Physicochemical Properties of Protoporphyrin IX by Metal Ions in Acetonitrile-Water Mixture Solution

Ki-Min Bark,[†] Jeong-Im Yang, Ho-Suk Lee, Jee-Bum Lee,[‡] Chul-Ho Park,[§] and Hyoung-Ryun Park[*]

Department of Chemistry, Chonnam National University, Gwangju 500-757, Korea. [*]E-mail: hrpark@chonnam.ac.kr
[†]Department of Chemical Education and Research Institute of Life Science, Gyeongsang National University, Chinju 660-701, Korea
[‡]Department of Dermatology, Chonnam National University Medical School, Gwangju 501-757, Korea
[§]Department of Cosmetic Science, Nambu University, Gwangju 506-706, Korea
Received March 15, 2010, Accepted April 23, 2010

The UV-vis absorption spectrum of protoporphyrin IX shows a very sharp and strong absorption maximum peak at 398 nm in acetonitrile-water mixture solution (1:1 v/v). When divalent metal ions such as Cu^{2+}, Zn^{2+}, and Ca^{2+} ion were added to protoporphyrin IX, metal protoporphyrin IX complexes were thereby produced. Cu-protoporphyrin IX complexes have the largest formation constant (K_f) among them. The fluorescence intensity of protoporphyrin IX was diminished by the presence of Cu^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, and Ni^{2+} ions as quenchers. However, Mg^{2+}, Mn^{2+}, and Ni^{2+} ions are hardly combined with protoporphyrin IX. Mg^{2+} ion does not take part in the fluorescence quenching process of protoporphyrin IX in acetonitrile-water mixture solution. According to the Stern-Volmer plots, fluorescence quenching by Cu^{2+}, Zn^{2+}, and Ca^{2+} ions involves static quenching, which is due to complex formation. On the contrary, dynamic quenching has a large influence on the overall quenching process, when Mn^{2+} and Ni^{2+} ions were added to protoporphyrin IX in aceto-nitrile-water mixture solution.

Key Words: Protoporphyrin IX, Metal complex, Formation constants, Divalent metal ions, Fluorescence quenching

Introduction

Porphyrin derivatives contain pyrrole rings, five membered heterocycles. These compounds have properties of a conjugated diene and of an amine. Delocalization of π electrons stabilizes the ring and their ultra-violet absorption maximum peak shifts to a longer wavelength. They can easily absorb visible light, and thus can utilize sunlight directly as a good photosensitizer. Recently, photodynamic therapy has been used in the management of various malignant tumors, including cancers of the skin, gastrointestinal tract, lung and uterus.[1] It is, however, necessary to use an appropriate photosensitizer and light source to enhance the efficiency of photodynamic therapy. One of the most important porphyrin derivatives is protoporphyrin IX, which has attracted considerable attention for use in photodynamic therapy of skin tumors.[2-4] Protoporphyrin IX is produced in the haem biosynthesis pathway of biological systems.[1,5] It is normally formed *in vivo* from the reactants glycine and succinyl-coenzyme A in the mitochondria and then converted into the actual photosensitizer, protoporphyrin IX.[6] When protoporphyrin IX is electronically excited by the absorption of light, the substance emits fluorescence strongly and photobleaches rapidly under formation of singlet oxygen. It induces cell damage that may lead to tumor cell death.[7-10]
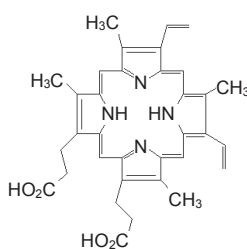
There are various divalent metal ions such as Cu^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, that are vital for biological functions, such as gene expression, apoptosis, enzyme regulation and neurotransmission.[11,12] These divalent metal ions have acidic properties, and may combine with Lewis-basic compounds to form acid-base complexes.[13-16] As a result, the photosensitizing ability of proto-porphyrin IX can be diminished in biological systems. Therefore, it is important to study the photophysical and photochemical properties of protoporphyrin IX, because this investigation can provide essential knowledge in our understanding of the photosensitizing activity of protoporphyrin IX. It is not easy to study its *in vivo* properties directly. Several studies have therefore been carried out using systems that mimic biological environments such as aerosol-OT (AOT) reverse micelle or aqueous-organic solvent mixture. Solvent mixture of H_2O-CH_3OH and H_2O-CH_3CN have been found suitable to emulate biological conditions, because they simultaneously show low polarity and a partially aqueous content, which is always present in biological systems.[17-19] In this work, we examined the formation of metal complex between protoporphyrin IX with several divalent metal cations such as Cu^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, and Ni^{2+}. In addition, the fluorescence quenching of protoporphyrin IX by metal ions was also studied, because the metal ions can cause fluorescence quenching.

Experimental Section

Protoporphyrin IX as well as metal ion compounds such as $CuCl_2 \cdot 2H_2O$ and $ZnCl_2$ were purchased from the Sigma Chemical Co. (St. Louis, U.S.A.) and were used without further purification.

The chemicals used as metal cations, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, and $NiCl_2 \cdot 6H_2O$ were obtained from the Junsei Chemical Co. (Japan, ACS reagent grade) and were used as received. Acetonitrile was purchased from Fluka Chemical Co (HPLC grade). Porphyrin IX was very slightly soluble in

Protoporphyrin IX

Scheme 1

Figure 1. UV-vis absorption spectra of the 4.36×10^{-5} M protoporphyrin IX reacted with various concentration of Zn^{2+} ion in acetonitrile-water mixture solution (1:1 v/v).

water, whereas it was soluble in acetonitrile. The solubility of protoporphyrin IX is also very low when added directly to acetonitrile-water mixture solution (1:1 v/v). Therefore, we prepared two kinds of solutions to prepare protoporphyrin IX containing metal ions in acetonitrile-water mixture solution; protoporphyrin IX was dissolved in acetonitrile and divalent metal ions were dissolved in distilled water, which was obtained by passing distilled water through a Barnstead (U.S.A.) Nanopure II deionization system. Each of the solutions was mixed in a test tube and then shaken using an ultrasonic cleaner. A low-concentration solution (below 3.6×10^{-5} M) was used as the sample in order to avoid any solute-solute interactions and self-absorption. The UV-vis absorption spectra of the solutions were then taken using a UV-vis Spectrophotometer (Uvikon, model 943, Italy). The formation constant (K_f) of the metal complexes was obtained using the equation proposed by Sanchez *et al.*, who reported a spectrophotometric method for determining the formation constant of the form $A_m B_n$ for metal complexes.[20] Apparent complex formation constants were calculated from the difference in absorbance at the new peak on the UV-vis absorption spectra after addition of divalent metal ions. The concentration of protoporphyrin IX was held constant while the concentration of divalent metal cation was raised from zero until no further change in the difference spectrum was detected. The divalent cation formation constant (K_f) can be obtained from the following expression,

$$\log \frac{1}{[B]} = \frac{1}{n} \log \left(\frac{1}{K_f} ma^{m-1} \right) + \frac{m}{n} \log \left(\chi^{1/m} - \chi^{1/m-1} \right)$$

where [B] is the metal cation concentration. m and n indicate reaction coefficient of the form $A_m B_n$ of metal complexes. χ is the ratio A_0/A, where A is the absorbance for a given concentration of divalent cation (A) at the new peak on the UV-vis absorption spectra after addition of divalent metal ions. A_0 is the absorbance for the saturating concentration by a divalent cation. The formation constant (K_f) was calculated by the plot of log $1/[B]$ as a function of log $(\chi^{1/m} - \chi^{1/m-1})$. The steady-state fluorescence emission spectra were obtained on a varian Cary Eclipse spectrofluorometer with 10 nm slits at room temperature.

Results and Discussion

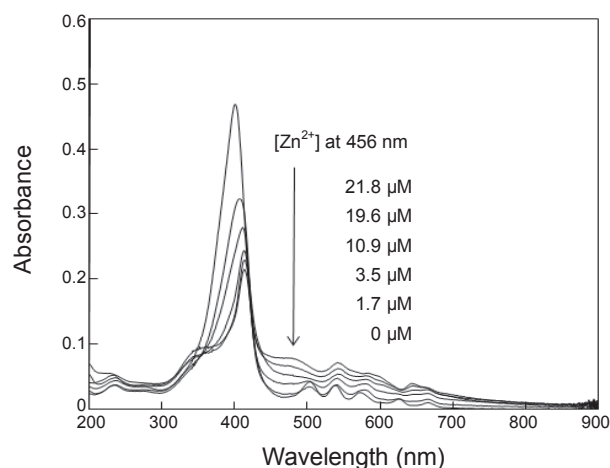To investigate the formation of the metal complex between

protoporphyrin IX and divalent metal ions in acetonitrile-water mixture solution (1:1 v/v), an aliquot of Zn^{2+} ion dissolved in neat water was added to 8.72×10^{-5} M protoporphyrin IX in acetonitrile. The solution was then examined by measuring UV-vis absorption spectra as a function of the reaction time. As shown in Figure 1, the UV-vis absorption spectrum of protoporphyrin IX reveals a very sharp and strong absorption maximum peak at 398 nm in acetonitrile-water mixture solution. However, the absorbance at this wavelength changed as the reaction occurred after mixing the two kinds of solutions. It did not change after treatment of the solutions with an ultrasonic cleaner for 2 hours. This implies that the reaction between metal ions and protoporphyrin IX under these experimental conditions proceeded very slowly, needing about 2 hours to complete.

Figure 1 shows different absorption properties of the UV-vis absorption spectrum of protoporphyrin IX in acetonitrile-water solution depending on the concentration of Zn^{2+} ion added. The UV-vis absorption spectra of 4.36×10^{-5} M protoporphyrin IX shifted to a longer wavelength as the concentration of Zn^{2+} ion increased. In addition to this, the absorbances at the absorption maximum peak of 398 nm were decreased and a new absorption peak appeared at 456 nm. This means that protoporphyrin IX reacted with Zn^{2+} ion in an acetonitrile-water mixture solution. Moreover, protoporphyrin IX has one isosbestic point at 422 nm on the UV absorption spectra obtained within the variation of the concentration of Zn^{2+} ion, which suggests that there are two kinds of chemical species and the reaction has reached equilibrium. One is protoporphyrin IX and another a metal protoporphyrin IX complex, because the other chemical species in the solution were not absorbed in the given wavelength region. These results are very similar to the case of Cu^{2+} and Ca^{2+} ions. However, in the case of Mg^{2+}, Mn^{2+}, and Ni^{2+} ions, the absorbance at the absorption maximum peak of protoporphyrin IX was found to be nearly the same value and the peak had not shifted, even though the concentration of the metal ions in the solution increased. From these results, we can suggest that protoporphyrin IX was combined with divalent metal ions such as Cu^{2+}, Zn^{2+}, and Ca^{2+} ions in acetonitrile-water mixture solution,

Physicochemical Properties of Protoporphyrin IX

*Bull. Korean Chem. Soc.* **2010**, Vol. 31, No. 6   1635

Table 1. Apparent complex formation constants of protoporphyrin IX with divalent cations in acetonitrile-water mixtures (1:1 v/v). The uncertainty is less than 7%

Divalent metal cation	Formation constant
Zn^{2+}	2.69×10^3
Ca^{2+}	4.47×10^3
Cu^{2+}	3.47×10^6
Mn^{2+}	very small
Mg^{2+}	very small
Ni^{2+}	very small

thereby producing metal protoporphyrin IX complexes. Mg^{2+}, Mn^{2+}, and Ni^{2+} ions, on the other hand, may have hardly combined with protoporphyrin IX.

The formation constant (K_f) of the metal complexes was determined using the equation proposed by Sanchez *et al.*[20] As shown in Figure 1, new absorption peaks appeared at 456 nm in the acetonitrile-water mixture solution, caused by the formation of the Zn-protoporphyrin IX complex. So, we used the wavelength of 456 nm as the analytical wavelength for the determination of the formation constant of the Zn-protoporphyrin IX complex. In the cases of the Cu^{2+} and Ca^{2+} ions, we used the wavelengths of 502 nm and 470 nm, respectively, as the analytical wavelength for the determination of their formation constants. The plot of log 1/[B] *vs.* log ($\chi^{1/m} - \chi^{1/m-1}$) showed a liner slope in the case of m = 1 and n = 1 under our experimental condition. Therefore, it can be assumed that the Zn^{2+} ion will form 1:1 complex with protoporphyrin IX. This result is very similar to the case of Cu^{2+} and Ca^{2+} ions and the formation constant (K_f) of the metal complexes are summarized in Table 1. Cu^{2+} ion was found to have large K_f values compared with the other metal ions. The Cu^{2+} ion is one of the most well known examples of an ion that forms square planar complexes.[21] Protoporphyrin IX occupies four coordination positions and thus behaves as if it were four molecules of pyrrole tied together. There are a number of circumstances in which square planar geometry is formed in the combination of Cu^{2+} ion and protoporphyrin IX. The Cu-protoporphyrin IX complex could be stabilized by the Jahn-Teller effect, which would be the reason why the Cu^{2+} ion was found to have a large formation constant compared with the other metal ions.

The steady state fluorescence emission spectra of 4.36×10^{-5} M protoporphyrin IX were measured as a function of the concentration of Zn^{2+} ion in acetonitrile-water mixture solution (1:1 v/v). As shown in Figure 2, the fluorescence emission spectrum of protoporphyrin IX contains three bands in acetonitrile-water solution: a strong peak at 631 nm and two small peaks at 582 nm as well as 694 nm. The fluorescence spectra of protoporphyrin IX were quite sensitive to the physicochemical properties of the environments. When Zn^{2+} ion was added to protoporphyrin IX in acetonitrile-water mixture solution, the fluorescence intensity of the small emission maximum peak at 582 nm increased, whereas that of the other emission maximum peaks such as 631 nm and 694 nm decreased. In particular, the emission maximum peak at 694 nm disappeared as the concentration of Zn^{2+} ion increased, supporting the conclusion that protoporphyrin IX combined with Zn^{2+} ion to form the Zn-protoporphyrin IX complex.
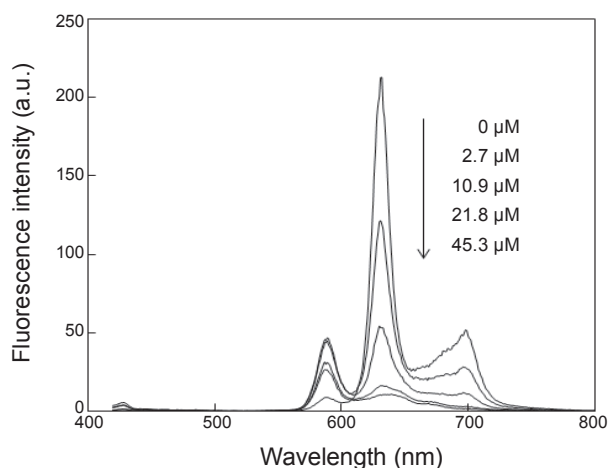
Figure 2. Fluorescence spectra (excitation at 399 nm) of the 4.36×10^{-5} M protoporphyrin IX reacted with various concentration of Zn^{2+} ion in acetonitrile-water mixture solution (1:1 v/v).
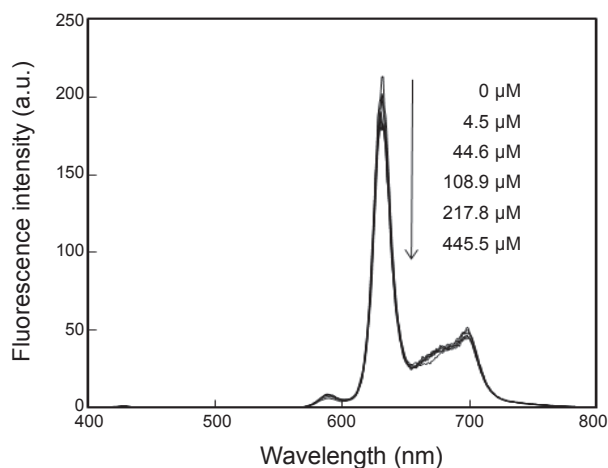
Figure 3. Fluorescence spectra (excitation at 399 nm) of the 4.36×10^{-5} M protoporphyrin IX reacted with various concentration of Mg^{2+} ion in acetonitrile-water mixture solution (1:1 v/v).

Furthermore, the fluorescence intensities of protoporphyrin IX decreased as the concentration of Zn^{2+} ion increased, which means that Zn^{2+} ion acted as fluorescence quencher. Fluorescence quenching refers to any process that decreases the fluorescence intensity of a given substance. Quenching processes involve a chemical interaction of the fluorophore and quencher.[22,23] One such process is static quenching, whereby a non-fluorescent ground-state complex is formed between a ground-state fluorophore and quencher. Another important quenching mechanism is collisional or dynamic quenching, which involves the collision and subsequent formation of a transient complex between an excited state fluorophore and a ground-state quencher. In addition to the Zn^{2+} ion, other metal ions such as Cu^{2+}, Ca^{2+}, Mn^{2+}, and Ni^{2+} also diminished the fluorescence intensities of protoporphyrin IX in acetonitrile-water mixture solution. However, the fluorescence intensities of protoporphyrin IX hardly changed, as shown in Figure 3, although the concentration of the Mg^{2+} ion increased, indicating that the Mg^{2+} ion did not take part in the fluorescence quenching process of protopor-
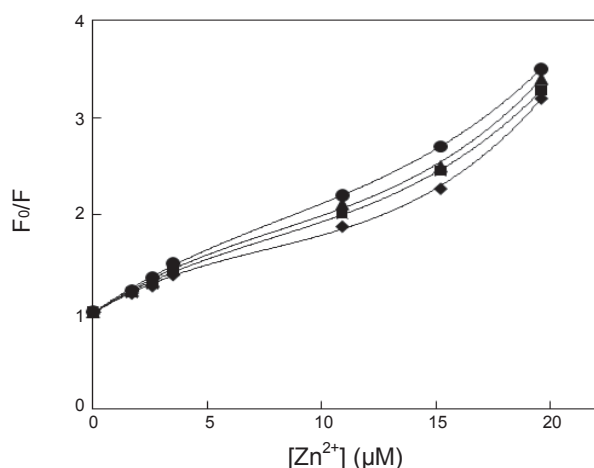
Figure 4. Stern-Volmer plots for the quenching of protoporphyrin IX fluorescence by Zn^{2+} ions at various temperature in acetonitrile-water mixture solution (1:1 v/v): 10 °C(●), 20 °C(▲), 40 °C(■), 50 °C(♦).

Figure 5. Stern-Volmer plots for the quenching of protoporphyrin IX fluorescence by Ni^{2+} ions at various temperature in acetonitrile-water mixture solution (1:1 v/v): 10 °C(●), 20 °C(▲), 40 °C(■), 50 °C(♦).
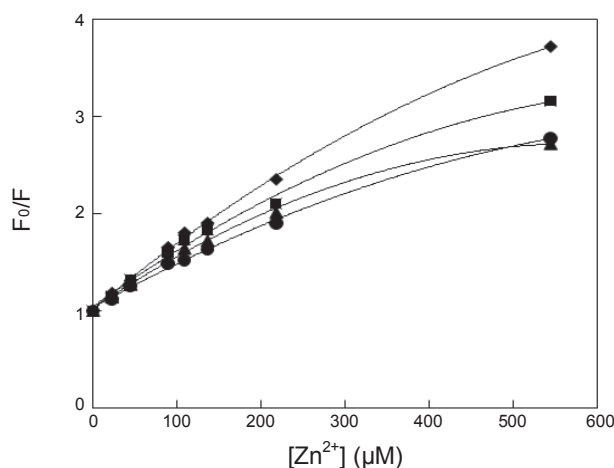
phyrin IX in acetonitrile-water mixture solution. The reason might be explained in terms of ionic potential, is defined as the charge to size ratio(q/r).[24] The Mg^{2+} ion has a relatively a large ionic potential and thus will be strongly hydrated by water. It is, therefore, difficult to form the metal complex by the hydrated magnesium cation. Static quenching was hardly observed in this experimental system. Moreover, the hydrated magnesium cation has a relative large size in solution, and consequently its diffusion makes slow progress. Dynamic quenching is diffusion controlled because the quencher must diffuse to the fluorophore during the lifetime of the excited state.[23] As a result, neither static quenching nor dynamic quenching were observed in the experimental system.

To investigate the fluorescence quenching process of protoporphyrin IX by the divalent metal cations, Stern-Volmer plots of protoporphyrin IX by Cu^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, and Ni^{2+} ion were studied. Figure 4 and 5 show the Stern-Volmer plots of protoporphyrin IX by Zn^{2+} and Ni^{2+} ions, respectively. As shown

in Figure 4, the change in the fluorescence intensity of protoporphyrin IX as a function of the concentration of Zn^{2+} ion, (Fo/F) *vs.* [Zn^{2+}], decreases roughly as the temperature of the sample solution increases. This suggests that static quenching has a large influence on the overall quenching process. Increased temperature is likely to reduce the stability of the complexes, resulting in lower static quenching constants. The result is quite similar to the cases of Cu^{2+} and Ca^{2+} ions. When Cu^{2+} ion was added to protoporphyrin IX, especially sensitive fluorescence quenching occurred, even at very low concentrations (below 1×10^{-5} M). This is related to the formation of the Cu-Protoporphyrin IX complex which is also sensitive to fluorescence quenching. It is also evidence of formation of the Cu-Protoporphyrin IX complex. On the other hand, the change in the fluorescence intensity of protoporphyrin IX as a function of the concentration of Ni^{2+} ion increases as the temperature of the sample solution increases. Because dynamic quenching depends upon diffusion, it is expected to be proportional to temperature. Therefore, dynamic quenching will occur more with increased temperatures. This suggests that when Mn^{2+} and Ni^{2+} ions are added to protoporphyrin IX in acetonitrile-water mixture solution, dynamic quenching has a large influence on the overall quenching process. The reason why fluorescence quenching of protoporphyrin IX by Mn^{2+} and Ni^{2+} ion progress predominantly by the dynamic quenching process, is unclear in these experimental results. Further studies will be performed to investigate these phenomena.

Conclusion

The UV-vis absorption spectrum of protoporphyrin IX shows a very sharp and strong absorption maximum peak at 398 nm in acetonitrile-water mixture solution (1:1 v/v). When metal cations such as Cu^{2+}, Zn^{2+}, and Ca^{2+} were added to protoporphyrin IX, the UV-vis absorption spectra of protoporphyrin IX were shifted to a longer wavelength and their shapes were also changed, indicating that protoporphyrin IX reacted with metal cations such as Cu^{2+}, Zn^{2+}, and Ca^{2+} ion. From these results, it can be concluded that protoporphyrin IX combines with divalent metal ions such as Cu^{2+}, Zn^{2+}, and Ca^{2+} ions in acetonitrile-water mixture solution and metal protoporphyrin IX complexes were thereby produced. On the other hand, Mg^{2+}, Mn^{2+}, and Ni^{2+} ions may hardly combine with protoporphyrin IX. The formation constant (K_f) of Zn-protoporphyrin IX and Ca-protoporphyrin IX complexes were found to be 2.69×10^3 and 4.47×10^3, respectively. However, that of Cu-protoporphyrin IX had the relatively large value of 3.47×10^6, possibly because Cu^{2+} ion enhances the stability of the metal complex by the Jahn-Teller effect.

The steady state fluorescence emission spectra of protoporphyrin IX were quite sensitive to the physicochemical properties of the environments. When Cu^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, and Ni^{2+} ion were added to protoporphyrin IX in acetonitrile-water mixture solution, the divalent metal ions act as a fluorescence quencher diminishing the fluorescence intensity of protoporphyrin IX. However, Mg^{2+} ions did not take part in the fluorescence quenching process of protoporphyrin IX in acetonitrile-water mixture solution. The quenching mechanism of Cu^{2+}, Zn^{2+}, and Ca^{2+} ions are somewhat different from that of the Mn^{2+} and Ni^{2+} ions.

Fluorescence quenching by Cu^{2+}, Zn^{2+}, and Ca^{2+} ions involves static quenching, which is due to complex formation. However, dynamic quenching has a large influence on the overall quenching process, when Mn^{2+} and Ni^{2+} ion were added to protoporphyrin IX in acetonitrile-water mixture solution.

References

1. Otake, M.; Nishiwaki, M.; Kobayashi, Y.; Baba, S.; Kohno, E.; Kawasaki, T.; Fujise, Y.; Nakamura, H. *British . J. Cancer* **2003**, *89*, 730.
2. Lee, J. B.; Choi, J. Y.; Chun, J. S.; Yun, S. J.; Lee, S. C.; Oh, J.; Park, H. R. *British . J. Cancer* **2008**, *159*, 61.
3. Berlin, N. I.; Neuberger, A.; Scott, J. J. *Biochemistry* **1956**, *64*, 80.
4. Kloeck, J.; Akkermans, W.; Beijersbergen van Henegouwen, G. M. J. *Photochem. Photobiol.* **1998**, *67*, 150.
5. Kenedy, J.; Pottier, R.; Pross, G. *Photochem. Photobiol. B* **1990**, *6*, 143.
6. Zensen, V.; Zankl, H. *Cancer Letters* **2003**, *202*, 35.
7. Georgakoudi, I.; Forster, T. H. *Photochem. Photobiol.* **1998**, *67*, 612.
8. Gross, G. *Intervirology* **1997**, *40*, 368.
9. Beutner, K. R.; Ferenczy, A. *Am. J. Med.* **1997**, *102*, 28.
10. North, J.; Neyndorff, H.; King, D.; Levy, J. G. *Blood Cells* **1992**, *18*, 129.
11. Falchuk, K. H. *Mol. Cell Biochem.* **1998**, *188*, 41.
12. AjaA.; Carol, P.; Sreejith, S. *J. Amer. Chem. Soc.* **2005**, *127*, 14962.
13. Park, H. R.; Seo, J. J.; Shin, S. C.; Lee, H. S.; Bark, K. M. *Bull. Kor. Chem. Soc.* **2007**, *28*, 1573.
14. Park, H. R.; Oh, C. H.; Lee, H. C.; Jung, B. I.; Bark, K. M. *Bull. Kor. Chem. Soc.* **2006**, *27*, 2002.
15. Lee. H. S.; Lee, J. B.; Yun, S. J.; Choi, J. Y.; Oh, J.; Park, H. R. *Bull. Kor. Chem. Soc.* **2006**, *27*, 1067.
16. Park, H. R.; Oh, C. H.; Lee, H. C.; Lim S. R.; Yang, K. Y.; Bark, K. M. *Photochem. Photobiol.* **2004**, *80*, 143.
17. Park, H. R.; Oh, C. H.; Lee, H. C.; Lee, J. K.; Yang, K.; Bark, K. M. *Photochem. Photobiol.* **2002**, *75*, 237.
18. Sanz-Nebot, V.; Valls, I.; Barbero, D.; Barbosa, J. *Acta Chem. Scand.* **1997**, *51*, 896.
19. Barbosa, J.; Berges, R.; Toro, I.; Sanz-Nebot, V. *Int. J. Pharm.* **1997**, *149*, 213.
20. Sanchez, J. C.; Leyva, J. A.; Ceba, M. R. *Analytica Chimica Acta* **1977**, *90*, 223.
21. Basolo, F.; Johnson, R. C. *Coordination Chemistry*; Science Review: London, UK, 1986; p 47.
22. Goodpaster, J. V.; McGuffin, V. L. *Anal. Chem.* **2000**, *72*, 1072.
23. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Plenum Press: New York, USA, 1983; p 257.
24. Huheey, J. E.; Keiter, E. A.; Keiter, R. L. *Inorganic Chemistry*, 4th ed.; Harper Collins College Publishers: New York, USA, 1993; p 130.