyesters based on citric acid (CA) as AB_3 monomer and glycerol (G) as A_3 monomer by step-by-step thermal polycondensation.

CA is a cheap and biocompatible compound that is used on large scale in the food and drug industries.[16] On the other hand, glycerol is a key component in the synthesis of phospholipids.[17–19] Therefore, one can suggest that polymers or copolymers based on

Additional Supporting Information may be found in the online version of this article.

CA and glycerol should be biocompatible materials and possess unique properties.

Except CA–polyethylene glycol–CA ABA-type linear–dendritic copolymers which were synthesized by our groups previously[20] and a crosslinked copolymer,[21] there are no reports for producing of hyperbranched polyesters based on CA and glycerol monomers.

To prove the efficacy of hyperbranched poly(CA–glycerol)s as drug-carrier agents, cisplatin (*cis*-dichlorodiammineplatinum (II), CDDP) was loaded and transported by these copolymers. *In vitro* studies were carried out to evaluate the efficiency of the obtained drug delivery systems for killing the cancer cells.

EXPERIMENTAL

Materials

CA monohydrate, glycerol, tetrahydrofuran (THF), and cyclohexane were purchased from Merck, Whitehouse Station, Germany. Cisplatin (CDDP) and dialysis tubing of the molecular weight cutoff of 1200 Da were purchased from Sigma-Aldrich, Dorset, United Kingdom. The C26 murine colon adenocarcinomas were obtained from the National Cell Bank of Iran (NCBI) Pasteur institute, Tehran, Iran. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder, Annexin-V FLUOS Staining Kit, was obtained from Sigma-Aldrich. RPMI 1640-modified medium, fetal bovine serum, and penicillin/streptomycin solution were obtained from Gibco Invitrogen (Carlsbad, CA). Phosphoric acid used as the mobile phase in high-performance liquid chromatography (HPLC) was purchased from Merck. Deionized water was used in all experiments. To measure the molecular weight of hyperbranched polyesters and study the relationship between CA/G molar feed ratio and molecular weight, gel permeation chromatography (GPC) experiments using Pump 1000 and PL aquagel-OH mixed-H 8 μm column connected to a differential refractometer, refractive index with water as the mobile phase at 25°C were performed. Pullulan standard samples were used for calibration. Thermal behavior of copolymers was investigated by thermal analyzer (model: DSC 60, Shimadzu, Japan) under dynamic atmosphere of an inert gas (i.e., N_2) at 30°C/min (room temperature).

Thermal Production of Hyperbranched Polyesters Containing CA and Glycerol (G) Monomers with Different CA/G Molar Feed Ratios, P(CA_x-G)

All hyperbranched polyesters were synthesized by using CA monohydrate as AB_3 monomer and glycerol (G) as A_3 monomer at different CA/G molar feed ratios through the melting polycondensation. The used amount of glycerol was 0.5 mL (6.6 mmol) and the amounts of CA monohydrate were 7 g (33 mmol), 11.09 g (52.8 mmol), and 16.64 g (79.2 mmol), corresponding to the CA/G molar feed ratios of 5, 8, and 12, respectively.

CA monohydrate and glycerol were mixed in a polymerization ampule equipped with gas inlet, vacuum inlet, and magnetic stirrer at 90°C, and heated to 110°C for 20 min under constant stirring. The temperature of polymerization ampule was increased to 120°C and mixture was stirred at this temperature for 30 min. Then mixture was stirred at 130, 140, 145, and 150°C for 40, 40, 50, and 60 min, respectively, under vacuum to remove the water formed during the reaction. The mixture was then kept at room temperature to cool down. Viscose compound was dissolved in THF and filtered to obtain clear solution. The solution was then concentrated under the reduced pressure and product was precipitated in cyclohexane several times. Precipitated compound was dialyzed against THF for 4 h. It (1 g) was dissolved in THF (10 mL) and then placed in a dialysis bag immersed in THF (100 mL). Then, the whole system was left at room temperature and at predetermined time intervals THF in the outside medium of dialysis bag was replaced with the fresh solvent. Dialysis continued for 4 h and then the contents of dialysis bag were put into a reaction flask.

Finally, THF was evaporated under the reduced pressure to obtain pure product as colorless and viscose compound. This reaction was performed in three different total times of 4, 6, or 8 h and different products namely P(CA_4-G), P(CA_6-G), and P(CA_8-G) were obtained, respectively.

Encapsulation of CDDP by P(CA_x-G) Copolymers

P(CA_8-G) (0.1 g, 1.67×10^{-2} mmol) was dissolved in 1 mL of distilled water and stirred for 1 h. Then, 100 mL of cisplatin aqueous solution (50 μg in 1 mL of distillated water) was added dropwise to the above solution. The solution was stirred at 37°C for 24 h in dark to obtain final product (P(CA_8-G)–CDDP) without any purification (yield, 97%). Yield of the reaction was determined using HPLC. Conditions for HPLC experiments are explained as follows.

P(CA_{12}-G)–CDDP was synthesized in the same manner as explained above. P(CA_8-G)–CDDP and P(CA_{12}-G)–CDDP were used for MTT assay experiments.

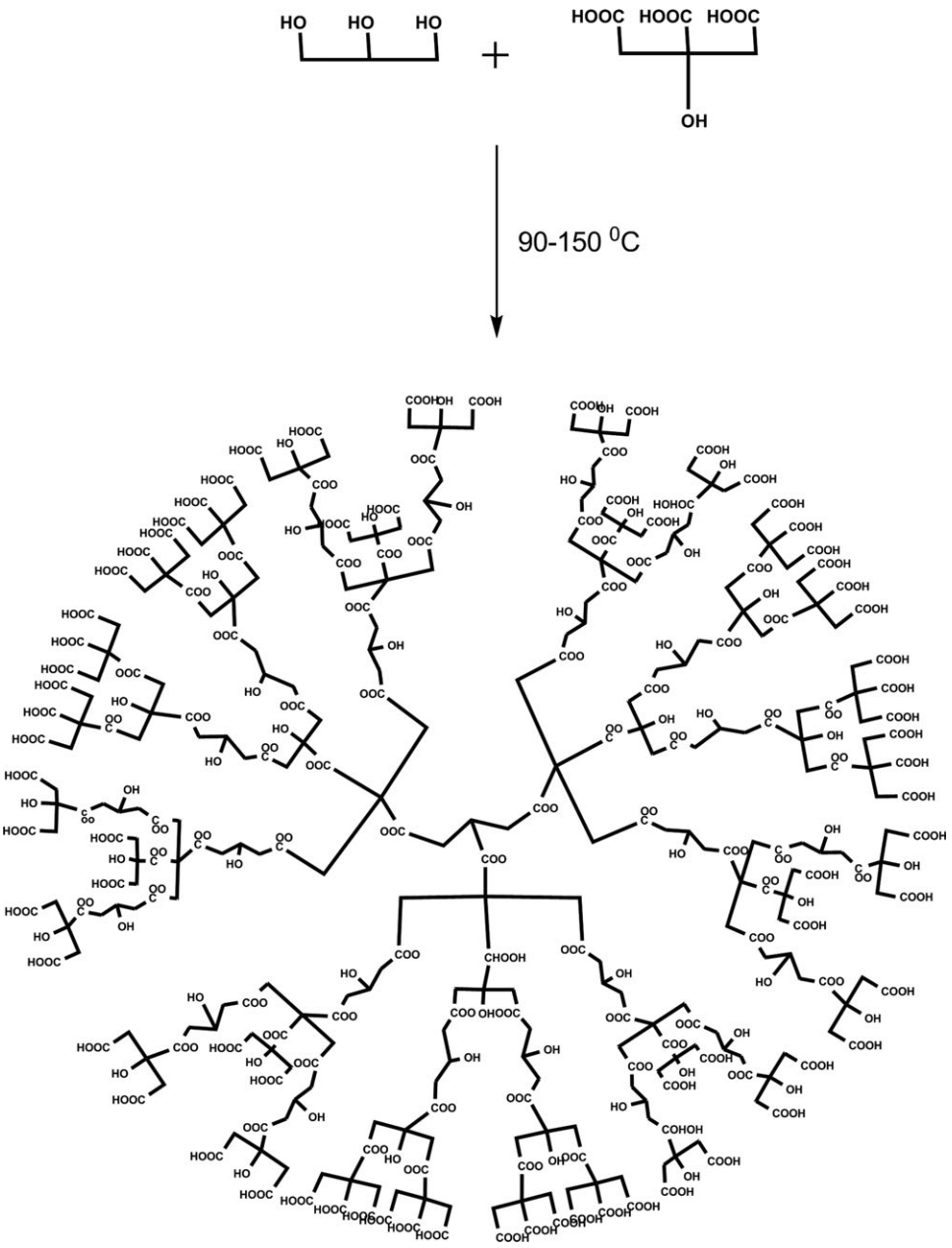
Loading Capacity

Loading capacity of P(CA-G) copolymers (to load CDDP molecules) was determined by HPLC. A water solution of P(CA-G) copolymers was prepared and filtered and it was added to a water solution of CDDP. Then, aliquot of 20 μL of mixture was analyzed by the HPLC to detect the free (not loaded) CDDP concentration. The loading capacity of the polymer was calculated as follows:

$$\mathrm{LC} = (C_i - C_f)/C_i \times 100$$

where LC is Loading capacity, C_i is the initial concentration of CDDP, and C_f is the final concentration of CDDP.

A simple and reproducible reversed-phase HPLC with a Knauer liquid chromatograph (Smart line; Knauer, Berlin, Germany) equipped with an ultraviolet detector (Wellchrom, K-2600; Knauer) and a reverse-phase C18 column (Nucleosil H.P.; 25 cm × 0.46 cm internal diameter, pore size mm; Knauer) using isocratic elution with UV absorbance detection was developed and validated for the determination of cisplatin loaded on nanomaterials. The mobile phase was 15 m*M* phosphoric acid solution and flow rate was 1.00 mL/min. The column effluent was detected at 210 nm. The retention time of free CDDP peak was appeared between 2 and 4 min and the run time was 15 min. Linear regression with an acceptable linear relationship between response (peak area) and concentration in the range of 1–64



Scheme 1. Synthesis of hyperbranched copolymers containing CA and glycerol (G) building blocks by thermal polycondensation.

μg/mL was observed. The regression coefficient was 0.9999 and the linear regression equation was $Y = 34{,}324X + 15{,}334$. Sample concentrations were calculated using the calibration curves.

Cell Culture

C26 murine colon adenocarcinoma was cultivated in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in a humidified incubator with 5% CO_2. The cells were maintained in an exponential growth phase by periodic subcultivation.

Cytotoxicity Assay

In vitro cytotoxicity of the nanomaterials was determined by MTT assay. The cells (2500 cells/well) were seeded in 96-well plates. P(CA-G) copolymers were then added to the wells in triplicates and incubated for 72 h. After the incubation period, 20 μL of MTT dye (5 mg/mL in PBS) was added to each well, and they were incubated in the dark at 37°C for 5 h. Then, media were removed and formazan crystals were dissolved in 200 μL dimethyl sulfoxide (DMSO) and 20 μL of glycine buffer. Then, the absorbance of each well was measured by an ELISA reader (Statfax-2100 Awareness Technology, USA) at 570 nm.

Cell viability was calculated using the following equation:

$$\text{Cell viability}(\%) = (\text{Ints}/\text{Intscontrol}) \times 100 \qquad (1)$$

where "Ints" is the colorimetric intensity of the cells incubated with the samples and "Intscontrol" is the colorimetric intensity of the cells incubated with the Media only (positive control).

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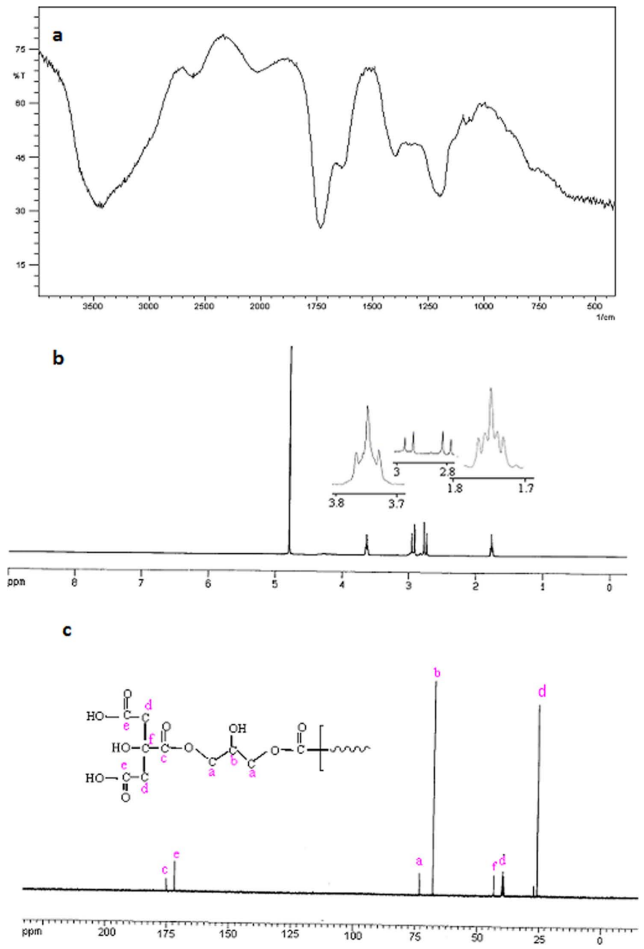

Figure 1. (a) IR spectrum of P(CA_5-G) hyperbranched polyester, (b) ^{1}H NMR spectrum of P(CA_8-G) hyperbranched polyester in D_2O, and (c) ^{13}C NMR spectrum of P(CA_5-G) hyperbranched polyester in DMSO-d_6. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

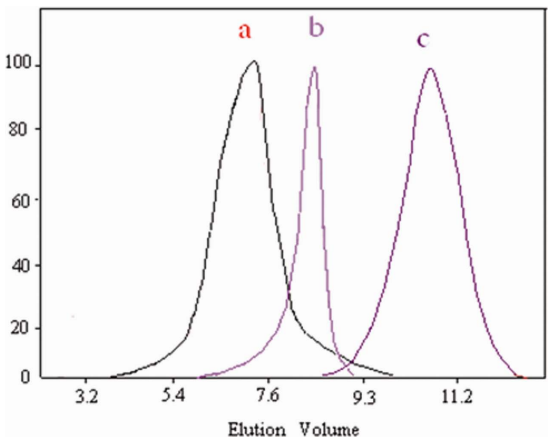

Figure 2. GPC images of (a) P(CA_{12}-G), (b) P(CA_8-G), and (c) P(CA_5-G) hyperbranched polyesters. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table I. The Obtained Molecular Weights for P(CA_5-G), P(CA_8-G), and P(CA_{12}-G) Hyperbranched Copolymers

Sample	P(CA_5-G)	P(CA_8-G)	P(CA_{12}-G)
Molecular weight (M_n)	3000	6000	8000

It is notable that owing to the absorption of vitamins, amino acids and ions to P(CA-G) copolymers, it is clear that the common *in vitro* examination method can yield erroneous cell viability values.

Outlier Detection

All MTT experiments were performed in triplicate or more, with the results expressed as mean ± standard deviation; standard deviation values are indicated as error bars in the MTT results plots. The results were statistically processed for outlier detection using a "T procedure" using MINITAB software (Minitab, State College, PA). One-way analysis of variance (ANOVA) with $P < 0.05$ was performed for each set of MTT assay test repeats. Outlier samples have then been excluded from the corresponding asset viability calculations.

In this method, a T-ratio is calculated as follows

$$T = \frac{X - \bar{X}}{S} \tag{2}$$

where X is the suspected outlier point (normally the smallest or the largest value in a set of measurements), $\bar{X}$ is the sample mean, and S is the (estimated) standard deviation. If the calculated value of T is equals to or exceeds a critical value, the outlier point is removed with a significance level of 0.05. In the latter case, assuming that the data were sampled from a normal distribution, there is at least a 95% chance that the suspected point is, in fact, far from other points.

RESULTS AND DISCUSSION

Synthesis of Hyperbranched Polymers

Scheme 1 shows $AB_3 + A_3$ polycondensation for synthesization of the hyperbranched polyesters according to the procedure explained in the **EXPERIMENTAL** section. In this synthetic route, condensation of CA as AB_3 monomer in the presence of the glycerol as B_3 monomer with different CA/G molar feed ratios has led to the formation of hyperbranched poly(CA–glycerol). Owing to their biocompatibility, water solubility, and high molecular weights, the synthesized hyperbranched polyesters were recognized as promising materials for biomedical applications.

Table II. The Obtained Molecular Weights for P(CA_{12}-G) Hyperbranched Copolymer Synthesized in Total Reaction Times of 4 and 8 h

Reaction time (h)	4	8
Molecular weight	8000	12,000

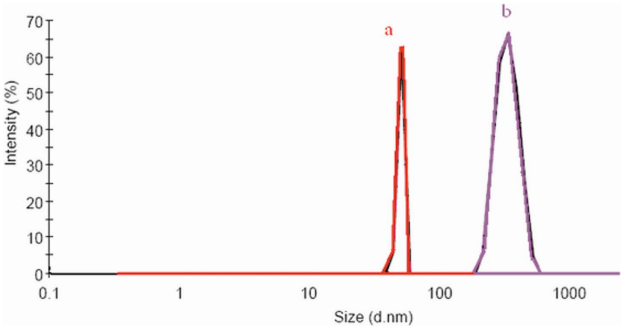

Figure 3. DLS diagrams of (a) P(CA_8-G) and (b) P(CA_{12}-G) hyperbranched polyesters. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

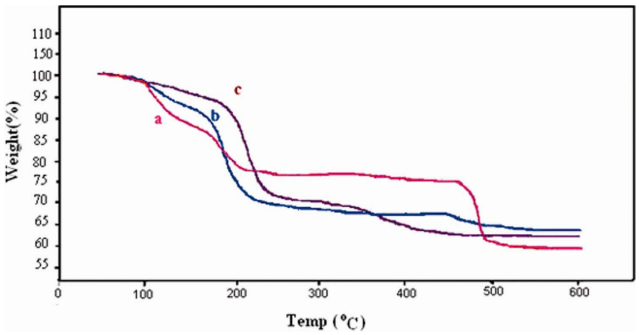

Figure 5. TGAs of (a) P(CA_5-G) hyperbranched polyester synthesized with total reaction time of 4 h, (b) P(CA_8-G) hyperbranched polyester synthesized with total reaction time of 4 h, and (c) P(CA_5-G) hyperbranched polyester synthesized with total reaction time of 4 h. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Characterization of Hyperbranched Polymers

Structure of the synthesized hyperbranched polyesters was characterized using different spectroscopy methods and thermal analysis.

IR spectra of hyperbranched polymers were recorded by a Nikolt 320 FTIR. In the IR spectrum of P(CA_5-G) [Figure 1(a)], absorption bands for acidic and esteric carbonyl groups are appeared at 1647 and 1731 cm^{-1}, respectively. Appearance of the vibration band for esteric carbonyl groups in the IR spectrum of copolymer proves condensation of glycerol and CA monomers.

^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX 400 (400 MHz) apparatus in DMSO-d_6 and D_2O as solvents.

Figure 1(b) (Supporting Information Figure 1) shows ^1H NMR spectra of P(CA_8-G) and P(CA_5-G) hyperbranched copolymers, respectively. Signals at 2.8–3 ppm are assigned to the AB system of methylene protons of CA building blocks. Signals of glycerol building blocks are appeared at 1.7 and 3.6 ppm for methine (—CH—) and methylene (—CH_2—) groups, respectively. Signal at about 4.85 ppm is corresponded to the hydroxyl functional groups of copolymer. Comparison of the integrated peak areas for protons in P(CA_5-G) and P(CA_8-G) spectra indicates that the average number of reacted CA monomers increases with increasing CA/G molar ratio (integration values not shown in the ^1H-NMR spectra).

Figure 1(c) and Supporting Information Figure 2 show ^{13}C NMR spectra of P(CA_5-G) and P(CA_8-G) hyperbranched copolymers, respectively. Signals attributed to the carbons of glycerol building blocks (a, b), CA building blocks (f, d), and esteric and acidic carbonyl groups (c and e respectively) are determined in these spectra.

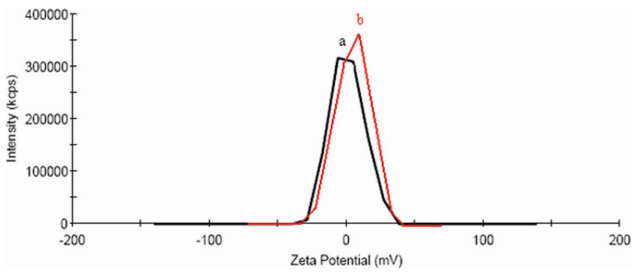

Figure 4. ζ-Potential values of (a) P(CA_8-G) and (b) P(CA_{12}-G) hyperbranched polyesters. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The obtained molecular weights (M_n) for the P(CA_5-G), P(CA_8-G), and P(CA_{12}-G) were about 3000, 6000, and 8000, respectively (Table I). These results indicate that the molecular weights of polyesters depend on the CA/G molar feed ratios and they increase with increased CA/G molar feed ratio (Figure 2).

Effect of the polymerization time on the molecular weight of P(CA_{12}-G) was also investigated. In this regard, hyperbranched polyesters using a CA/G molar feed ratio equal to 12 and total reaction times of 4 and 8 h were synthesized and analyzed by using GPC. As summarized in Table II, the molecular weights (M_n) of synthesized hyperbranched polyesters depend on the total reaction times directly. Molecular weight (M_n) of the synthesized hyperbranched polymers increased from 8000 for 4 h reaction time to 12,000 for 8-h reaction time, indicating that polycondensation is progressing efficiently and transesterification is negligible.

Size of copolymers in water was determined using dynamic light scattering (DLS) (Zetasizer ZS, Malvern Instruments, Westborough, USA).

Figure 3 shows DLS diagrams for P(CA_8-G) and P(CA_{12}-G) hyperbranched polyesters. These diagrams clearly indicate that there is a direct relationship between size of hyperbranched polyesters and CA/G molar feed ratios. According to the DLS experiments, sizes of P(CA_8-G) and P(CA_{12}-G) hyperbranched polyesters are around 80 and 500 nm, respectively. Increasing the size of the synthesized hyperbranched polymers with increased CA/G molar feed ratios indicates that there is a direct relationship between this ratio and degree of polymerization.

ζ-Potentials of hyperbranched polyesters were measured in water to evaluate their surface charges. Figure 4 shows ζ-potential values for P(CA_8-G) and P(CA_{12}-G) hyperbranched polyesters. Interestingly, the surface charge for polyesters is almost neutral which is opposite to the usual cases in which acidic

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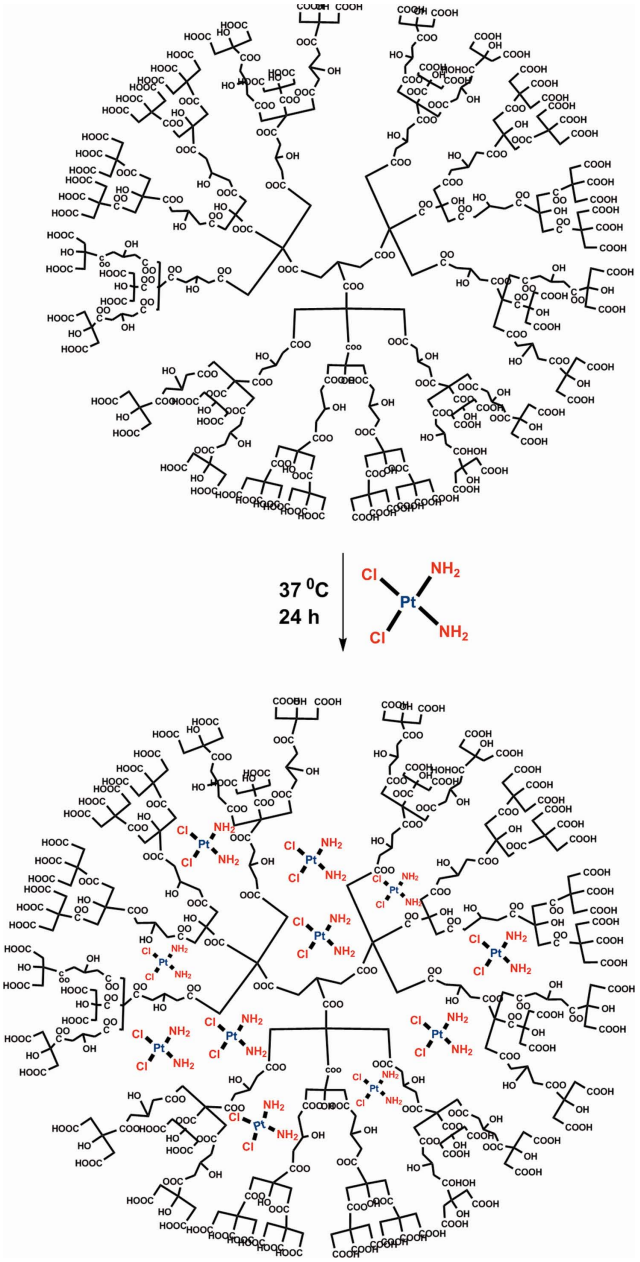

Scheme 2. Loading of CDDP by synthesized P(CA_x-G) hyperbranched polyesters. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

functional groups in natural pH change to carboxylate groups and therefore surface charge is negative. More experiments are needed to understand this unusual result.

Table III. Loading Capacity of P(CA_8-G) and P(CA_{12}-G) Hyperbranched Copolymers for Delivering CDDP Determined by HPLC

Sample	First concentration of CDDP (mg/mL)	Free CDDP measured by HPLC (mg/mL)	Loading capacity (%)
P(CA_8-G)	50	6.01	87.9
P(CA_{12}-G)	50	6.4	87.2

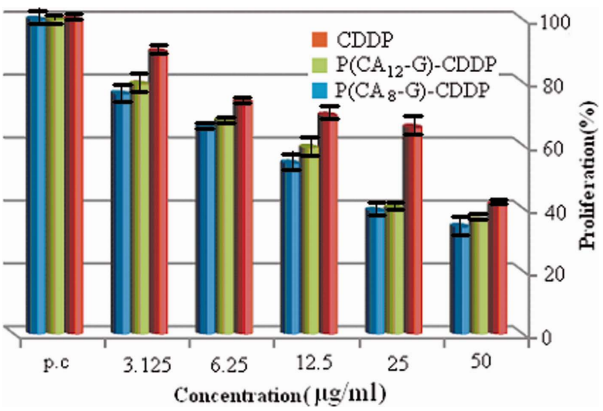

Figure 6. *In vitro* cytotoxicity of free cisplatin (CDDP), P(CA_8-G)–CDDP, and P(CA_{12}-G)–CDDP systems against tumor cells C26 evaluated by using MTT assay. The horizontal axis shows concentration of the CDDP loaded in the hyperbranched polymers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5 shows thermogravimetric analysis (TGA) thermograms for P(CA_5-G) and P(CA_8-G) hyperbranched polyesters synthesized with total reaction times of 4 h, and P(CA_5-G) synthesized with total reaction time of 6 h. The weight loss of hyperbranched polyesters occurs in three stages at 95–110, 170–230, and 380–500°C which are attributed to the evaporation of water, breaking of ester bonds, and decomposition of sample, respectively. Percentages of the weight loss in the second stage for thermograms a, b, and c are 10.5, 19.5, and 20.5% respectively. This result again shows that the growth of the synthesized hyperbranched polyesters directly depends on the CA/G molar feed ratio used in polymerization and also the total reaction time of polymerization.

Hyperbranched Copolymers as Drug Delivery Systems

Although the development of pharmaceutical biotechnologies has led to an increasing number of new drugs, these products still possess many intrinsic limitations to large-scale applications, such as low biocompatibility and poor solubility. As all intrinsic properties for a drug are fixed after synthesis, the design of an appropriate delivery system can be used as a promising way to overcome such problems. For example, some *cis*–platinum complexes are widely used for the treatment of a wide spectrum of cancers such as lung, ovarian, head and neck cancer.[22] Cisplatin performs its antitumor activity by forming stable DNA–cisplatin complexes through intrastrand crosslinks.[23] This results in interference with normal transcription and DNA replication mechanisms, leading to apoptosis.[22] However, toxicity and poor water solubility of cisplatin limit its high cancer activity.

To prove the usefulness of the synthesized hyperbranched polyesters as drug delivery systems, cisplatin was loaded by these copolymers and P(CA_x-G)–CDDP drug delivery systems were obtained (Scheme 2).

Investigation of the Loading Capacity of Hyperbranched Copolymers

The loading of cisplatin into the P(CA_8-G) and P(CA_{12}-G) hyperbranched polyesters was investigated by HPLC. The standard curve of free cisplatin was obtained and used for calculating drug loading

Table IV. Obtained IC_{50} values for CDDP, P(CA_8-G)–CDDP, and P(CA_{12}-G)–CDDP

Sample	P(CA8-G)-CDDP	P(CA12-G)-CDDP	CDDP
IC_{50} (μg/mL)	15.01	16.8	37.9

into the P(CA_8-G) and P(CA_{12}-G) hyperbranched polyesters. According to the HPLC results (Table III), the loading capacity of P(CA_8-G) and P(CA_{12}-G) hyperbranched polyesters was 87.9 and 87.2%, respectively. High loading capacity for these copolymers promises them as new vectors to deliver cisplatin efficiently.

Cytotoxicity of Drug Delivery Systems Against C26 Cell Line

In vitro cytotoxicity of free cicsplatin (CDDP), P(CA_8-G)–CDDP, and P(CA_{12}-G)–CDDP was evaluated against C26 cell line by using MTT assay. To measure cytotoxicity, tumor cells C26 were separately incubated in a plate with different concentrations of CDDP, P(CA_8-G)–CDDP, and P(CA_{12}-G)–CDDP. The time of incubation was 72 h for all samples. The MTT assay results are shown in Figure 6. Based on these results, loading of cisplatin into the P(CA_8-G)–CDDP and P(CA_{12}-G)–CDDP influences their cytotoxicity against cancer cells strongly. P(CA_8-G)–CDDP and P(CA_{12}-G)–CDDP drug delivery systems show higher toxicity as compared to free cisplatin. It can be found that the toxicity of both P(CA_8-G)–CDDP and P(CA_{12}-G)–CDDP drug delivery systems is comparable in all concentrations. However, the best concentration is 25 μg/mL in which toxicity of P(CA_8-G)–CDDP and P(CA_{12}-G)–CDDP drug delivery systems against cancer cells is much higher than that for free CDDP. IC_{50} doses for free cisplatin, P(CA_8-G)–CDDP and P(CA_{12}-G)–CDDP drug delivery systems were calculated using MTT assay. As summarized in Table IV, cisplatin loaded in polyesters has lower IC_{50} value than free cisplatin. In fact, a decrease in the IC_{50} dose shows an increase in drug toxicity. As both CA and glycerol are included in the metabolic processes in cells and have key roles in this cases, it seems that their copolymers interact with the cell membranes much better and transfer the loaded drugs from the cell membranes in higher rates. These results prove the efficacy of the synthesized hyperbranched polyesters as biocompatible cargos to transport anticancer drugs.

CONCLUSIONS

Hyperbranched polyesters with CA and glycerol building blocks could be synthesized through a step-by-step melting polycondensation. The synthesized hyperbranched copolymers are able to load and deliver anticancer drugs efficiently. Anticancer drug delivery systems resulted from these copolymers are powerful vectors for killing cancer cells *in vitro*.

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