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Effects of Copper-Cyclam and Copper-Cyclam/Polymer Complexes on HeLa Cells

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Abstract

Cyclam-based ligands and their metal complexes have shown antitumor activity. The aim of this study is to prepare positively charged, water soluble Cu(II)-cyclam and Cu(II)-cyclam/poly(acrylic acid) (PAA) complexes which will be used as a chemotherapic agent. In the first part of the study, cyclam reacted with copper(II) to form Cu(II)-cyclam complex. To increase the antitumor activity of Cu(II)-cyclam complex, the complex was linked to PAA and Cu(II)-cyclam/PAA complex was prepared. The Cu(II)-cyclam and Cu(II)-cyclam/PAA complexes were characterized by fouriertrasnform infrared spectroscopy (FTIR), nuclear magnetic resonance (^{1}H-NMR) and atomic absorption spectrophotometer (AAS). To observe, antitumor effects of Cu(II)-cyclam and Cu(II)-cyclam/PAA complexes to HeLa cell lines, 3-[4,5-Dimetiltiazol-2-]-2-5-diphenyl tetrazolium bromide (MTT) test was performed. DNAs of the HeLa cells were isolated and agarose gel electrophoresis was performed to investigate DNA cleavage. To determine the biocompatibility of the complexes, they were interacted with the human serum and analyzed by SDS-PAGE electrophoresis. FTIR and NMR data proved the formation of complexes. The complex prepared with a Cu(II)-cyclam/PAA mol ratio of 1/1 was used in cell culture experiments. According to the MTT test results 100 µg/ml Cu(II)-cyclam and 100 µg/ml Cu(II)-cyclam/PAA were able to kill 12.4% and 51.4% of the cells in the culture, respectively. It was shown that 250 µg/ml Cu(II)-cyclam/PAA complex was able to cleave the DNA effectively. There were no significant effect of neither Cu(II)-cyclam, nor Cu(II)-cyclam/PAA and PAA on the human serum proteins.

Key Words: 1,4,8,11-tetra-azacyclotetradecan (cyclam), metal-chelate ligand, cancer therapy, HeLa cell lines, PAA.

INTRODUCTION

Several metal complex agents have already been introduced into clinical tumor therapy. Effective anti-tumor drug, cisplatin, has been found to make a covalent 1,2-intrastrand adducts at N7 of guanine base of DNA [1]. Screening new metal complexes with antitumor activity are research interests. It has been reported that macrocyclic complexes with tetraazamacrocyclic ligands, such as cyclam, cyclen or bicyclam exhibit antitumor activity [2]. It has been reported that macrocyclic metal complexes with amine or carboxylates as chelators which have cleaving or binding interaction to DNA [3].

Transition metal complexes incorporating macrocyclic ligands are well-known to represent kinetically inert, thermodynamically stable systems.

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During the past three decades, the field of macrocyclic complexes has expanded greatly [4]. The synthetic flexibility of macrocycles is now used in preparing low molecular weight compounds of bio-inorganic relevance [5]. There is also much interest in the metal complexes of macrocycles due to their diverse potential use, which, since recently, includes radiometal uptake in cancer threapy [6]. Historically, tetramacrocycles of varying ring size and degrees of insaturation were among the first azamacrocycles synthesized [7] and a large amount of literature has been gathered on the complexes of these tetradentate macrocycles. Particularly, the ligand 1,4,8,11-tetra-azacyclotetradecan ([14]ane N4), cyclam has played an important role in many different areas of inorganic chemistry. The properties of the cyclam complexes should correspond, to those of the most typical classic coordination compounds, deviating only in ways that reflect the cyclic structure of the ligands. This macrocyclic and its congeneres are considered to be the ultimate prototypic macrocyclic ligands. In particular, the preparations of the macrocyclic diimide ligand 5,7-dioxo-1,4,8,11-tetra-azacyclo-tetradecan (cyclamdione) and its Cu(II) and Ni(II) complexes have been described by Hayer et al. [8].

Cyclam and cyclamdione have different properties, i.e., cyclam metal complex formation does not involve deprotonation on the N atoms, instead cyclamdione accommodates certain metal ions within the macrocycle N4 cavities with simultaneous dissociation of the two amide protons. Cyclamdione possesses the novel ligand properties of both saturated macrocycle tetraamine and oligopeptides. In general, work on this type of molecule refers to the structural aspects and to the effects that the large hole size and the chelate ring sequence have on the stability of the macrocycle [9,10].

Metal ions play an important role in biological systems and without their catalytic presence in trace or ultratrance amounts many essential co-factors for many biochemical reactions would not take place. However, they become toxic to cells when their concentrations surpass certan optimal (natural) levels. Copper is an essential metal. Catalytic copper, becomes of its mobilization and redox activity, is believed to play an central role in the formation of reactive oxygen species (ROS), such as $O_2^{-}\cdot$ and $\cdot OH$ radicals, that bind very fast to DNA, and produce damage by breaking the DNA strands or modifying the base and/or deoxyribose leading to carcinogenesis [11].

The synthesis and investigation of metal-containing polymers are important since these systems offer advantages over non-polymeric metal complexes and metal salts in a variety of chemical reactions [12]. One of the most versatile and studied polymer is poly(acrylic acid) (PAA). Polymer metal chelates, moreover, are of great significance in different fields of chemistry, e.g. catalytic reactions, biochemistry, medicine, etc [13]. The aim of this study is to prepare positively charged, water soluble Cu(II)-cyclam and Cu(II)-cyclam/PAA complexes which will be used as a chemotherapic agent.

MATERIALS AND METHODS

Materials

Lineer polyacrylic acid (AM_W =30x10^3) and cyclam (M_W: 200) and flourescein, were purchased from Aldrich (USA). 3-[4,5-Dimetiltiazol-2-]-2-5-diphenyl tetrazolium bromide (MTT) was purchased from Aldrich (USA). MTT concentration was 5 mg/ml in RPMI-1640 without phenol-red. HeLa cells was purchased from Sap Institue of Culture Collection (Ankara, TURKEY). Cell culture flask and other plastic materials were purchased from Corning (USA). The growth medium, which is Dulbecco Modified Medium (DMEM), supplemented fetal calf serum (FCS), Trypsin EDTA, EZ-DNA were purchased from Biological Industries (Israel) and

other reagents used were analytical grade and used as received.

Cell Culture

HeLa was the first aneuploid epithelial-like cell line to be derived from human tissue and maintained continuously by serial cell culture. HeLa cells were cultured in DMEM-F12 medium supplemented with 10% FCS and 1% antibiotics (100 units of penicillin and 100 mg streptomycin) in a humidified incubator at 37ºC and in 5% CO_2 atmosphere. The cells were subcultured twice a week, using a dissociation medium, Trypsin-EDTA, pH 7.4 as buffer system [14].

Synthesis of Cu(II)-cyclam complex

Equimolar quantities of $Cu(ClO_4)_2.6H_2O$ and cyclam were mixed in methanol solution to react for 3 h in a water bath (*ca.* 60ºC). The resulting solution was dried by rotary evaporator and the single crystals were obtained from water [15]. Cu(II)-cyclam complex and the molecular structure of complex was given in Figure 1a. The complex was characterized by FTIR (FTIR 8000, Shimadzu, Japan).

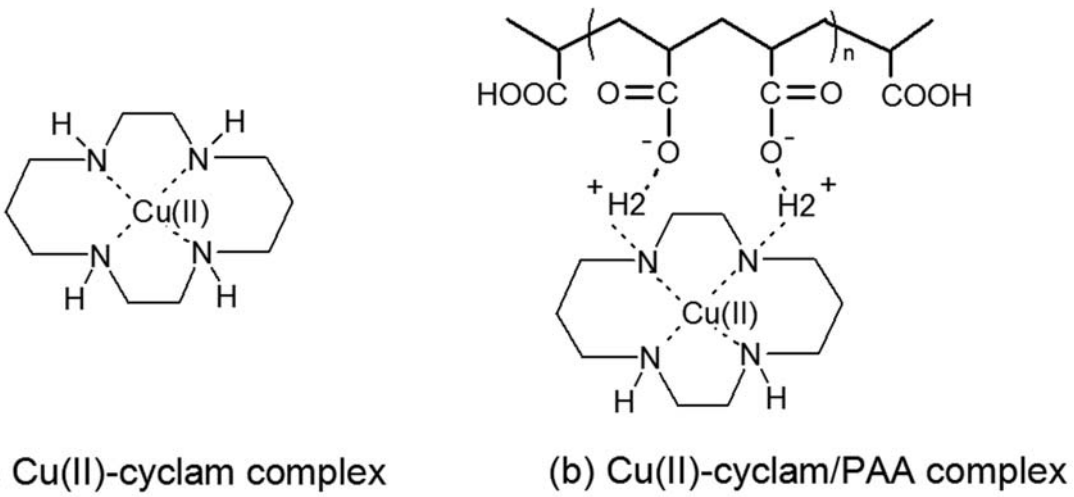
Synthesis of Cu(II)-cyclam/PAA complex

To increase the antitumor activity of Cu(II)-cyclam complex; the complex was attached to PAA and Cu(II)-cyclam/PAA (Figure 1b) complex was prepared. In the preparation of Cu(II)-cyclam/PAA complexes, the mole ratio of PAA was kept constant ($64.1x10^{-6}$) and mole ratio of Cu(II)-cyclam complex was changed between $1.9x10^{-4}$-$6.4x10^{-6}$. Cu(II)-cyclam/PAA complexes were precipitated by acetone. The amount of Cu(II) in the supernatants was measured by AAS (Unicam, 929, England). The Cu(II)-cyclam/PAA complex was characterized by FTIR and ^{1}H-NMR (Bruker, AC250, USA).

MTT-assay

The MTT-assay for cell injury is based on the ability of mitochondrial dehyrogenases of viable cells to reduce MTT-assay to a purple formazan product (insoluble in water) which can be quantified spectrophotometrically after solubilization in isopropanol containing 0.08 M HCl. The absorbance of these solutions was measured at 570 nm.

Cytotoxicity evaluation after Cu(II)-cyclam and Cu(II)-cyclam/PAA complexes exposure for complex concentrations 0 and 250 µg/ml were assessed by MTT-assay. HeLa cells were plated at 50 $x10^3$ cell/ml on 96-well plate. Twenty-four hours after seeding, complex solutions were added to the medium and the cultures were incubated at 37ºC. After the incubation renewed the medium and 13 µl/well MTT-assay solution was added (5 mg/ml in



(a) Cu(II)-cyclam complex (b) Cu(II)-cyclam/PAA complex

Figure 1. (a) Cu(II)-cyclam and (b) Cu(II)-cyclam/PAA complexes.

RPMI-1640 without phenol-red) and incubated for 4 h at 37ºC in dark. Then, the solutions were replaced by 100 µl isopropanol-HCl in order to dissolve the formazan crystals. The optical density was read on a microplate reader (Coulter, USA) at 570 nm. The results were expressed as the percentage of MTT-assay reduction, assuming the absorbance of control cells as 100% [14].

Agarose and SDS-PAGE electrophoresis

DNAs of the HeLa cells were isolated and agarose gel electrophoresis was performed to investigate DNA cleavage. HeLa cells were plated at $80x10^3$ cell/ml on 6-well plate. Twenty-four hours after seeding, complex solutions were added to the medium and the cultures were incubated for 24 h. After the incubation, cells were harvested and DNAs are isolated by EZ-DNA kit. Isolated DNAs are run on 2 % agarose gel.

To determine the biocompatibility, complexes are interacted with human serum and, then, SDS-PAGE electrophoresis was performed. Human serum (diluted with 0.9% NaCl, 1/10), the complexes (0-250 µg/ml) and PAA were interacted (in 0.9% NaCl buffer) for 2 h at 37ºC. At the end of the incubation time, 15 µl samples were loaded onto 8% SDS-PAGE gel and run for 4 h at 100 V. After the electrophoresis, the gel was photographed.

Cu(II)-cyclam and Cu(II)-cyclam/PAA complexes uptake

For in vitro Cu(II)-cyclam and Cu(II)-cyclam/PAA complexes uptake, the complexes were labelled with fluorescein in order to follow uptake of the complexes by HeLa cells. Cu(II)-cyclam/fluorescein (the mol ratio is 1/1) complex was formed at room temperature in 2 h and then Cu(II)-cyclam/fluorecein complex attached to PAA. Uptake of the resulting complexes by HeLa cells were photographed by fluorescence and optical microscopy and counted the total number of the cells with a hemocytometer (C.A. Hausser & Son Phila, USA) by fluorescence and optical light.

RESULT AND DISCUSSION

Infrared spectra of Cu(II)-cyclam and Cu(II)-cyclam/PAA complexes

Cu-cyclam complexes were prepared and chemical structure of the complex was examined by FTIR in the region of 4600-400 cm^{-1} and the samples were dispersed in KBr. The NH band is observed in cyclam at high enery (1519 cm^{-1}) which confirms the existence of hydrogen bonding. Cu(II) coordination weakens the NH bonding and as a consequence, the NH band should appear at lower energy and could be masked by the CH, CC, CN bands. The band at 437 cm^{-1}, observed only in the spectrum of the complex, can be ascribed by Cu-N interactions and a deformation because of the metal atom [16].

The Cu(II)-cyclam/PAA complex was characterized by FTIR in the 4600-400 cm^{-1} region. Cu(II)-cyclam and Cu(II)-cyclam/PAA were dispersed in KBr. In the spectrum, carbonyl from PAA appeared at 1610-1560 cm^{-1} and also at 1561 cm^{-1} from Cu(II)-cyclam/PAA complex. Cu-N band of Cu(II)-cyclam and Cu(II)-cyclam/PAA appeared at 437 cm^{-1}. In the literature, amine salt bands usually appear at 2700-2250 cm^{-1} (NH(II) stretching). In Cu(II)-cyclam complex, amine salt band also appeared at 2389 cm^{-1}. In addition, amine bands appeared at 1230-1100 cm^{-1} in Cu(II)-cyclam and Cu(II)-cyclam/PAA complex.

AAS

In the preparation of Cu(II)-cyclam/PAA complexes, the mole ratio of PAA was kept constant ($64.1x\ 10^{-6}$). Mole ratio of Cu(II)-cyclam/PAA complex changed between 0.2-5 was formed. The complex was precipitated by acetone. The amount of Cu(II) in the supernatant was measured by AAS. According

Table 1. AAS data of Cu(II)-cyclam/PAA complex

PAA/Cu(II)-cyclam (mol/mol)	*Non-attached Cu(II)-cyclam/ initial Cu(II)-cyclam (% mol)	Attached Cu(II)-cyclam/PAA (% mol)
1/5	9.39	47.32
1/2	40.79	82.19
1/1	77.65	78.30
1/0.5	80.00	40.31
1/0.2	78.24	15.80

* Reactions yield.

to AAS results; reaction yield was calculated as; the mole ratio of initial Cu(II)-cyclam to Cu(II)/cyclam in the final solution and the mol ratio of Cu(II)-cyclam to PAA.

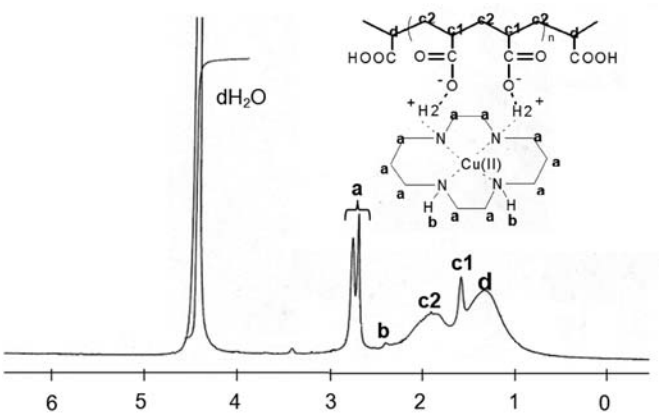


Figure 2. ^{1}H-NMR spectrum and chemical formula of Cu(II)-cyclam/PAA complex.

^{1}H-NMR spectrum Cu(II)-cyclam/PAA complex

A representative ^{1}H-NMR spectrum and chemical formula of Cu(II)-cyclam/PAA complex are presented in Figure 2 which shows that (in H_2O at 27ºC), δ ppm: (a) CH_2 2,7 (double peak) and (b) NH 2.4 (broad peak) for Cu(II)-cyclam unit. (d) CH and CH_2 1.3 (double partner peak) for acrylic acid unit. (c1) CH and (c2) CH_2 1.6-1.3 (attached Cu(II)-cyclam and acrylic acid).

According to ^{1}H-NMR spectrum of Cu(II)-cyclam/PAA complex (Table 2), the ratio of the attached to non-attached end group integral areas of Cu(II)-cyclam was calculated to find the attachment ratio of acrylic acid. The formulation is :

$$\frac{Am_{c1} + Am_{c2}}{Am_d} = \frac{0.0863 + 0.0478}{0.1318} = 1.017$$

$$\% \frac{Am_c/Am_d}{\Delta Am_c} = \frac{1.017}{2.017} 100 = 50.4\%$$

ΔAm_c: integral areas of attached acrylic acid (CH and CH_2) groups for Cu(II)-cyclam/PAA complex.

Am_d: integral areas of non-attached acrylic acid (CH and CH_2) groups for Cu(II)-cyclam/PAA complex.

Table 2. The result of ^{1}H-NMR spectrum of Cu(II)-cyclam/PAA complex.

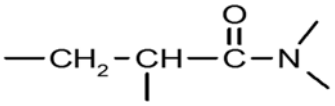
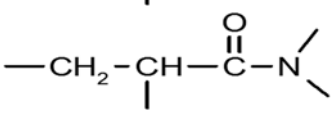
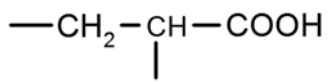
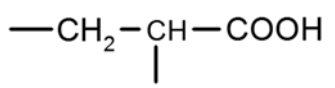
Functional groups	Monomer units		Chemical shifts (ppm)	Integral areas
CH_2 (a)	Cu(II)-cyclam	Cu(II)-cyclam/PAA	2.785-2.722 (double)	0.0717
NH (b)	Cu(II)-cyclam	Cu(II)-cyclam/PAA	2.409	0.0094
CH (c1)		Cu(II)-cyclam/PAA	1.610 (single)	0.0478
CH_2 (c2)		Cu(II)-cyclam/PAA	1.927-1.867 (broad)	0.0863
CH(d)		PAA	1.359-1.337 (broad)	0.0439
CH_2 (d)		PAA		0.0879

Table 3. In-vitro cyctotoxicity (HeLa cell line) of Cu(II)-cyclam, Cu(II)-cyclam/PAA complex and PAA.

Conc. (mg/ml)	Absorbance	Cell viability (%)
Control	0.539	100
PAA		
250	0.518	87.7
Cu(II)-cyclam		
10	0.527	97.7
50	0.503	93.9
100	0.471	87.4
250	0.396	73.4
Cu(II)-cyclam/PAA		
10	0.492	91.3
50	0.357	66.2
100	0.263	48.7
250	0.178	33.0

According to ^{1}H-NMR spectrum of 1/1 mole ratio of Cu(II)-cyclam/PAA complex; the ratio of the attached for Cu(II)-cyclam complex to carboxyl group of acrylic acid monomer was found to be 50%.

Anti-tumor effects of Cu(II)-cyclam and Cu(II)-cyclam/PAA complexes

To observe the antitumor activity of Cu(II)-cyclam and Cu(II)-cyclam/PAA complexes on HeLa cell lines, MTT-assay was performed. In this study, HeLa cells were treated different concentrations of Cu(II)-cyclam, Cu(II)-cyclam/PAA complexes and PAA. MTT-assay results were given in Table 3. As an example of the results; the toxicities of 250 mg/ml PAA, Cu(II)-cyclam, Cu(II)-cyclam/PAA complexes were found to be 12.3%, 26.6%, and 67%, respectively. As expected, more toxicities were observed using Cu(II)-cyclam/PAA complex. According to these results, Cu(II)-cyclam/PAA complex was found to behave as a good antitumor agent (probably due to higher positive charge and effects of polymer/metal chelate complex) on HeLa cell lines.

In-vitro complexes uptake

In this part of the study, Cu(II)-cyclam, Cu(II)-cyclam/PAA complexes were labelled with fluorescein in order to follow uptake of the complexes by the HeLa cell. Figure 3 gives optical and fluorescence microscopy photographs of labelled Cu(II)-cyclam and Cu(II)-cyclam/PAA complexes. According to the counting of the cells with a hemocytometer by optical and fluorecence microscopy (ratio of fluorecence cells count /optical cells count); the transfection ratio of Cu(II)-cyclam is around 34.2%, Cu(II)-cyclam/PAA is around 58.6%.

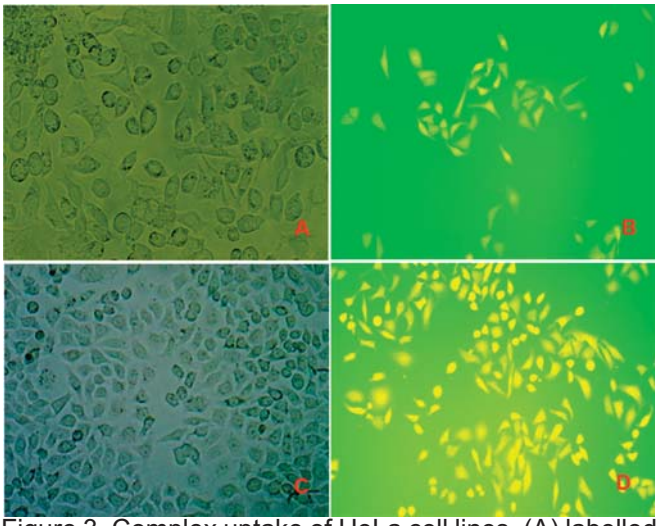


Figure 3. Complex uptake of HeLa cell lines, (A) labelled Cu(II)-cyclam (optical microscopy); (B) labelled Cu(II)-cyclam (fluorescence microscopy); (C) labelled Cu(II)-cyclam/PAA (optical microscopy); (D) labelled Cu(II)-cyclam/PAA (fluorescence microscopy), (10X20 magnificant).

DNA cleavage

Metal-chelate complexes can catalyze the hydrolysis of diphosphorester under physiological conditions, which would effectively cleave DNA [17,18]. We planned to investigate whether Cu(II)-cyclam, Cu(II)-cyclam/PAA complexes have interactions with DNA, so we treated the complexes and PAA with HeLa cells. The DNAs isolated from cells were analyzed by agarose gel electrophoresis (Figure 4). Line 1 is 250 mg/ml Cu(II)-cyclam/PAA, line 2 is 100 mg/ml Cu(II)-cyclam/PAA, line 3 is 250 mg/ml PAA, line 4 is 250 mg/ml Cu(II)-cyclam, line 5 is 100 mg/ml Cu(II)-cyclam, line 6 is control (not interacted with any complex). According to the electrophoresis result, 250 mg/ml concentration of Cu(II)-cyclam/PAA complex can cleave DNA. Neither PAA (Line 3) nor Cu(II)-cyclam (Line 3-4) complex can not cleave the cell DNAs.

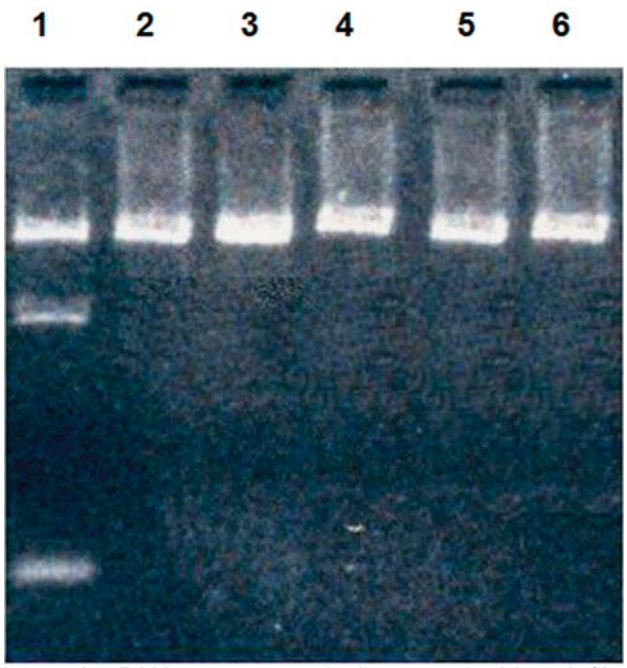


Figure 4. DNA agarose gel photograph: Line 1 (250 mg/ml) and Line 2 (100 mg/ml) Cu(II)-cyclam/PAA; Line 3 (250 mg/ml) PAA; Line 4 (250 mg/ml) and Line 5 (100 mg/ml) Cu(II)-cyclam; Line 6 Control.

Protein electrophoresis

To determine the biocompatibility of the complexes, different concentrations of the complexes and PAA were interacted with human serum and, then, SDS-PAGE electrophoresis was performed (Figure 5). In this experiment: 1/10 diluted (with 0.9% NaCl) human serum, different concentrations of the complexes and PAA were interacted and electrophoresis was performed. Then, gel was photographed. Line 1 is control (1/10 diluted human serum), line 2 is 100 µg/ml Cu(II)-cyclam/PAA, line 3 is 250 µg/ml Cu(II)-cyclam/PAA, line 4 is only Cu(II)-cyclam/PAA, line 5 is 250 µg/ml PAA, line 6 is 100 µg/ml Cu(II)-cyclam, line 7 is 250 µg/ml Cu(II)-cyclam. Line 2 and 3; it is likely to be; Cu(II)-cyclam/PAA complex has more positive charge, that's why probably the complex is binding the negatively charged human serum protein. However, during the electrophoresis, electric current weakened the interaction between Cu(II)-cyclam/PAA complex and human serum protein [19]. Line 4 is only Cu(II)-cyclam/PAA, that is why it is not apparent. PAA (Line 5) and Cu(II)-cyclam (Line 6 and 7) have no significant effects to human serum proteins.

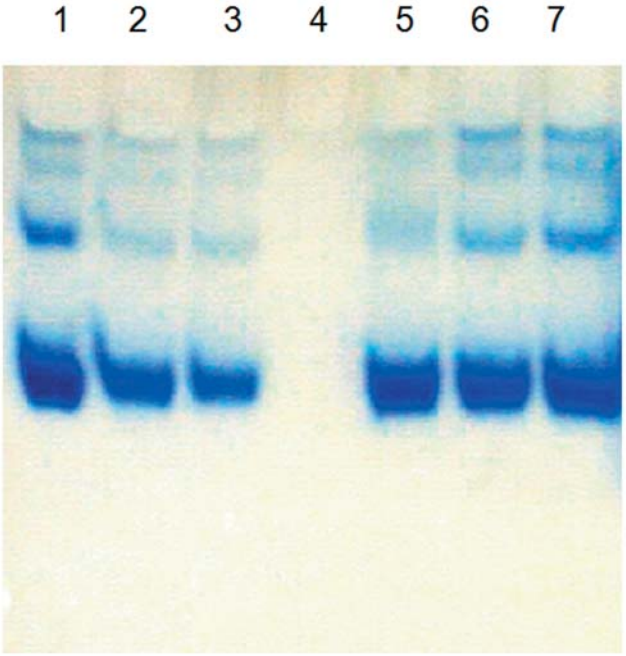


Figure 5. Protein gel electrophoresis: (1) control (1/10 diluted human serum); (2) 100 µg/ml Cu(II)-cyclam/PAA complex (3) 250 µg/ml Cu(II)-cyclam/PAA complex; (4) non-interacted Cu(II)-cyclam/PAA with human serum; (5) 250 µg/ml PAA; (6) 100 µg/ml Cu(II)-cyclam complex; (7) 250 µg/ml Cu(II)-cyclam complex.

CONCLUSIONS

Cyclam-based ligands and their metal complexes have shown antitumor activity. Water soluble Cu(II)-cyclam and Cu(II)-cyclam/PAA complexes which will be used as a chemotherapy agent. Cyclam reacted with copper(II) to form Cu(II)-cyclam complex. To increase the antitumor activity of Cu(II)-cyclam complex; the complex was linked to polyacrylic acid (PAA) and Cu(II)-cyclam/PAA complex was prepared. The Cu(II)-cyclam and Cu(II)-cyclam/PAA complexes were characterized by FTIR, ^{1}H-NMR and AAS. To observe, antitumor effects of Cu(II)-cyclam and Cu(II)-cyclam/PAA complexes to HeLa cell lines, MTT test was performed. DNAs of the HeLa cells were isolated and agarose gel electrophoresis was performed to investigate DNA cleavage. To determine the biocompatibility of the complexes, they were interacted with the human serum and analyzed by SDS-PAGE electrophoresis. FTIR and NMR data proved the formation of

complexes. The complex prepared with a Cu(II)-cyclam/PAA ratio of 1/1 mole ratio was used in cell culture experiments. According to the MTT test results, 100 µg/ml Cu(II)-cyclam and 100 µg/ml Cu(II)-cyclam/PAA were able to kill 12.4% and 51.4% of the cells in the culture, respectively. It was shown that 250 µg/ml Cu(II)-cyclam/PAA complex was able to cleave very effectively of the DNA. There were no significant effect of neither Cu(II)-cyclam, nor Cu(II)-cyclam/PAA and PAA on the human serum proteins.

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