Notes

Comparison of a Polymerized Liposome with Nonpolymerizable Ones

### Yong-Chan Chung*, Jong-Moon Jeong†, and Ji-Hwan Hwang†

Department of Chemistry, and †Department of Biology, The University of Suwon, Suwon 445-890, Korea

Received August 8, 1997

Liposome has been extensively examined in many areas such as biological membrane model,[1] controlled and targeted drug delivery,[2] solar energy conversion,[3] and development of cytotoxic reagent.[4] The inherent instability of conventional liposome has limited further application in many fields. Accordingly, various kinds of polymerized liposomes (PL), either surfactant- or lipid-type, are introduced to overcome the instability problem, inducing polymerization by radical or photo initiation method.[5] A different type of polymerizable lipid was reported not long ago, and the mildness of the polymerization method could satisfy some of the requirements for biological applications.[6] We have already reported the permeation control results of this PL,[7] and the structure and the synthetic strategies are same as that shown in the literature.[6,7] The fact that some of the basic bilayer properties of the PL, nonetheless, had not been reported yet intrigued us to compare the PL and NL (nonpolymerizable liposome) in the points of resistance to chemical and thermal perturbations, together with the possibility of long-term storage. Additionally, real encapsulation of two representative proteins and difference in encapsulation efficiency between two kinds of liposomes are reported, which is definitely a prerequisite step toward biological applications.

Results and Discussion

The polymerizable phospholipid, 1,2-bis[12-(lipoyloxy) dodecanoyl]-sn-glycero-3-phosphorylcholine (DLL), was synthesized by the literature method.[6] The DLL monomer
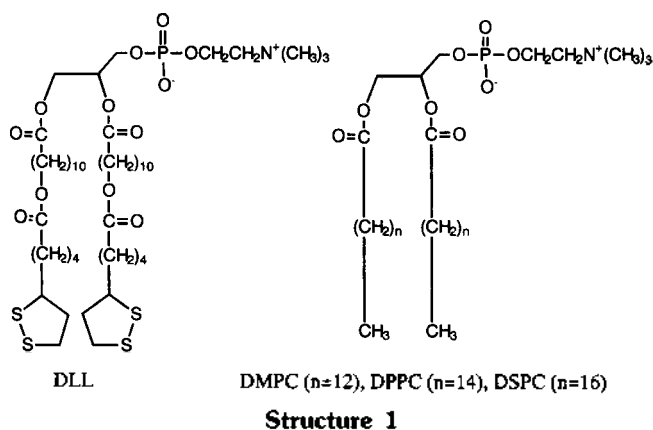
synthesized could be stored in chloroform at 4 °C for a long period and care was taken in handling the DLL solution not to prepolymerize before dispersion in aqueous solution. The aqueous liposome solution was prepared by addition of an aliquot of 0.02 M phosphate buffer (pH 6.4) into a dry lipid film, followed by freeze-thawing and vortex mixing 5 times. Then it was extruded at above the phase transition temperature through a membrane filter with an appropriate pore size under argon pressure 5 times,[8] and polymerization of liposomal solution was initiated by addition of cysteine solution as an initiator, together with adjusting pH to 8.4 by dilute NaOH solution. The liposomal solution was shaken overnight and then the pH was reduced back to 6.4 with dilute HCl solution. The progress of polymerization could be easily monitored by TLC as stated in the literature.[6] Nonpolymerizable liposome solution from DMPC (Dimyristoyl phosphatidylcholine), DPPC (Dipalmitoyl phosphatidylcholine), and DSPC (Distearyl phosphatidylcholine) could be prepared directly from extrusion without any intervening polymerization process. As all of liposomal solutions were ready, comparisons were made in the area of turbidity, DSC
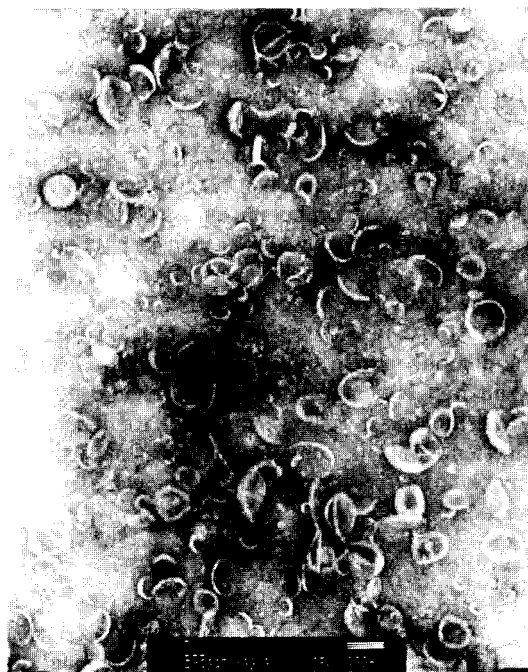
DLL   DMPC (n=12), DPPC (n=14), DSPC (n=16)

Structure 1

*Author to whom correspondence should be addressed.

Figure 1. Transmission electron micrograph of polymerized liposome stained with 2% uranyl acetate.

Notes

Bull. Korean Chem. Soc. 1998, Vol. 19, No. 7   781

(Differential Scanning Calorimetry), polarization plus microviscosity, and the protein encapsulation. Formation of the vesicular structure from PL was confirmed by TEM (Transmission Electron Microscopy) as in Figure 1 in which 2% uranyl acetate was used for staining.[9]

As a first step, turbidity of the liposome solution was measured at 330 nm in presence of various surfactants like SDS (Sodium Dodecylsulfate), Triton X-100, SDC (Sodium Deoxycholate), and three kinds of Polyethyleneglycol (M_w= 2,000, 4,000, 10,000). Turbidity was actually occurring from light scattering of liposome solution and surfactants were expected to break apart the lipid components, resulting in a clear solution. 330 nm was selected due to the most significant absorbance change after addition of surfactants. As in Figure 2, turbidity of the PL of DLL was not affected even at more than 1.5% (w/v) of SDS. In contrast, DMPC showed a sharp drop of turbidity with a trace of SDS, and DPPC, DSPC, although not included, also showed similar shapes in turbidity profiles. When Triton X-100 was used for SDS, similar trend was observed. SDC also could not reduce the turbidity of the PL, but it was not as effective as SDS or Triton X-100 in disrupting all of nonpolymerizable liposomes. Polyethyleneglycols with 3 different molecular weights were not effective at all for both polymerized and nonpolymerized ones. In view of all of the experimental results of turbidity, PL of DLL, which was a huge cross-linked polymer, was solid enough to survive the disruption of SDS or other detergents.

In the second phase of comparison, gel to liquid crystalline phase transition temperature (T_m) was detected by flu-

orescence polarization method. 2 mol % of 1,6-diphenyl-1,3,5-hexatriene (DPH) in THF was added into the extruded liposome, LUV (Large Unilamellar Vesicle), and polarization intensity was measured by the polarizer of Perkin-Elmer LS-50B spectrometer (E_x: 350 nm, E_m: 420 nm). As shown in Figure 3, liposomes of DMPC, DPPC, DSPC showed sudden decreases of polarization intensity at around their T_m's known in the literature.[9] But the polarization of PL remained the same and addition of excess surfactants like SDS (3%), or Triton X-100 (3%) did not change intensity in polarization scan. Presumably, cross-linked lipid chains of PL were not able to transform itself from trans to gauche form along the carbon-carbon bond at the temperature range measured. After the first heating scan, second and third ones were repeated, and no apparent variation of polarization for PL was observed. DPH is well known as a very sensitive probe to dynamic properties of hydrocarbon chain, so that the polarization results support the fact that bilayer structure of the PL of DLL maintains a very stable structure even under the high temperature and the high concentration of disrupting surfactants.

Microviscosities of various liposomes were calculated from the polarization data, and the PL of DLL showed almost constant values as in Table 1.[10] Once again, the microviscosity data showed that the bilayer of PL kept very stable and robust bilayer against temperature change.

The phase transition could be also detected by DSC. In Figure 4, DMPC, DPPC, DSPC showed expected endothermic peaks at around their reported transition temperatures as indicated in parentheses. But PL of DLL didn't show any transition up to 80 °C after 3 heating and cooling cycle. In addition to the nonvariant characteristics of polarization and
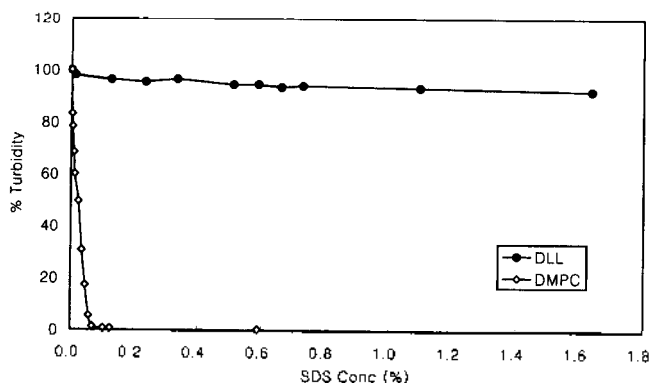

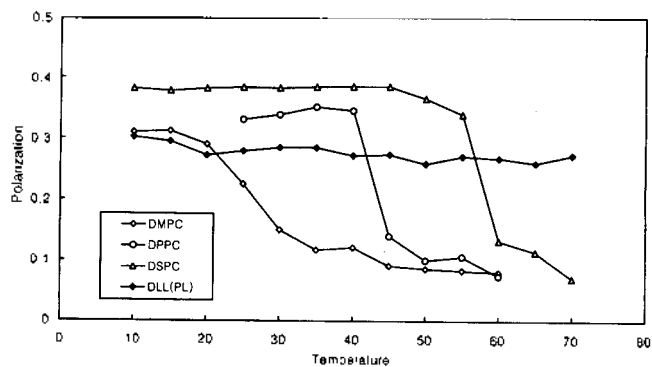Figure 2. Turbidity of liposome solution with various conc. of SDS.

Figure 3. Polarization of various liposomal solution.

Table 1. Microviscosities of Various Liposomes

Temp (°C)	η, cP			
	DMPC	DPPC	DSPC	DLL
30	1.6	15	61	6.8
40	1.2	18	77	6.0
50	0.8	1.0	31	5.3
60	0.75	0.68	1.5	6.1

For more about the calculation of microviscosity, see ref 10.
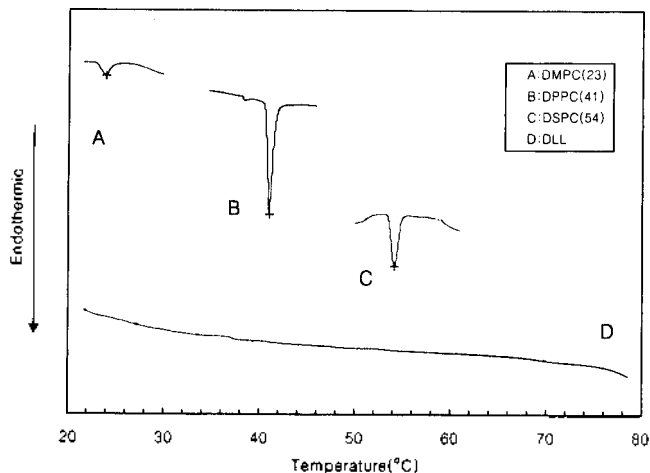
Figure 4. DSC profile of various liposomes.

782   *Bull. Korean Chem. Soc.* **1998**, Vol. 19, No. 7

Notes

microviscosity results, DSC further supports the thermal stability of the PL. Thermal spectrum was measured by DSC of MAC Science Co. and 30 μL of turbid liposomal solution in a sealed aluminum pan was used for each sample and same amount of buffer was put at the reference side.

For the purpose of storage, the PL solution with encapsulated FID (Fluorescein Isocyanate Dextran, M_w=4,400) which showed very high fluorescence intensity was freeze-dried and kept in a freezer for a month. The dry lipid was reconstituted by adding same amount of buffer, and freeze-thawing and vortex mixing (×5). The mixture was gel-filtered to remove unentrapped FID and FID permeation through the bilayer was measured by dialysis method.[11] In another way of storage, frozen PL solution with the same encapsulated FID was also stored in a freezer for a month, and release kinetics were monitored by the same dialysis method. As in Table 2, the recovered PL from either freeze-dried or freezing method kept the molecules very well, and showed similar rate constants as freshly made PL. Ideal carrier is supposed to have this kind of property suitable for long-term storage of encapsulated molecules and our PL is quite close to the demand in this respect.

According to the previous permeability data, permeability of our PL was not much affected by temperature, and the permeation rate was too fast to capture small molecule like carboxyfluorescein within the time scale of dialysis method.[7] In case of big molecules like BSA (Bovine Serum Albumin), the permeation rate was so low that leakage of encapsulated molecule was not observed.[7] To induce more permeation, surfactants like SDS, or Triton X-100 was added to a final concentration of 0.1% (w/v) and the moderate-sized FID (M_w=4,400) was used as a marker to compare the permeation rates with and without surfactants. As per the data in Table 2, the permeation rates of FID were not also much changed by the presence of surfactants, and thus it could be assumed that the powerful surfactants could not modify the bilayer property of our PL at all.

As we intend to apply our PL to microencapsulation as an delivery vehicle, two enzymes, Ovalbumin (45 kDa) and BSA (Bovine Serum Albumin, 66.2 kDa), were encapsulated with the PL or NL of DMPC by the same procedure as CF encapsulation, differing in that more basic Tris-HCl buffer solution (pH 7.4) was used, no thiol reagent was used as an initiator, and polymerization was proceeded without pH adjustment to maintain milder polymerization condition. In consideration of fair comparison of PL and NL, same molar amount of DLL or DMPC was used in liposomal preparation. Unencapsulated enzymes were removed by repetitive centrifugation, removal of the supernatant solution,

and resuspension with same buffer (×2).

The encapsulated amount of enzyme was checked by SDS-PAGE (Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis). As shown in Figure 5, the lane 1 indicates molecular markers of various molecular weights, lane 2 and lane 5 are for protein solution of Ovalbumin and BSA, respectively, without any liposomes. Lane 3 and lane 6 are for proteins encapsulated with PL, and the lane 4 and lane 7 are for proteins encapsulated with NL. Those encapsulated with PL clearly showed darker bands than nonpolymerized ones (#3 vs #4, and #6 vs #7), and this results demonstrate more efficient encapsulation by PL than NL.

Probably, the rigid and robust structure of the PL has kept the proteins safely during the washing step, whereas the NL could have lost quite an amount of the encapsulated enzyme due to the unstable structural features. Because the encapsulation efficiency of most liposome solution is very low, ca. 5%, more efficient encapsulation can greatly reduce the amount of pricy proteins needed for encapsulation, and, as such, PL is superior to NL in this practical point alone. Continuous effort is also being exerted on enhancing the encapsulation efficiency of PL at this moment. As for the application to microencapsulation system, more reliable, stable, and biocompatible delivery vehicle is needed and our PL may satisfy some of those requirements, as shown in the superior capabilities of PL to NL in protein encapsulation. Further applications using PL is now undergoing, and the results will be presented in due time.

Overall, PL of DLL are stable enough to survive chemical, and thermal perturbations, and the permeability is not affected by surfactants or thermal energy. In addition to the quite contrasting bilayer properties of PL and NL, the result of protein encapsulation highly encouraged us to keep pursuing the ultimate goal of developing efficient drug delivery system. To control the permeability of small molecule, it seems more effective to use partially polymerized liposome or PL prepared with a polymerizable lipid which has the polymerizable group at one chain end. Thus, the cross-link density can be reduced, and more flexible bilayer chain may permit surfactant- or temperature-sensitive passage of encapsulated molecules. Coupled with the stability, precise fine tuning of bilayer permeability of PL will mostly fulfill the demanding requirements of DDS (Drug Delivery System). As a more advanced delivery tool, tagging an antibody which can recognize the antigenic site on the head group of our PL is one of our next coming goal.

Table 2. FID Permeation Coefficients with Surfactants at 45 °C[a]

Surfactants[b]	10^7 P, cm/hr		
	DLL	R-DLL-1[c]	R-DLL-2[d]
SDS	12	11	8.2
Triton X-100	6.2	12	9.4
NS[e]	14	10	13

[a] For more information about the calculation of permeation coefficient see ref 11. [b] 0.1% (w/v) of concentration. [c] Recovered DLL from freeze-dried method. [d] Recovered DLL from freezing method. [e] NS=No surfactants.
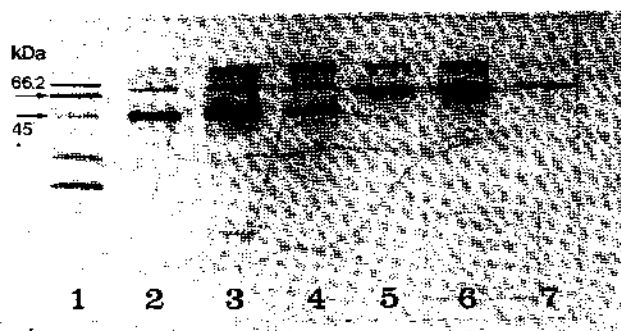

Figure 5. SDS-PAGE of ovalbumin and bovine serum albumin encapsulated in polymerized and nonpolymerized liposome.

Notes

Bull. Korean Chem. Soc. **1998**, Vol. 19, No. 7   783

Experimental Section

General Methods. Solvents were purified by extensive distillation and drying process, and water was distilled and deionized by Barnstead system. All the reagents were purchased from either Aldrich or Sigma Chemical Co. unless specified. Turbidity was scanned by Hewlett-Packard HP 8453 diode-array spectrophotometer. Fluorescence polarization intensity was measured by Perkin-Elmer LS-50B Luminescence spectrometer equipped with temperature control. DSC was measured by MAC science DSC 3100 at Ajou university, Suwon, Korea. Freeze-drying of sample was carried out with Labconco freeze-dryer. TEM picture was taken at Inter-University Center for Natural Science Research Facilities at Seoul National University.

Preparation of Liposome. DLL was synthesized by the procedures described in reference 5. For the brief description, the sealed sample of DLL in chloroform (30 mg/mL) was stored at 4 °C, and an aliquot was taken out with a microsyringe for liposome preparation. The same amount of DMPC, DPPC, and DSPC are prepared in chloroform solution (30 mg/mL) and stored at 4 °C. Usually, an aliquot of DLL solution (3 mg) was dried to a uniform lipid film by blowing with nitrogen and drying under high vacuum for 3 hrs. 1 mL of 0.02 M phosphate buffer (pH 6.4) was added and vortex mixed with brief sonication. The milky solution was freeze-thawed 5 times by freezing in a cold trap (-80 °C) and thawing at 45 °C. The solution was extruded 5 times through 0.1 μm membrane at 45 °C. The pH of the solution was raised to 8.4 by addition of 40 μL of 0.3 M NaOH solution, and 10 μL of 0.01 M cysteine solution was added as an initiator. The whole mixture was shaken overnight for polymerization, followed by reduction of pH to 6.4 by addition of 45 μL of 0.3 M HCl. This PL solution showed no precipitation even after standing for a week unlike NL which had some precipitation after overnight standing.

Turbidity of 2 mL of liposomal solution was measured at 330 nm after each addition of 100 μL of 2.5% (w/v) surfactant that is SDS, Triton X-100, DCP, and 3 kinds of ethylene glycols (M_w=2,000, 4,000, 10,000). The turbidity was corrected for added volume of surfactant solution by the following equation, (Corrected turbidity)$\times$(2 mL)= (Turbidity measured)$\times$(2 mL+Volume of added surfactant).

Polarization and DSC. 15 μL of 4×10^{-3} M DPH in THF was added into 1 mL of liposomal solution (LUV), and polarization intensity was measured at the optimum wavelength (E_x: 350 nm, E_m: 420 nm) from 15 °C to 70 °C. The polarization intensity was automatically calculated by the operating program of the Perkin-Elmer LS-50B luminescence spectrometer equipped with polarizer. As for the measurement of DSC, 30 μL of milky liposomal solution was loaded into a aluminium pan and sealed with a cap under pressure. As a reference, same amount of sealed buffer solution was used. The sample was scanned at a rate of 1.0 deg/min from 15 °C to 80 °C, and the third heating scan was recorded with peak-finding for transition temperature (T_m) after 3 heating and cooling cycles.

Permeation. The liposome solution prepared with 1 mM FID in 0.02 M phosphate buffer (pH 6.4) was gel-filtered through a column (1.5$\times$4.0 cm) packed with Sephadex

G-50 to remove the unencapsulated marker, eluting with the same buffer. A fraction of 1.2 mL was collected up to 60 fractions by Bio-Rad model 2110 fraction collector and then fractions containing encapsulated marker were combined and put into the Spectrapore No 2 dialysis tubing (1.5$\times$5 cm, M_w cutoff=12,000-14,000). The dialysis bag was put into a container with 500 mL of buffer, while controling the temperature at 45 °C precisely. 100 μL of the sample inside the bag was periodically taken out for 100 hrs and diluted with 2 mL of buffer for each sample taken. The fluorescence intensity was measured at E_x: 490 nm and E_m: 520 nm, and the permeation coefficient was calculated according to the methods in reference 12.

Protein Encapsulation. The protein molecular markers were purchased from Bio-Rad Co. (Richmond, CA, USA) and their individual sizes are phosphorylase b: 97.1 kDa, bovine serum albumin: 66.2 kDa, ovalbumin: 45 kDa, carbonic anhydrase: 31 kDa, soy bean trypsin inhibitor: 21.5 kDa, and lysozyme: 14.4 kDa, respectively. The molecular size is in increasing order from bottom to top in lane #1 of Figure 5.

A 20 mg of ovalbumin (Junsei) or bovine serum albumin (Sigma) was dissolved in 1 mL of 10 mM Tris-HCl buffer (pH 7.4) for encapsulation by PL or NL. Following the above procedure for encapsulation, the unencapsulated proteins were separated by centrifugation at 8,000 g for 5 minutes, and the clear supernatant solution was carefully removed by a micropipette. The same molar amount (20 μmole) of DLL (20 mg) for PL and DMPC (13 mg) for NL was used for the comparison of encapsulation. The residual pellet was resuspended with 1 mL of Tris buffer with vortex mixing and this washing procedure was repeated once more.

After resuspension of the liposome solution, an aliquot was used for SDS-PAGE.[12] Polyacrylamide gel was prepared at 12.5% (w/v) in water and 2 μL of loading buffer containing 5 mM EDTA, 20% (v/v) glycerol, 5% (w/v) sodium dodecylsulfate, 200 mM dithiothreitol, and 50 mM Tris-HCl (pH 6.8) was mixed with 10 μL of the protein-encapsulated liposome solution. After heating the mixture at 95 °C for 5 minutes, it was loaded onto the well of gel plate. Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) sodium dodecylsulfate, pH 8.3) was used as conducting solution, and 40V was applied for 5-6 hrs at room temperature. The gel plate after electrophoresis was removed, stained for 10 hrs with a solution containing 0.05% (w/v) Coomassie brilliant blue R-250, 25% (v/v) methanol, and 18% (v/v) acetic acid, and destained with a solution of 20% (v/v) methanol and 10% (v/v) acetic acid.

References

1. Papahadjopoulos, D., Ed.; *Liposomes in Biology and Medicine*, New York Academy of Sciences: New York, 1978.
2. Gregoriadis, G. *N. Engl. J. med.* **1976**, *295*, 765.

784   *Bull. Korean Chem. Soc.* **1998**, Vol. 19, No. 7

Notes

3. Fendler, J. H. *Membrane Mimetic Chemistry*; J. Wiley & Sons: New York, 1982.

4. (a) Goormaghitigh, E.; Chatelain, P.; Caspers, J.; Ruysschaert, J. M. *Biochim. Biophys. Acta* **1980**, *597*, 1 (b) Henry, N.; Fantine, E. O.; Bolard, J.; Garnier-Suillerot, A. *Biochemistry* **1985**, *24*, 7085 (c) Nagawa, Y.; Regen, S. L. *J. Am. Chem. Soc.* **1991**, *113*, 7327.

5. Fendler, J. H. *Science* **1984**, *223*, 878.

6. Sadownik, A.; Stefly, J.; Regen, S. L. *J. Am. Chem. Soc.* **1986**, *108*, 7789.

7. Chung, Y-C. *Bull. Korean Chem. Soc.* **1997**, *18*, 1041.

8. Hope, M. J.; Bally, M. B.; Webb, G.; Cullis, P. R. *Biochim. Biophys. Acta* **1985**, *812*, 55.

9. Krisovitch, S. M.; Regen, S. L. *J. Am. Chem. Soc.* **1992**, *114*, 9828.

10. Shinitzky, M.; Barenholz, Y. *J. Bio. Chem.* **1974**, *249*, 2652, Jain, M. K.; Wagner, R. C. *Introduction to Biological Membranes*; J. Wiley & Sons: New York, 1980; pp 82-83.

11. Johnson, S. M.; Bangham, A. D. *Biochim. Biophs. Acta* **1969**, *193*, 82.

12. Laemmli, *Nature* **1970**, *227*, 680.

Adsorption and Preconcentration of Nickel Ion Using Silica-Dimethylglyoxime Adsorbent

Chan-il Park and Ki-Won Cha*

Department of Chemistry, Inha University, Incheon 402-751, Korea
Received September 18, 1997

Separation and preconcentration techniques are of great importance owing to the limited sensitivity and selectivity of modern instrumental methods of trace analysis. Recently, adsorbents obtained by modified of chelating agents on silica gel have gained much attention. Those adsorbents are very useful in the analysis of environmental samples, particularly natural waters. The wide range of preconcentration of metal ions,[1-10] separation of metal ions,[11,12] solvent cleanup from undesired metal ions and selective extraction of metal ions from different solvent system[13] have been studied using those adsorbents.

Dimethylglyoxime has been used in gravimetric analysis[14] and in trace analysis for the separation and preconcentration of Nickel(II) ion by extraction and precipitation. This work was devoted to the preparation and examination of the analytical properties of a adsorbent obtained by modifying of dimethylglyoxime on the silica gels. And, we have studied the separation and preconcentration of Ni(II) from the synthetic metal solution using silica-dimethylglyoxime adsorbent.

Experimental

A Shimadzu AA-670 atomic absorption spectrophotometer was used for metal ions determination. An NOVA-310 digital pH-meter was used for pH measurement. Silica gel 60 (70-230 mesh) was a product of Merck Co. The metal ion stock solutions were prepared by dissolving the 1000 ppm AAS standard solutions of Ni(II), Co(II), Zn(II), Fe (II) and Pb(II) obtained from Junsei Chemical Co. The other chemicals were of analytical-reagent grade. The following buffered solutions were prepared: hydrochloric acid-potassium hydrogen phthalate (pH 1-3); acetic acid-sodium acetate (pH 3-6); potassium dihydrogen phosphate-sodium hydroxide (pH 7); and sodium borax-hydrochloric acid (pH 8-9).

Synthesis of adsorbent ; Silica gel was refluexed with 6 M HCl for about 12hr to remove any contaminating metals such as iron. It was then washed with deionized water and dried at 130 °C. The dried silica gel was refluxed with dimethylglyoxime in ethanol (20%) at 70-80 °C for 4hr. The solid thus obtained was filtered and dried under vacuum.

The adsorption of metal ions were performed in the batch and column methods. Excess metal ion (50 mL, 20 μg/mL) was shaken with 0.5 g of adsorbent for 24hr. The pH of solution was adjusted prior to equilibration over a range of 1-8 with buffer solutions. After equilibrium, the solution was filtered through Whatman #2 filter paper. The filtrate was diluted with distilled water and the concentration of metal ions were determined by AAS. In the column method, 10 g of the adsorbent was placed into a column (20 mm in inner diameter) and washed with 100 mL volume of water. The sample was passed through the column at a rate of 0.5 ± 0.1 mL min^{-1}. The adsorbed metal ions were desorbed with a suitable eluent.

Result and Discussion

pH effects on metal ion adsorption. The chemical stability of the adsorbent was evaluated by measuring the change of adsorption capacity for Ni(II) after a successive contact of silica-dimethylglyoxime adsorbent with an acidic solutions in various concentration range. When this silica-dimethylglyoxime adsorbent was contacted with an acidic solution weaker than 1.0 M HNO$_3$ or 1.5 M HCl for 24hr, the decomposition was negligibly small and no significant decrease in the adsorption capacity for Ni(II) was observed. From these results, it is believed that this adsorbent has sufficient stability.

The effect of pH on adsorption of Fe(II), Co(II), Zn(II), Ni(II) and Pb(II) in pH 1.0-8.0 was examined by a batch