Romanian Biotechnological Letters *Vol. 18, No.4, 2013*

ORIGINAL PAPER

Antitumor activity of polyelectrolytes-metal complexes

Received for publication, March 20, 2013
Accepted, May 5, 2013

MESUT KARAHAN[A*], S. ARDA OZTURKCAN[B], ZEYNEP MUSTAFAEVA[C]
[a]*Üsküdar University, Faculty of Engineering and Natural Sciences, Department of Bioengineering, 34662 Uskudar-Istanbul TURKEY, E-mail: mesut.karahan@uskudar.edu.tr Fax: +90 216 474 1256 Phone: +90 216 400 2222*
[b]*Yildiz Technical University, Faculty of Arts and Sciences, Department of Chemistry, 34220 Esenler-Istanbul Turkey, E-mail: turkcana@hotmail.com*
[c]*Yildiz Technical University, Faculty of Chemical and Metallurgical Engineering, Department of Bioengineering, 34220 Esenler-Istanbul Turkey, E-mail: zmustafaeva@yahoo.com*

Abstract

The interactions between anionic polyelectrolytes [polyacrylic acid, poly(methyl vinyl ether-co-maleic anhydrate)] and copper ions in aqueous solution (pH 7.0) were investigated by dynamic light scattering, UV-visible and fourier transform infrared spectroscopic techniques. Viscosity was also measured by ubbelohde automatic viscometer. Observations indicate that the ratio of ingredients, the nature of the metal ions and their environmental conditions affect the formation of soluble and insoluble Polyelectrolyte-Metal complexes. These binary complexes were tested in vitro as potential antitumor agents with Human Embryonic Kidney 293 cells. Depending on the ratio and sequence of mixing of components in the formation of Polyelectrolyte-Copper complexes, it was observed that the most stable water soluble polycomplexes prepared exhibited a high antitumor activity in the ratio of n_{Cu}^{2+}/n_{AA}: 0.4 and n_{Cu}^{2+}/n_{MVE-MA}: 0.5, respectively.

Key words: Biopolymer, copper ions, polyelectrolyte-metal complex, antitumor activity.

Introduction

Polyelectrolyte-Metal (PE-M) complexes, subject of many studies, are functional biopolymer systems that represent a specific class of PE-M compounds. Electrostatic interactions are the main forces in PE-M complex formation. The interactions of PE with M ions in aqueous solutions were found to be dependent on pH, ionic strength as well as the ratios of both M ions and PE. Transition M ions self-bind to water-soluble polymer ligands to complex, the M ions on the surface of the polymer. In recent studies, it has been shown that M ions (Cu^{2+}, Zn^{2+}, Fe^{2+}) complexed with functional polymers can play an important role in biological processes such as antitumor activity and immunoadjuvant properties (ZHAO & al. [1]; KARAHAN & al. [2, 3]; DING & al. [4]; ETAIW & al. [5]; ADRIANOW & al. [6]). For example, iron deficiency causes hemopoiesis, lymphopoiesis, morphological changing in the thymus as well as reduction of T and B dependent areas of spleen cells. As such, PE-M complexes can be used for drug delivery purposes *in vivo* biomedical studies. Carrier PE can also link to microbial and viral antigens to form a stable complex (or conjugate), shown to increase by several orders of magnitude the immune response to the organism affording effective immune protection (MUSTAFAEV [7]; MAN'KO & al. [8]; KENDRICH & al. [9]; LIU & al. [10]). Copper-Chitosan complexes have been investigated for their antitumor properties (ZHENG & al. [11]). Chitosan contains multiple amino, hydroxyl, and acetamide groups and it forms complexes with many M ions (MUZZARELI & al. [12]; VARMA & al. [13]). It is known that copper complexes interact with DNA, leading to chemically-induced cleavage of DNA and, thus, antitumor activity (HIRANO [14]; QIN & al. [15]; LIANG [16]) and M complexes are becoming increasingly important as biochemical, analytical,

antimicrobial, and anticancer agent reagents (PUNNIYAMURTHY & al. [17]; ROUTLER & al. [18]; GAO & al. [19]; TUMER & al. [20]; GOLCU & al. [21]; LI & al. [22]; MILLER [23]).

The aim of the present study is to understand the interaction two polyelectrolytes with copper ions in aqueous solution at pH 7.0 using dynamic light scattering, UV-visible and Fourier transform infrared (FT-IR) spectroscopic techniques and viscosity measurement alongside studies to investigate its antitumor activity.

Materials and Methods

Ultra-pure water was obtained from Millipore MilliQ Gradient System. Metal salt $CuSO_4.5H_2O$ was purchased from Merck (Darmstadt, Germany) and was dissolved in ultra-pure water. Tumor HEK 293 Cell line were obtained from ATCC/LGC Standards. Cell Proliferation Assay Kit (96 wells) were obtained from Cayman Chemical Company**.**

Spectrophotometric measurements for PE-M complexes were made on UV-Visible spectrophotometer Shimadzu UV-1700 Pharma Spec. FT-IR spectra were recorded with Shimadzu IR Prestige-21. The viscosity measurements were performed at a constant temperature of 25 °C with an Ubbelohde automatic viscometer (Schott Gerate, Berlin, Germany).

Dynamic Light Scattering Method

The average size, size distribution and zeta potential of PE and PE-M complexes were were investigated by using photon correlation spectroscopy with a Zetasizer Nano ZS instrument (Malvern Instruments, UK) equipped with 4.0 mV He–Ne laser at a wavelength 633 nm at a temperature 25 ^oC. Scattered light was detected at an angle 173^o using non-invasive back scattering (NIBS) technique. All solutions were filtered with 0.2 μm RC-membrane filters (Sartorius) before DLS measurement.

Preparation of PAA and poly(MVE-MA)

Polyacrylic acid (PAA) was synthesized and fractionated according to the methodology described in the literature (MILLER [23]). Polymer was prepared by radical polymerization of acrylic acid in toluene with benzoyl peroxide as the initiator. PAA was fractionated from 3% to 4% solution in methanol by fractional precipitation with ethyl acetate; (molecular weight 100 kDa). Poly(methyl vinyl ether-co-maleic anhydrate) [poly(MVE-MA)] was purchased, Mw:41 kDa Gantrez AN – 139 BF.

Preparation of PE-M Complexes

15 mg of the $CuSO_4.5H_2O$ was added into 1 mL of ultrapure water. Then, 3 mg of PE [PAA and poly(MVE-MA)] was dissolved in 1 mL of ultrapure water. These solutions were mixed and stirred at 20 ^0C for 24h. Then, the pH was adjusted to 7.0 by adding 1 N NaOH into solution. In order to follow the complexation of copper ions with the PE, the following measurements were performed.

The measurements were taken by means of dynamic light scattering (DLS), UV-Vis, Fourier transform infrared spectroscopy (FTIR) and viscosity , the mole ratio of copper ions to the PAA and poly(MVE-MA) was kept constant at $n_{Cu^{2+}} / n_{AA}$= 0.1, 0.2, 0.3, 0.4 and $n_{Cu^{2+}} / n_{MVE-MA}$= 0.1, 0.2, 0.3, 0.4, 0.5. The $n_{Cu^{2+}}/n_{AA}$ and $n_{Cu^{2+}}/n_{MVE-MA}$. Ratios were calculated using the following equation (1).

$$n = CN_A/M \quad (1)$$

Where n is the number of the molecules in 1 mL, M is the molecular weight of components, N_A is the Avogadro number and C represents concentration in g/100mL.

Potential of Antitumor Activity

The HEK 293 cell line was plated in a 96-well micro plate (100 µL/well) at a density of $1x10^5$ per well in ATCC growth medium. Background control wells contained the same volume of complete culture medium and were included in each assay. The micro plate was incubated for 24 h at 37 ^{0}C.

Cell proliferation assays were performed to count the number of living cells after adding 2-(2-methoxy-4-nitrophenyl)-3-(4-nitro-phenyl)-5-(2,4-disulfo-phenyl)-2*H*-tetrazolium (WST-8) and 1-methoxyphenazine methosulfate (1-methoxy-PMS). The cells were treated with copper complexes of differing ratios for 48 h. Cell viability was determined by WST-8 and 1-methoxy PMS, and presented as percentage of living cells. The inhibition ratio was quantified by the following equation (2);

$$IR\%= (C_0\text{-}C/C_0)\ 100\% \qquad (2)$$

Where C_0 is the absorbance value from the control group (without adding copper-PE complexes), C is the absorbance value from copper-PE complexes.

Results and Discussions

Complex formation can be seen in Figure 1 and Figure 2. In these spectra newly formed and vanished bands are evident for complex formation between Cu^{2+} and polymers, (PAA) and poly(MVE-MA) respectively. It is notable that in the regions 1550-1580 cm^{-1}, 1400 cm^{-1} and 1150-1170 cm^{-1} show nearly the same data as in the two PE-Cu^{2+} complex structures.

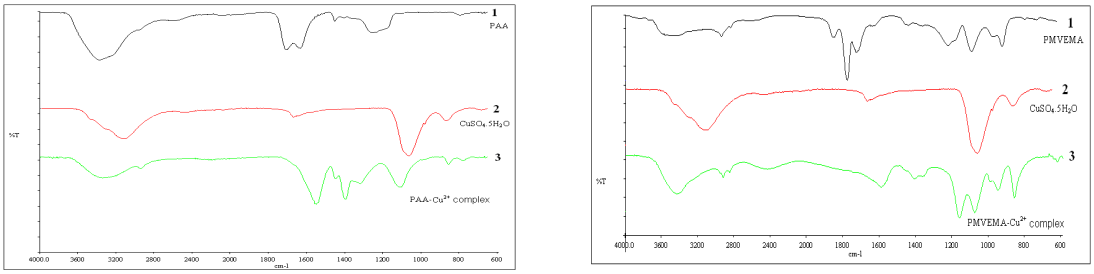
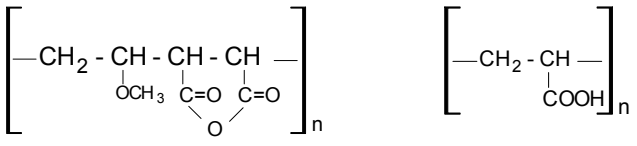


Figure 1. FT-IR analysis of these three structures shows differentiation in the carbonyl and finger print region.

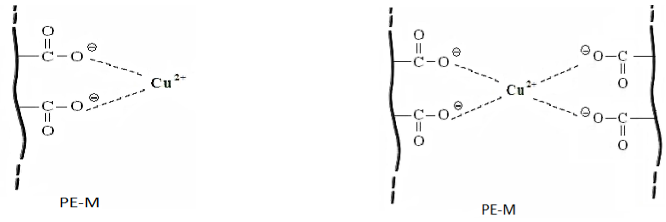
There are newly formed three bands (1550 cm^{-1}, 1400 cm^{-1}, 1100 cm^{-1}) in the spectra of PAA-Cu^{2+} complex structure.

Figure 2. FT-IR analysis of these three structures shows differentiation in the carbonyl and finger print region, again.

There are newly formed three bands (1580 cm^{-1}, 1400 cm^{-1}, 1170 cm^{-1}) in the spectra of poly(MVE-MA)-Cu^{2+} complex structure.



(a) (b)



(c) (d)

Figure 3. Structure of poly(MVE-MA) (a); PAA (b); PE-M (c) and PE-M-PE (d).

Different amounts copper ions binded to PAA and the absorbance was measured at 400 nm (MUSTAFAEV [7]). The purpose of these measurements was to define the binary complex ratio of water solubility. As shown in Figure 4, different proportions of copper were added to PAA and adjusted to pH 7.0 to initiate PE-M complex formation, confirmed with 400 nm absorbance values, $n_{Cu}^{2+}/n_{AA} \leq 0.4$. When the ratio to the PE-M binary complex of M increases, the rate increased slowly (water-soluble portion), a ratio $n_{Cu}^{2+}/n_{AA} > 0.4$ was observed to occur after the crash. It can be concluded that water soluble complex of PAA-M complex forms until the ratio n_{Cu}^{2+}/n_{AA}

≤ 0.4.

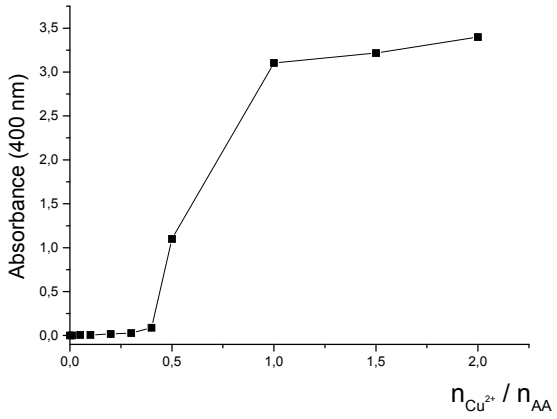
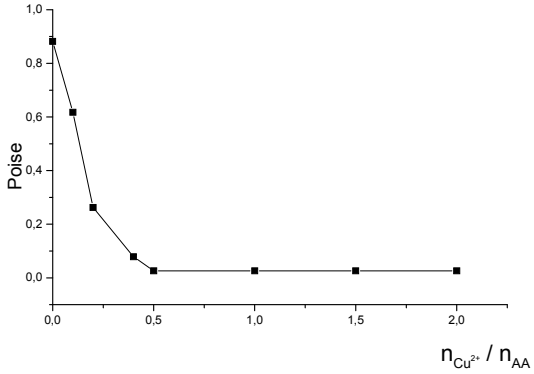


Figure 4. PAA-Cu^{2+} binary complex, depending on the ratio of optical density 400 nm: $0 \leq n_{Cu}^{2+}/n_{AA} \leq 2$.

PAA is a negatively charged, linear polymer at pH 7.0. The viscosity of a mixture of PAA falls with the addition of copper but after a certain rate remains constant. The reason for this is that when M ions are added to the polymer, it moves to the compact form. After a certain ratio of M ion was added, there was no further change in the structure and the complex viscosity remained constant.

Copper ions with decreasing hydrodynamic diameter were added to the polymer that have been, after beginning the increment has continued steadily. As the copper ions were added to the PE, the viscosity, and hydrodynamic diameter reduced and the compact form occurred as shown in Figure 5 (once measurement was done in water). If the ratio of copper increases, then the hydrodynamic diameter due to increased interaction between the PE of copper ions also increased without causing interference to the hydrodynamic diameter (Figure 6).



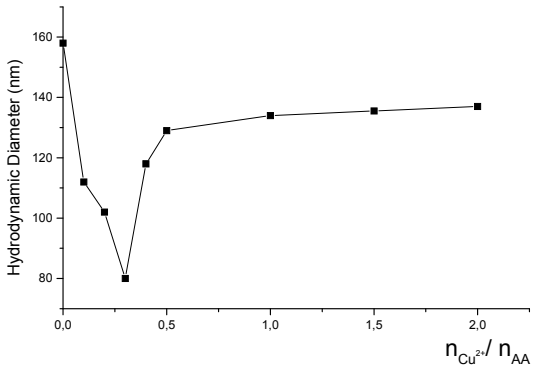


Figure 5. The rate of PAA-Cu^{2+} binary complex due to the viscosity.

Figure 6. The rate of PAA-Cu^{2+} binary complex due to the hydrodynamic diameter.

Figure 7 shows that as the $n_{Cu}{}^{2+}/n_{AA}$ ratio increases, the zeta potential approaches a positive (+) value. This indicates that the compact form occurred. As the ratio of copper ions increases, the interaction between polymers compacted ensuring a fall in the zeta potential towards a more negative shift. With the continuing addition of copper ions, the zeta potential remained stable and showed no interaction with the polymer.

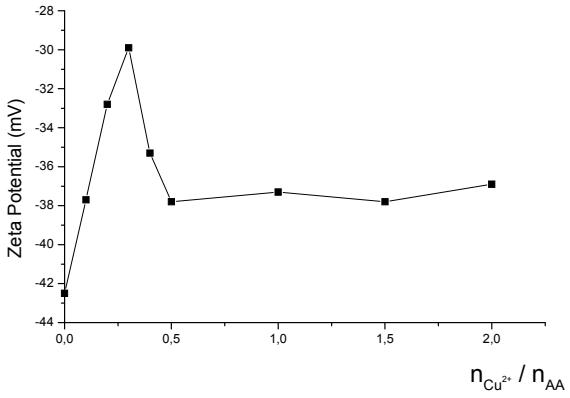
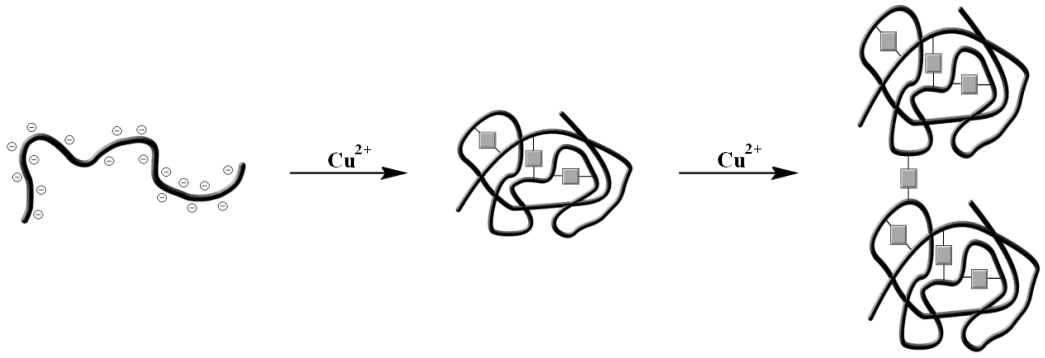


Figure 7. The rate of PAA-Cu^{2+} binary complex due to the zeta potential.

As a result, copper ions were added to the linear polymer to create the compact form. Increasing amounts of copper ions forming compact polymers were interconnected however, in the more complex structures, copper ions had no change. The results obtained in the light of PAA-Cu^{2+} binary complex structure of the model are shown (Scheme 1).



Scheme 1. Three-dimensional proposed structure model for PAA-Cu^{2+} binary complex. Filled square binding between COO^- groups in Cu^{2+} presence.

Various amounts of copper ion solution as well as PAA a certain rate were added to poly(MVE-MA) and adjusted to pH 7.0 in the system until the crash was not seen ($0 \leq n_{Cu^{2+}}/n_{MVE\text{-}MA} \leq 0.5$). As seen in Figure 8, different proportions of copper ions added to poly(MVE-MA) (at pH 7.0), PE-M complexes as confirmed by 400 nm absorbance values $n_{Cu^{2+}}/n_{MVE\text{-}MA} \leq 0.5$. The rate reduces slowly while (water-soluble portion), $n_{Cu^{2+}}/n_{MVE\text{-}MA} > 0.5$ however this point, the rate increases were larger. These binary complexes data indicate that the collapse had occurred at $n_{Cu^{2+}}/n_{MVE\text{-}MA} \leq 0.5$ indicating water soluble poly(MVE-MA)-M complex formation.

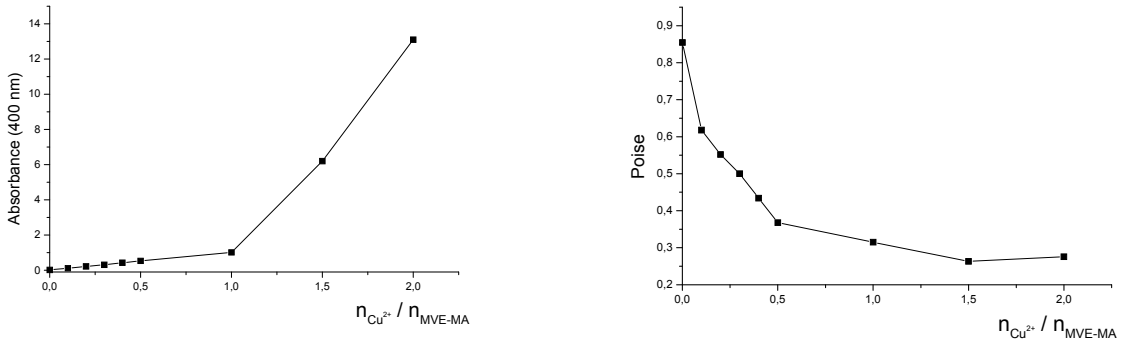
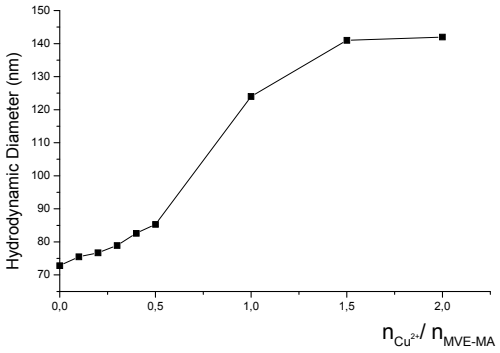


Figure 8. Poly(MVE-MA)-Cu^{2+} binary complex, depending on the ratio of optical density 400 nm: $0 \leq n_{Cu^{2+}}/n_{MVE\text{-}MA} \leq 2$.

Figure 9. The rate of poly(MVE-MA)-Cu^{2+} binary complex due to the viscosity in water.

Looking at the viscosity curve before the fall, then $n_{Cu^{2+}}/n_{MVE\text{-}MA} \geq 1.5$ was continuing stabile. Poly(MVE-MA) at pH 7.0 is a negatively charged linear polymer. With the addition of copper ions the polymer was a compact form. With further incremental increase of copper ions, the viscosity decreases. With continuing addition of copper ions, the structure of the complex did not change and the viscosity remained constant (Figure 9).

Increasing the copper ions ratio of increased the hydrodynamic diameter of the poly(MVE-MA) -Cu^{2+} complex. These ions became more compact while at the same time causing the polymer to bind. The diameter did not change because excess copper ions did not interact (Figure 10).



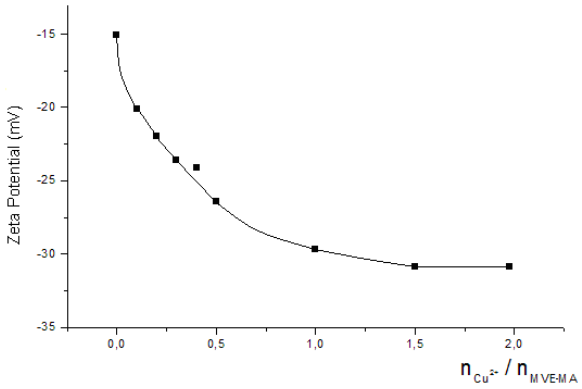
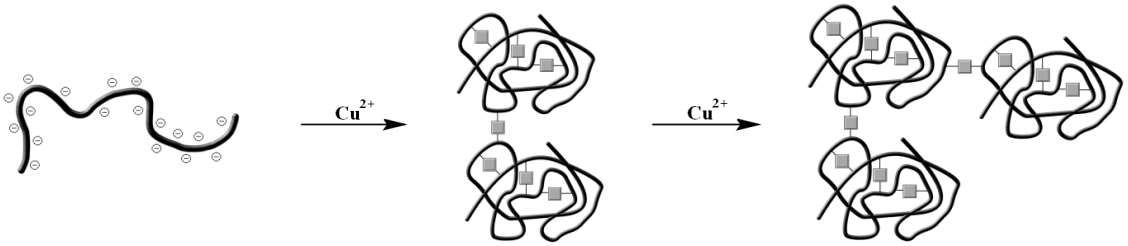


Figure 10 . The rate of poly(MVE-MA)-Cu^{2+} binary complex due to the hydrodynamic diameter.

Figure 11. The rate of poly(MVE-MA)-Cu^{2+} binary complex due to the zeta potential.

An incremental change in n_{Cu}^{2+}/n_{MVE-MA} rate resulted in a zeta potential shift towards a negative (-) value. By providing interaction between polymers the copper ions connected to each other. After adding copper ions the zeta potential remained constant and continued interaction with the polymer was not observed (Figure 11). As a result, poly(MVE-MA) binary complex was formed via a different mechanism than PAA (Scheme 2).



Scheme 2. Three-dimensional proposed structure model for poly(MVE-MA)-Cu^{2+} binary complex. Filled square binding between COO^- groups in Cu^{2+} presence.

The UV light absorption data indicated that there was a water soluble complex formation of PAA-M (n_{Cu}^{2+}/n_{AA}: 0.4) with a poly(MVE-MA)-M and (n_{Cu}^{2+}/n_{MVE-MA}: 0.5) complex ratio.

The proliferation assay results showed that all of the PAA and poly(MVE-MA) copper complexes inhibited the proliferation of the Human Embryonic Kidney (HEK) 293 tumor cell line (Table 1). In these experiments, the copper complexes of PAA were found to be more effective to HEK 293 tumor cell lines. The PAA-Cu (0.4) (n_{Cu}^{2+}/n_{AA}: 0.4) and poly(MVE-MA)-Cu (0.5) (n_{Cu}^{2+}/n_{MVE-MA}: 0.5) exhibited the highest antitumor activity, but PAA and poly(MVE-MA) were not effective. Of the four ratios tested, PAA-Cu (0.4) displayed a better antitumor efficiency. $CuSO_4$ exhibited antitumor activity at 10^{-3} mol/L, but when the copper ion was diluted to 10^{-4} or 10^{-5} mol/L, the percentage of inhibition decreased sharply to 3 or 5%.

Table 1. Inhibition of cell proliferation by Cu^{2+}, PAA, poly(MVE-MA), PE-Cu^{+2} complexes

Compound (mol/L)	Tumor Cell Line 293		
	10^{-3}	10^{-4}	10^{-5}
$CuSO_4$	88	5	3
PAA	30	4	3
PAA- Cu^{2+} (0.1)	82	33	6
PAA- Cu^{2+} (0.2)	83	35	6
PAA- Cu^{2+} (0.3)	86	38	7
PAA-Cu^{2+} (0.4)	90	47	12
Poly(MVE-MA)	24	2	2
Poly(MVE-MA)-Cu^{2+} (0.1)	80	30	5
Poly(MVE-MA)-Cu^{2+} (0.2)	81	31	6
Poly(MVE-MA)-Cu^{2+} (0.3)	84	32	6
Poly(MVE-MA)-Cu^{2+} (0.4)	85	42	10
Poly(MVE-MA)-Cu^{2+} (0.5)	88	43	11

Conclusions

PE can form stable binary complexes with copper ions. As the complex forms via copper ions, the polycomplex particles become friable structures in which protein molecules are practically exposed to the solution. On the basis of the obtained results, we can suggest hypothetical models. The study showed that the copper complexes of PE have antitumor activity, as indicated by the response of HEK 293 cells. Of the ratios tested, $n_{Cu^{2+}}/n_{AA}$: 0.4 and $n_{Cu^{2+}}/n_{MVE\text{-}MA}$: 0.5 displayed a better antitumor activity. Also, PAA and PAA-M showed greater antitumor activity than poly(MVE-MA) and poly(MVE-MA)-M . These results are important because they demonstrate the potential for developing antitumor agent and a new type of synthetic immunogens.

Acknowledgements

These projects were supported by grant from Turkish Republic Prime Ministry State Planning Organization (Project No: 25-DPT-04-07-05) and Yildiz Technical University, Coordination of Scientific Research Projects (Project No: 2011-01-02-GEP06).

References

1. X-Z. ZHAO, T. JIANG, L. WANG, H. YANG, S. ZHANG, P. ZHOU, Interaction of curcumin with Zn(II) and Cu(II) ions based on experiment and theoretical calculation, *J. Mol. Struct.*, **984**, 316, 325 (2010).
2. M. KARAHAN, Development of functional biopolymer systems containing metal, *Chemistry,* Yildiz Technical University, Istanbul, 2009.
3. M. KARAHAN, Z. MUSTAFAEVA, C. OZEROGLU, Investigation of Ternary Complex Formations of Polyacrylic Acid with Bovine Serum Albumin in the Presence of Metal Ions by Fluorescence and Dynamic Light Scattering Measurements, *The Protein Journal*, **29**, 336, 342 (2010).
4. N. DING, W. LIN, W. SUN, Z. SHEN, A novel hyperbranched aromatic polyamide containing bithiazole: Synthesis, metal complexation and magnetic properties, Science China Chemistry, **54**, 320, 325 (2011).
5. SE-DH. ETAIW, A. S. SULTAN, M.M. EL-BENDARY, In vitro and in vivo antitumor activity of novel

3D-organotin supramolecular coordination polymers based on CuCN and pyridine bases, *J. Organomet. Chem.*, **696**,1668, 1676 (2011).
6. A. ANDRIANOV, A. MARIN, D. DECOLLIBUS, Microneedles with Intrinsic Immunoadjuvant Properties: Microfabrication, Protein Stability, and Modulated Release, *Pharm, Res.*, **28**, 58, 65 (2011).
7. M.I. MUSTAFAEV, *Biopolymers*. Turkey: Tubitak (1996).
8. B.M. MAN'KO, E.A. SOKOLAVA, P.J. GAJIEV, M.I. MUSTAFAEV, *Immunologiya*, **1**, 75 (1991).
9. M.J. KENDIRCH, M.T. MAY, J. PM, D. DK, *Metal in Biological Systems*. New York: Ellis Harwood (1992).
10. C. LIU, M. WANG, T. ZHANG, H. SUN, DNA hydrolysis promoted by di- and multi-nuclear metal complexes, *Coord. Chem. Rev.*, **248**, 147, 168 (2004).
11. Y. ZHENG, Y. YI, Y. QI, Y. WANG, W. ZHANG, M. DU, Preparation of chitosan–copper complexes and their antitumor activity, *Bioorg. Med. Chem. Lett.*, **16**, 4127, 4129 (2006).
12. R.A.A. MUZZARELI, A. FERRERO, M. PIZZOLI, Light-scattering, X-ray diffraction, elemental analysis and infrared spectro- photometry characterization of chitosan, a chelating polymer, *Talanta*, **19**, 1222, 1226 (1972).
13. A.J. VARMA, S.V. DESHPANDE, J.F. KENNEDY, Metal complexation by chitosan and its derivatives: a review, *Carbohydr. Polym.*, **55**: 77, 93 (2004).
14. S. HIRANO, Industrial Biotechnological Polymers Technomic, Lancaster. (1995)
15. C. QIN, Y. DU, L. XIAO, Z. LI, X. GAO, Enzymic preparation of water-soluble chitosan and their antitumor activity, *Int. J. Biol. Macromol.*, **31**, 111, 117 (2002).
16. F. LIANG, C. WU, H. LIN, Copper complex of hydroxyl-Substituted triazamacrocyclic ligand and its antitumor activity, *Bioorg. Med. Chem. Lett.*, **13**, 2469, 2472 (2003).
17. T. PUNNIYAMURTHY, M. MADHAVA REDDY, S.S. KALRA, J. IQBAL, Cobalt(ii) Schiff base catalyzed biomimetic oxidation of organic substrates with dioxygen, *Journal of Pure Applied Chemistry*, **68**, 619, 622 (1996).
18. S. ROUTIER, J-L. BERNIER, J-P. CATTEAU, Synthesis, DNA Binding, and Cleaving Properties of an Ellipticine−Salen·Copper Conjugate, *Bioconjugate Chem.*, **8**, 789, 792 (1997).
19. J. GAO, F.R. WOOLLEY, R.A. ZINGARO, In Vitro Anticancer Activities and Optical Imaging of Novel Intercalative Non-Cisplatin Conjugates, *J. Med. Chem.*, **48**, 7192, 7197 (2005).
20. M. TUMER, H. KOKSAL, S. SERIN, M. DIGRAK, Antimicrobial activity studies of mononuclear and binuclear mixed-ligand copper(II) complexes derived from Schiff base ligands and 1,10-phenanthroline, *Transition. Met. Chem.*, **24**, 13, 17 (1999).
21. A. GOLCU, M. TUMER, H. DEMIRELLI, R.A. WHEATLEY, Cd(II) and Cu(II) complexes of polydentate Schiff base ligands: synthesis, characterization, properties and biological activity, *Inorg. Chim. Acta.*, **358**, 1785, 1797 (2005).
22. Y-H. LI, B-D. WANG, Z-Y. YANG, Infrared and DNA-binding on ultraviolet and fluorescence spectra of new copper and zinc complexes with a naringenin Schiff-base ligand, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, **67**, 395, 401 (2007).
23. M.L. MILLER, *Encycl. Polym. Sci.*, **1**, 445 (1978).