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Analysis of plasmid DNA damage induced by melanin with capillary electrophoresis

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Abstract

Dilute linear poly(*N*-isopropylacrylamide) (PNIPAM) in Tris–Mes–EDTA (TME) buffer was used as sieving matrix for capillary electrophoresis (CE) of plasmid DNA and plasmid topological isomers induced by melanin in uncoated capillary. At the optimized condition of 0.1% (w/v) PNIPAM in TME buffer, base line separation of the plasmid DNA ladder (2–12 kbp) was achieved within 15 min. Three positive clones with inserts of 468, 1147 and 1566 bp can be distinguished from the plasmid pUC 18 vector within 13 min. The migration order of the plasmid topological isomers in the dynamic coating matrix was confirmed by the enzymatically prepared and UV-induced plasmids. The covalently closed circular form appeared firstly, followed by the linear plasmid form and then the open circular form. The effect of bacterial melanin obtained from *Pseudomonas maltophilia* AT18 on plasmid pUC 18 was investigated by CE in uncoated capillary in vitro. Plasmid pUC 18 incubated with either melanin or copper ions alone sustained little DNA damage. The combination of melanin with Cu(II) can cause the plasmid pUC 18 conformational changes from covalently closed circular form to open form. Understanding the damage effect of melanin with copper ions on DNA would be important for the melanin-related application, such as photoprotective antioxidant in protecting the skin from cancer, pathophysiology research in clinic. The investigation of melanin induced plasmid conformational changes by CE in uncoated capillary also revealed that the application of the dynamic coating matrix could be extended to the study of plasmid conformational changes in other plasmid-based biological technologies.

Keywords: Plasmid; Melanin; DNA damage; Dynamic coating; Poly(*N*-isopropylacrylamide)

1. Introduction

Plasmids are circular bacterial DNA sequences that replicate independently of the chromosome. Many plasmids encode unique metabolic functions such as resistance to antibiotics, heavy metals or UV radiation [1]. New approaches in gene therapy focus on the use of plasmid DNA for in vivo delivery, such as naked DNA vaccines and lipid-mediated gene transfers [2,3]. In recombinant technology, a DNA fragment that contains a gene of interest is inserted into the purified and altered plasmids is referred as genetic vectors. These plasmids may exist in several forms differing in topology. The prominent topological isomers of plasmids are the covalently closed circular (CCC) form, the open circular (OC) form and the linear form. The CCC form is lost by single or double strand breaks resulting in OC form or linear form structures as illustrated in Fig. 1. In most bacterial plasmid preparation, the majority of plasmids exist in the CCC form, however, the CCC form can convert to OC or nicked form by radiation, photolysis, nucleases or reactive oxygen species (ROS, such as $^{\bullet}OH$, $O_2^{\bullet -}$ and H_2O_2) generated by several physical and chemical agents [4]. In recent years, studies on plasmid conformational changes induced by exogenous damage have gained prominence because of their relevance in the development of new reagents for biotechnology and medicine.

Melanin is the most common biological pigment which is produced by special organisms and tissues. In mammals it is found in many sites including inner ear, eye, hair and skin where melanin is synthesized by melanocytes [5]. Melanin is considered to protect skin from solar carcinogenesis, as it is able to remove ROS which are generated in the skin in response to UV

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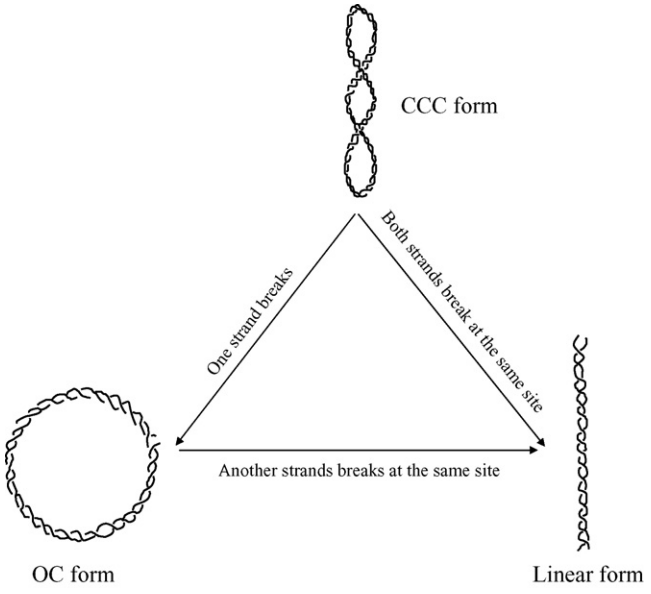

Fig. 1. Different topological forms of plasmid. CCC form, covalently closed circular form; OC form, open circular form.

radiation [6,7]. However, as melanin is a relative weak absorber of UV, its importance as sunscreen is a subject of debate [8,9]. Melanin has also been shown to both cause and prevent oxidative base damage in DNA. Melanin itself can produce DNA damage at high concentration [10] or bind some metal ions to produce ROS [11,12]. This could have many consequences in the melanocyte including effects on antioxidant defiance. Copper is a bioessential element with relevant oxidation states +1 and +2. Coordination compounds of copper have been extensively used in metal-mediated DNA cleavage through the generation of hydrogen abstracting activated oxygen species [13]. The impact of copper on the antioxidant defense system and oxidative damage to cellular components has been a topic of considerable interest [14].

The usual methods employed to identify the DNA damage are comet assay and agarose gel electrophoresis (AGE). The comet assay is applied to analyze DNA damage in individual cells. It is a powerful and versatile technique that relies on microscopic visualization or imaging of DNA after single cells are embedded in agarose, lysed and electrophoresed [15,16]. AGE provides a better overview of the sample components present. However, it is not suitable for reliable quantification of the specific DNA forms, particularly, if small amounts of DNA are to be analyzed [17]. In addition, form quantification based on the signal intensity of stained bands in an agarose gel may not be linear and requires experience and adequate equipment for densitometry [18,19]. Since the conformation and the quality of plasmid are critical to their use both as therapeutic agents and in plasmid-related biological technologies, it has become increasingly desirable to develop efficient separation methods to determine plasmid forms distribution for process monitoring in plasmid production. Capillary electrophoresis (CE) in polymer-containing matrices has been evolved as an important tool to analyze and quantify plasmid form distribution. Unlike linear ds-DNA separations, where a number of different polymers have been developed and tested in CE with varying degrees of success [20], little attention was paid to the use of matrices for plasmids DNA separation, although the electrophoretic behavior of plasmids differs greatly from the behavior of familiar and widely studied linear DNA [21]. In the sparse literature on CE of plasmid DNA only few matrices such as polyacrylamide [22], hydroxyethyl cellulose [1,21,23,24], hydroxypropylmethyl cellulose [18,25,26] and polydimethylacrylamide [27] are used for successful separations. Most of these studies have employed coated capillaries to suppress electroosmotic flow. As the dynamic coating matrix reduces cost and improves convenience associated with the use of bare capillaries, its potential for the separation of plasmid is very attractive [1,24,27].

In the present work, poly(*N*-isopropylacrylamide) (PNIPAM) in Tris–Mes–EDTA (TME) buffer was used as dynamic coating matrix for the separation of plasmid conformational changes induced by melanin purified from bacteria *Pseudomonas maltophilia* AT18 in the presence of Cu^{2+} in vitro. The bacterial melanin was reported to protect DNA from UV irradiation damage by efficiently scavenge ROS generated by UV [28]. In this paper, we found melanin can cause plasmid conformational changes even at a relative low concentration in the presence of Cu^{2+}.

2. Experimental

2.1. Chemicals and materials

N-Isopropylacrylamide was obtained from Aldrich (Milwaukee, WI, USA) and was used without further purification. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ethidium bromide (EtBr) and ammonium persulfate were purchased from Sigma (St. Louis, MO, USA). 2-(*N*-moropholino)ethanesulfonic acid (Mes) was from AMRESCO (Solon, OH, USA). Tris(hydroxylmethyl)aminoethane (Tris), EDTA were analytical grade from Shanghai Reagents Co. (Shanghai, China). Supercoiled DNA ladder, plasmid pUC 18 (2686 bp), *Bam*HI restriction endonuclease was from TaKaRa Biotech. Co. (Dalian, China). The supercoiled ladder was diluted to 40 μg/ml with water before using.

2.2. Sample preparation

2.2.1. Cloning of different size of ds-DNA into the pUC 18 vector

The restriction enzymes and T4 DNA ligase (TaKaRa Biotech. Co., Dalian, China) were used for DNA cloning. DNA fragments of 468, 1147 and 1566 bp were ligated into the multiple clone site of plasmid pUC 18 to construct a recombinant plasmid according to procedure provided by the manufacture. After the corresponding positive clones were selected, plasmid DNA was prepared from each positive clone with the kit from Shenenergy (Shanghai, China). The size of the vector of each clone was analyzed by restriction digestion with *Eco*RI. The DNA sizing of the *Eco*RI digest was done with the Agilent DNA 7500 kit (Agilent Technologies). The result confirmed that the selected clones contained the target sequences.

2.2.2. Enzymatic digestions

The linear form of the plasmid was generated by digestion with *Bam*HI restriction endonuclease at 37 °C for 1 h according to the procedure provided by the supplier.

2.2.3. UV radiation of plasmid pUC 18

Twenty microliters of pUC 18 at 10 ng/μl were added into 250 μl microcentrifuge tube. The sample was placed under the UV lamp (365 nm, 25 W, Hubei) and irradiated for 5, 10 and 15 min, respectively. The irradiated sample was then stored at −4 °C before analysis.

2.2.4. Isolation of melanin and its reaction with plasmid pUC 18

Melanin was obtained from *P. maltophilia* AT18 [29]. Cell-free filtrate was deposited in NaOH and HCl for the purification of melanin as described by Gibson and George [30]. The optical density at 400 nm was determined and compared to a standard curve of synthetic melanin to get the melanin concentration.

The reaction mixture (40 μl) solution essentially contained 400 ng plasmid pUC 18, 400 μM cupric chloride and varying amounts of melanin. After incubation at 37 °C for 3 h, the mixture solution was stored at −4 °C prior to CE separation.

2.3. Preparation of the sieving matrix

The linear PNIPAM was prepared by free-radical solution polymerization as reported in our previous work [31]. Briefly, 6 g of *N*-isopropylacrylamide was dissolved in 100 ml double-distilled water and filtered (0.22 μm). After bubbling with high-purity nitrogen for 3 h, the monomer solution was polymerized by addition of 70 μl of 100% (v/v) TEMED and 180 μl of 20% (w/v) ammonium persulfate. The bottle was sealed, and the solution was stirred slowly at room temperature for 2 h. The bottle was then transferred to a refrigerator at 4 °C for 48 h. After the polymer solution was purified by dialysis against water for 48 h using dialysis tubings with a molecular mass cut off 12,000 Da, the solid PNIPAM was obtained from the dialyzed solution by lyophilization. The viscosity-average molecular weight of PNIPAM was 6.5×10^6.

The sieving matrices were prepared by dissolving the PNIPAM in TME (40 mM Tris, 60 mM Mes and 2 mM EDTA, pH 6.2) buffer to desired concentration, respectively. The TME buffer was filtered with 0.22 μm filter member prior to use. Shortly before the analysis, EtBr was added to the buffer with the final concentration of 3 μg/ml.

2.4. Capillary electrophoresis instrumentation and procedures

A laboratory-built CE system with laser-induced fluorescence detection was similar to that described in previous work [32]. Briefly, a neodymium–yttrium–aluminium garnet (Nd–YAG) laser with 532 nm output (Quantel, France) was used for excitation. A long wave pass filter at 600 nm was used to block stray light. The signal from the photomultiplier tube (PMT) was fed into a Boxcar averager (M162/165, EG&G, PAR) and then transferred through a 12-bit A/D combining converter and stored in a personal computer. A 40-cm long (32 cm to the detector) uncoated capillary (Yongnian Optical Fiber Co., Hebei, China) with 75-μm inner diameter (i.d.) and 365-μm outer diameter (o.d.) was used for the separation. Before each run, the capillary was flushed with 1 M NaOH, 1 M HCl and water for 5 min, respectively. Both cathode and anode reservoirs were filled with sieving buffers. The sieving matrix was then pumped into capillary under high–pure nitrogen pressure. The capillary tube was then assembled onto the holder and a pre-run at constant electric field strength of 150 V/cm was used to stabilize the system. Injection of the plasmid DNA fragment was performed electrokinetically at the negative electrode and detection was at the positive electrode.

3. Results and discussion

3.1. Separation of the supercoiled plasmid ladder and the pUC 18 cloning vector

As there are great differences of electrophoretic migration behaviors between the plasmid and the widely studied ds-DNA, optimum conditions such as polymer concentration or electric field strength cannot be predicted from the accumulated knowledge resulting from the linear fragments [21,23]. The separation condition for plasmid DNA should at first be optimized with respect to peak resolution and time of analysis. Fig. 2 shows the effect of PNIPAM concentration on the separation performance of the supercoiled plasmid ladder. A solution of 0.1% (w/v) PNIPAM in TME buffer was suitable for separation of most plasmids in the supercoiled DNA ladder, all of the eight plasmids ranging from 2 to 12 kbp were separated within 15 min in an uncoated capillary with the smaller plasmids having shorter migration times (Fig. 2b). When the concentration of PNIPAM was decreased to 0.05% (w/v), the smaller plasmids could not achieve base line resolution (Fig. 2a). If the concentration of PNIPAM was increased to 0.25% (w/v), the larger DNA plasmids above 6133 bp were no longer separable (Fig. 2c and d). The optimized concentration of 0.1% (w/v) was nearly a factor of 35 below the measured entangled threshold of this polymer, 3.5% (w/v) [31]. Under the optimized condition, the reproducibility of migration time was excellent (R.S.D. < 2%), the separation efficiency of most plasmids was larger than 10^6 plates/m except the plasmids of 5023, 10085 and 11849 bp, respectively (the efficiency N was calculated from $N = 5.54(t/w)^2$, where t and w are the migration time and full peak width at half maximum height, respectively), as illustrated in Table 1. These results demonstrated the good dynamic coating ability of PNIPAM, which would be much of importance for its application in the efficient separation of the plasmid. The possible dynamic coating mechanism of PNIPAM was discussed in our previous work [33].

The determination of relative size of plasmid by CE from the migration time can be applied in many plasmid-based technologies. One of the applications is the screening for clones that contain target insert(s) on a cloning vector in recombinant technology. This analysis was traditionally performed by agarose or

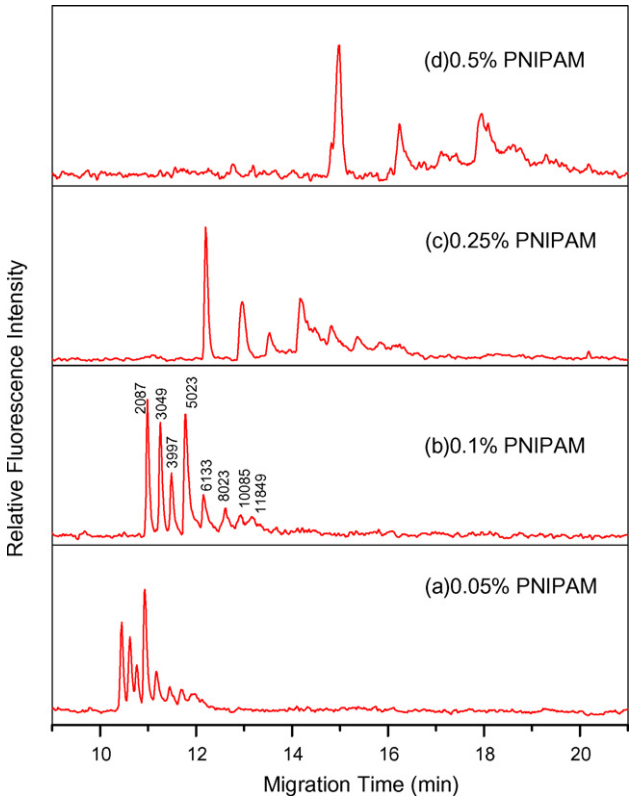

Fig. 2. Electropherogram of supercoiled DNA ladder with different concentration of PNIPAM in TME buffer: (a) 0.05% (w/v) PNIPAM, (b) 0.1% (w/v) PNIPAM, (c) 0.25% (w/v) PNIPAM, (d) 0.5% (w/v) PNIPAM; capillary: 75/365 μm i.d./o.d., and 32/40 cm efficient/total length; inject condition: −8.40 kV × 12 s; separation electric field strength: 150 V/cm.

polyacrylamide gel electrophoresis. As shown in Fig. 3, plasmid preparations from three positive clones with inserts of 468, 1147 and 1566 bp in comparison to that of the pUC 18 vector were clearly distinguished from each other within 13 min. In order to estimate the relative size of plasmid, a calibration curve was constructed by plotting plasmid size (Y, bp) against the migration time (X, min). The regression equation $Y = -22423.33 + 2292.47X$ with the regression coefficient (R^2) of 0. 998 was obtained for plasmid size ranging from 2087 to 5023 bp. The calculated molecular weight of these four plasmids were 2473, 2913, 3873 and 4650 bp, respectively. The determined molecular weight was within 10% of the expected

Table 1
Migration time reproducibility ($n = 5$) and peak efficiency of plasmid DNA separated in 0.1% PNIPAM TME buffer in uncoated capillary

Fragment size (bp)	Migration time (min)	R.S.D. (%)	Theoretical plates (10^6/m)
2087	10.68	1.44	1.67
3049	11.15	1.43	1.48
3997	11.49	1.38	1.46
5023	11.98	1.33	0.82
6133	12.15	1.37	1.17
8023	12.61	1.71	1.10
10085	12.92	1.47	0.74
11849	13.16	1.64	0.02

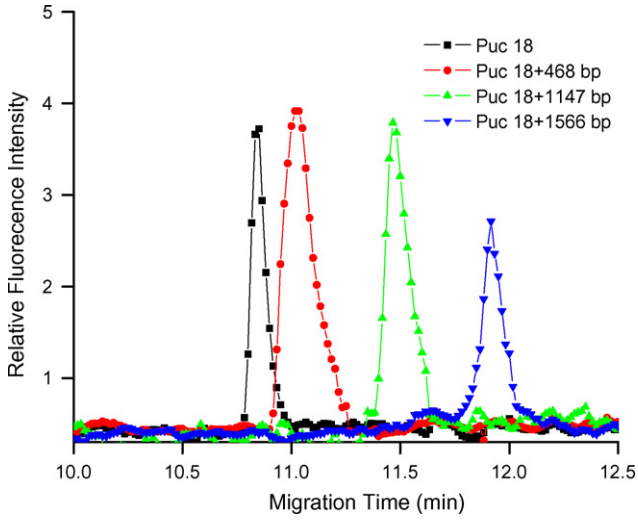

Fig. 3. Electropherogram of the supercoiled form of the plasmids with different size inserts on pUC 18 cloning vector in 0.1% (w/v) PNIPAM TME buffer. Other conditions as in Fig. 2.

molecular weight. This example revealed that this assay can be adopted in uncoated capillary for direct screening of positive clones in the process of vector constructions. Compared with the conventional AGE method of plasmid size screening, CE of the plasmid was much faster and consumed less plasmid.

3.2. Separation of different topological forms of plasmid

Plasmids prepared from bacteria exist predominately in the CCC form and the major impurities due to degradation are linear and OC forms. Several methods have been employed to induce the plasmid cleavage in order to obtain the different topological forms of plasmid DNA. Nackerdien et al. have used laser irradiation to get the various forms of the plasmids [22]. Mao et al. have applied a special restriction endonuclease to get the isomers of the plasmids [26]. The γ [1] and UV [18,27] radiation was also an effective method to induce strand breakage in the plasmid DNA. In the present work, the *Bam*HI restriction endonuclease and UV radiation was adopted to obtain the linear and OC form of the plasmid pUC 18, respectively.

Although the supercoiled plasmid ladder was separated well in 0.1% (w/v) PNIPAM TME buffer. It was found that the different topological forms of plasmid pUC 18 were separated better in higher concentration of PNIPAM. As shown in Fig. 4, the different topological forms of plasmid pUC 18 had been separated by CE in 0.25% (w/v) PNIPAM solution within 20 min. Peak 1 on the electropherogram was identified to be the CCC form of pUC 18 by comparing its migration time with that of the supercoiled DNA ladder in Fig. 2c under the same electrophoretic condition. The linear form of pUC 18 (peak 2) were identified by the separation of the complete digestion of pUC 18 with the restriction enzyme *Bam*HI (Fig. 4b). After the pUC 18 samples were exposed to the UV light for 5 min, a broad peak (peak 3) at a longer migration time appeared. The smallest mobility of this peak may correspond to the OC form, as this "floppy" molecular shape impeded movement through the polymer solution [26].

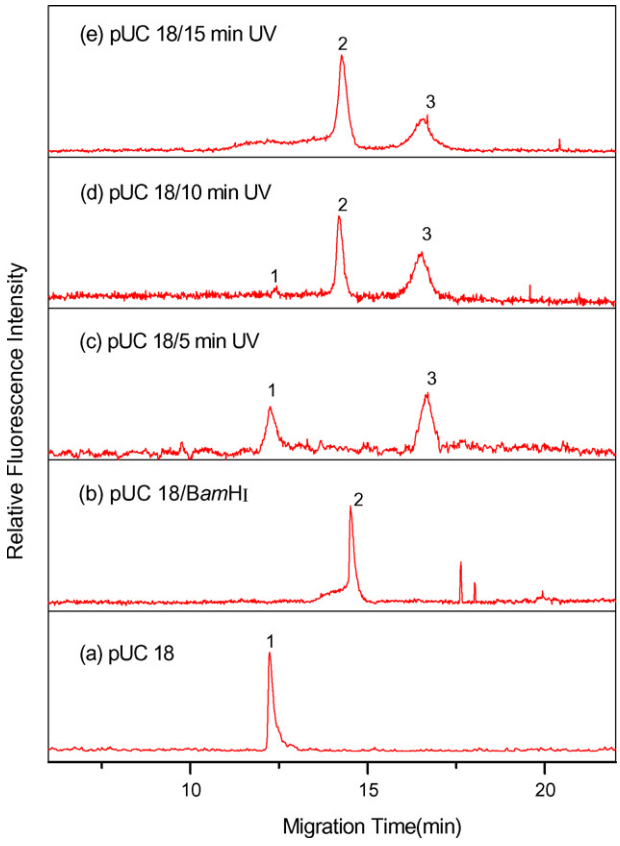

Fig. 4. Electropherogram of the different topological forms of plasmid pUC 18 in 0.25% (w/v) PNIPAM TME buffer: (a) pUC 18, (b) pUC 18/*Bam*HI, (c) pUC 18 irradiated with UV for 5 min, (d) pUC 18 irradiated with UV for 10 min and (e) pUC 18 irradiated with UV for 15 min; peak 1: CCC form, peak 2: linear form, peak 3: OC form. Other conditions as in Fig. 2.

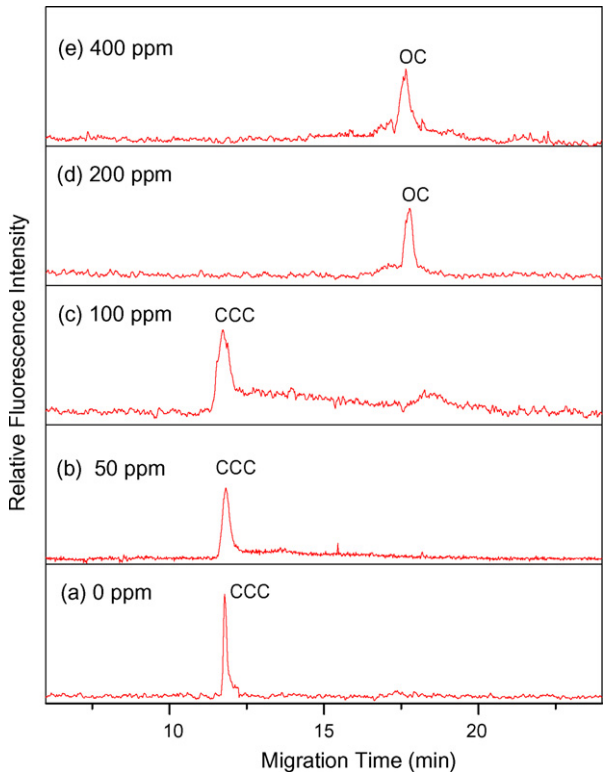

Fig. 5. Electropherogram of the plasmid pUC 18 after incubuation with melanin and $CuCl_2$ for 3 h at 37 °C in 0.25% (w/v) PNIPAM TME buffer: (a) 10 μM $CuCl_2$, (b) 10 μM $CuCl_2$ + 50 ppm melanin, (c) 10 μM $CuCl_2$ + 100 ppm melanin, (d) 10 μM $CuCl_2$ + 200 ppm melanin and (e) 10 μM $CuCl_2$ + 400 ppm melanin; other conditions as in Fig. 2.

When the exposure time increased to 10 min, the CCC form of pUC 18 decreased while the linear form increased (Fig. 4d). This result suggested that part of the OC form was transferred into the linear form. After 15 min UV exposure, no CCC form could be detected in the sample, but only linear and OC form (Fig. 4e). Therefore, the migration order of these three forms was CCC form, linear form and OC form, which consisted with the report of Schmidt et al. [18] and Ding et al. [27]. There was no general agreement on the migration order of these three forms in CE found in literature reports [21,22,26]. These disagreements may be ascribed to the different electrophoretic conditions (such as dye, polymer varieties, polymer concentration, etc.) that were used.

3.3. The damage effect of melanin on the plasmid DNA

Melanin is reported to have the ability to act as a UV absorber, cation exchanger, amorphous semiconductor and novel drug-binding biopolymer, to protect against irradiation by X-rays and γ-rays and to have antioxidant and antiviral activities [34]. Most of the functions of melanin were regarded to its ability to scavenge ROS as an antioxidant. In fact, melanin has been used commercially in photoprotective creams because of the relative simple and inexpensive purification procedure from bacteria [35]. But several studies have suggested that melanin was also a ROS generator. Melanin itself can produce DNA damage at high concentration [10] or at a relative low concentration in the presence of certain metal ions [11,12]. This finding may be very important for its application in the field of antioxidant defense.

Melanin obtained from *P. maltophilia* AT18 was used to investigate its effect on the plasmid pUC 18 in the presence of the most common and bioessential element copper(II). As shown in Fig. 5, the melanin alone up to 400 ppm had no effect on plasmid conformation (data not shown). However, when 10 μM cupric chloride was added to the sample, the CCC form of the pUC 18 began to convert into OC form with increasing amount of melanin. When the concentration of melanin was increased to 100 ppm, the peak corresponding to CCC form was much broader compared to the one in Fig. 5a and b. A minor broad peak appeared at longer migration time in Fig. 5c may be the OC form by comparing the migration time of the peak with Fig. 4. The CCC form had almost transferred into OC form in the presence of 200 and 400 ppm of melanin (Fig. 5d and e), no linear form was detected in these plasmid samples. The result indicated that melanin at relative low concentration along with Cu^{2+} can cause plasmid pUC 18 single-strand breaks. This result was similar with the investigation by Husain and coworkers using AGE [11]. Understanding the damage effect of melanin with copper ions on DNA would be important for the melanin-related

application, such as photoprotective antioxidant in protecting the skin from cancer, pathophysiology research in clinic, etc.

4. Conclusions

Dilute PNIPAM solution in uncoated capillary had been proved to be an effective sieving matrix for plasmid analysis. A particular plasmid can be sized by comparing the migration time with that of the plasmid DNA ladder at the same electrophoretic condition. One of these applications was to screen recombinants from the cloning vector. For the separation of the plasmid topological isomers, the major isomers can be identified by the migration time in uncoated capillary. The CCC form appeared firstly, followed by the linear plasmid form and then the OC form. Melanin at relative low concentration along with Cu^{2+} can cause plasmid pUC 18 single-strand breaks. Understanding the damage effect of melanin with copper ions on DNA would be important for the application of melanin. The investigation of effect of melanin with copper ions on plasmid conformational changes can be extended to the study of plasmid conformational changes in other plasmid-based biological technologies. Ongoing research is to investigate the combination effect of melanin with other metal ions on plasmid.

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