UREA biosensor based on magnetic nano particles (Co_3O_4, Fe_3O_4) for the estimation of urea concentration in blood and urine samples

A. ALI[a*], M. AHMAD[a], G. ABBAS[b], M. N. AKHTAR[a], M. ATIF [c,d]
[a]*Department of Physics, COMSATS Institute of Information Technology, Lahore, 54000-Pakistan*
[b]*Department of Physics, COMSATS Institute of Information Technology, Islamabad, 44000-Pakistan*
[c]*Department of Physics and Astronomy, King Saud University, Riyadh, Saudi Arabia*
[d]*National Institute of Laser and Optronics, Nilore, Islamabad, Pakistan*

In this study, a potentiometric urea biosensor through the immobilization of urease enzyme onto chitosan (CH)/Co_3O_4 and CH/Fe_3O_4 hybrid nano-biocomposites have been fabricated on glass filter paper. A copper wire of diameter 250 μm has been attached with nanoparticles in order to extract the voltage output signal. A physical absorption method has been adopted to immobilize the surface of CH/Co_3O_4 and CH/Fe_3O_4 hybrid nano-biocomposites. Urea biosensor based on magnetic nanoparticles (MNPs) was utilized for the estimation of urea concentration in blood and urine. Blood and urine samples of 25 healthy and 25 sick volunteers were collected and after that urease/ Fe_3O_4-CH/Cu biosensor electrode or urease/Co_3O_4-CH/Cu biosensor electrode with 20μL urease immobilization was used for estimation of blood and urine urea. The potentiometric sensitivity was measured over the concentration range 0.1 - 6.00 ppm; and the limit of detection is 0.073 ppm. The response time, efficiency and accuracy of this biosensor is 280 seconds, 50 samples and 94 - 99%, respectively. The concentration of urea in 100 times diluted blood and urine sample was found to be 4.1×10^{-4} and 3.84×10^{-4} M, respectively. The magnetic study shows that coercivity of both the samples is found to be a few oersteds which make them very promising candidates for a variety of applications in biomedical as well as recording technology.

(Received July 1, 2015; accepted September 9, 2015)

Keywords: Urea biosensor; Magnetic nanoparticles; Urea estimation; Blood sample; Urine sample.

1. Introduction

A biosensor is a device that combines a sensor and a biochemical reaction. The device consists of three parts: bioactive, transducer and detector. The bioactive is a molecule that reacts specifically with analyzes and results a compound or ion which is detected by transducers. The kinds of electrochemical transducers are conductometry, amperometry and potentiometry [1]. Therefore, the biosensor is a high selectivity device; hence in this work the construction of potentiometric urea biosensor was developed. Urea is considered to be one of the final products of protein metabolism. In clinical analysis and dairy industry, urea is a very important and its extra amount in blood from its allowed range provides a base for the dysfunction of the kidney. Hence, its analysis is significant and has been carried out regularly in various laboratories [2, 3]. Urea is a universal compound and present in various organic fluids in the human body. It transmits directly into milk via diffusion. Hence, second major biological sample used for the study of urea concentration is milk [4]. To predict the state of woman usually a periodic monitoring of urea in milk can be used including animal's health and the protein requirement in its food [5]. Besides milk, urea presence in agricultural land as a pollutant due to excessive use of fertilizers is also widely identified. For the determination of urea concentration various methods are used including gas chromatography, calorimetric and flour metric analysis, without the use of biocatalyst [6]. However, these methods required the sample pretreatment before measurement which is a major drawback in their versatility of applications. Alongside, these methods cannot be used for field monitoring. Therefore, different types of devices are developed based on biocatalyst "urease" to investigate urea also known as urea biosensors and are of vital significant. Guilbault et al. [7] developed the first urea biosensor after monitoring many clinical and biochemical analysts. In order to fabricate a urea biosensor, urease is normally immobilized over a substrate. Then this immobilized urease led the catalysis of the urea conversion into ammonium and bicarbonate ions due to enzyme substrate reaction. Most of the biosensors have been created in order to determine urea in the biological samples specifically spectrometry [8-10], potentiometry [11-14], conductometry [15-17], coulometry [18], amperometry [19] and inductometry [20]. Hence this detection done via electrochemical mode is highly accepted and adaptable among these methods including the use of electrochemical urea biosensor. The immobilization of urease over electrodes is the key factor in the development of electrochemical urea biosensors

keeping in view their sensitivity and reproducibility. The detection of urea is of great importance in biomedical and clinical analysis applications. Indeed, an interest of urea concentration in blood and a reduced level of urine is a strong sign of renal failure. The normal urea level in urine is 12-20 g per 24 h [21]. The determination of urea is generally performed with enzyme-based biosensor. For investigating kidney and liver diseases, urea is an important tool parameter to be frequently analyzed in clinical laboratories. For estimation of urea, the biological samples such as blood serum, urine and milk are routinely used. Human health is badly affected if the urea concentration is above the optimal level in above stated constituents. In human blood the normal range of urea is between 1.7 - 8.3 mmol/L. If urea level in blood is increased up to 100 mM/L, it affects normal functioning of kidney [22]. In the present study, the proposed urea biosensor was applied to measure urea in human blood and urine which were diluted 10 times and 100 times by using standard addition method.

The results of urea level in blood and urine of healthy and sick people is presented in this paper.

2. Experimental section

2.1 Materials

All the chemicals and reagents used in the current study were of analytical–reagent grade. Using distilled water all these solutions were prepared. Urea, chitosan, glutaraldehyde, acetic acid, phosphate buffer (pH 7-8.5) and sodium hydroxide were obtained from Merck and were used without further purification and treatment. Urease was isolated by Schizosaccharomyces Pombo 3054 (7mg/mL).

2.2 Method

The schematic image for the fabrication of working electrode based on Co_3O_4 and Fe_3O_4 are shown in Figs. 1 and 2. The working electrode was made by dispensing Co_3O_4-CH-urease nano-biocomposite on a copper wire mounted on a glass fibber filter. The impurities can be eliminated by centrifugation or filtration of samples before sample measurement. The following steps were followed for making the working electrode:

i. CH sol-gel was prepared in 1% CH_3COOH and 1 M hydrochloric acid (HCl) solutions and kept on stirring for 24 hours for better homogeneity.

ii. Co_3O_4 and Fe_3O_4 magnetic nanoparticles were mixed with deionized water and stirred for 1 hour. Finally, the Co_3O_4 and Fe_3O_4 magnetic nanoparticles were found suspended in the sol-gel of CH.

iii. The suspension was dispensed on a copper wire (d = 250 µm) mounted on a glass fiber filter.

iv. The Co_3O_4 and Fe_3O_4 magnetic nanoparticles based on biosensing electrode were developed by drop-wise dispersion of sol-gel solution on the suspended copper wire.

v. Finally, the Co_3O_4 and Fe_3O_4 magnetic nano particles based electrode were immobilized with urease enzyme using physical adsorption method.

vi. Urea level was determined in urine samples of 25 healthy and 25 sick people. For obtaining biosensor data the urine samples were diluted 10 times and 100 times. Mean values, standard deviation and frequency distribution of the laboratory data and biosensor data were obtained.

vii. Blood urea level was determined by taking the samples of 25 healthy and 25 sick people. biosensor data was obtained. Blood sample was 10 times and 100 times diluted for biosensor data measurements. Mean values, standard deviation and frequency distribution of the laboratory data and biosensor data were obtained.

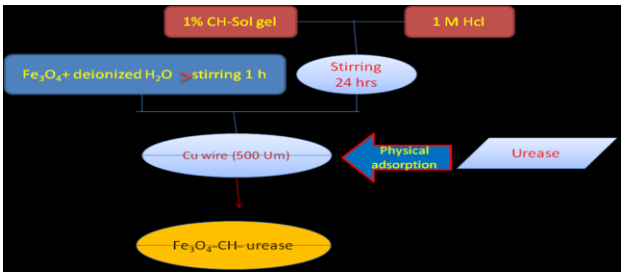

Fig. 1: Schematic image of working electrode based on Fe_3O_4.

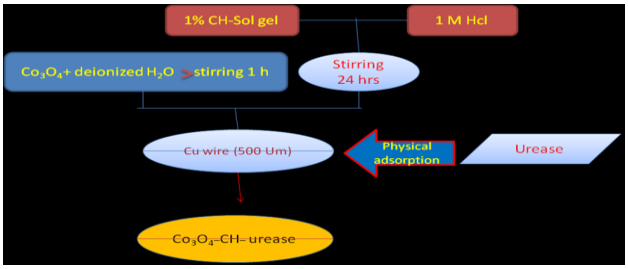

Fig 2: Schematic image of working electrode based Co_3O_4.

3. Results and discussion

3.1 Investigation for biosensing

Urea biosensor based on magnetic nano particles (MNPs) was applied for the estimation of urea concentration in blood and urine samples. Blood and urine samples of 25 healthy and 25 sick volunteers were collected and urease/ Fe_3O_4-CH/Cu biosensor electrode or urease/ Co_3O_4-CH/Cu biosensor electrode with 20 µL urease immobilization was used for estimation of blood and urine urea.

The concentration of urea in 100 times diluted blood sample was found to be 3.84×10^{-4} M and in real sample was 10.24×10^{-2} M by using standard addition method. The concentration of urea in 100 times diluted urine is 4.1×10^{-4} M and real sample is 1.44×10^{-2} M. Urea

biosensor was used to measure urea 0.1- 80 mM levels in blood samples and urine samples. These levels were compared with the data from a medical laboratory. The comparison is shown in Figs. 3-6.

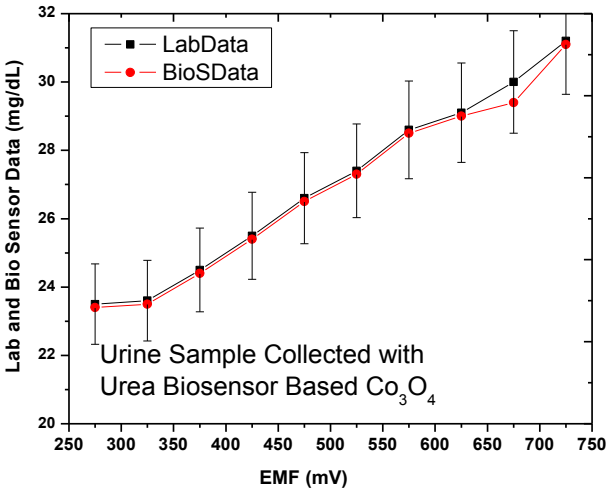

Fig. 3: Lab and Biosensor Data (mg/dL) vs EMF (mV) for urine Sample Based on Co_3O_4.

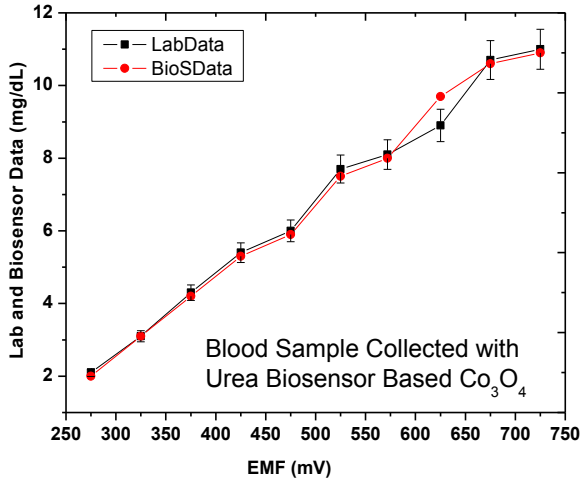

Fig. 4: Lab and Biosensor Data (mg/dL) vs EMF (mV) for blood Sample Based on Co_3O_4.

Blood urea levels obtained by biosensor have a relative error 0.1 to 2.5. Urine urea levels obtained by biosensor have a relative error 0.1 to 2.1. In both blood and urine samples, 100 times dilution showed less relative error as compared to 10 times dilution.

Human health is badly affected if the urea concentration is above the optimal level in above stated constituents. In human blood the normal range of urea is between 1.7 - 8.3 mmol/L. If urea level in blood increases up to 100 mM/L, it affects normal functioning of kidney [23]. The proposed urea biosensor was utilized in human blood which was diluted 10 times and 100 times by using standard addition method.

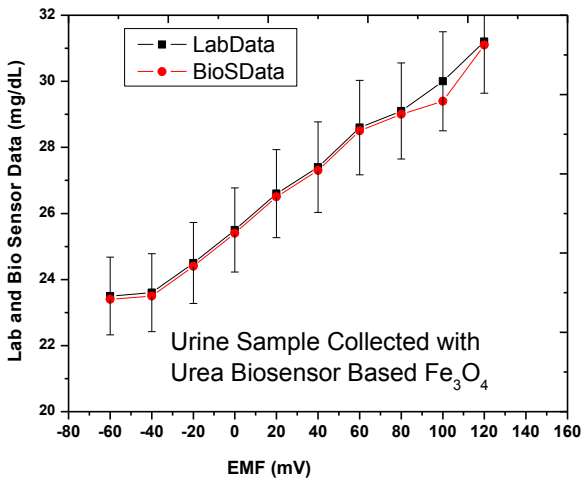

Fig. 5: Lab and Biosensor Data (mg/dL) vs EMF (mV) for URINE Sample Based on Fe_3O_4.

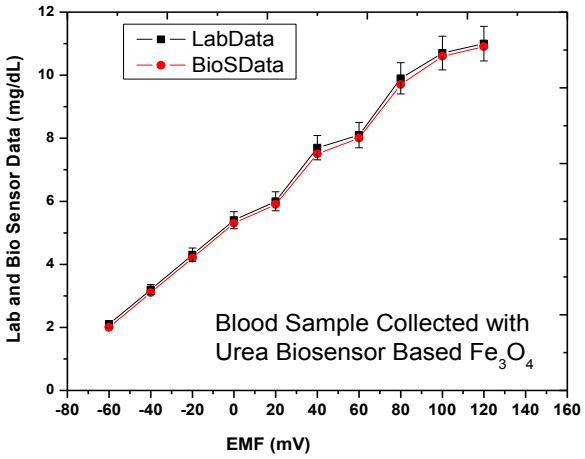

Fig. 6: Lab and Biosensor Data (mg/dL) vs EMF (mV) for BLOOD Sample Based on Fe_3O_4.

The relative error is different for all samples depending on concentration and type of impurities. 100 times diluted sample contains less impurity than 10 times diluted sample. In urine samples, it may be due to impurities like calcium oxalate, epitheland blood cell [24-25], which hinders the porosity of the hybrid nanocomposite. Therefore, the interaction of urea with enzyme was decreased. In both samples, the concentrations of impurities were less in the sample diluted 10 times than 100 times.

Urea level in blood was measured by collecting 50 blood samples of 25 healthy and 25 sick people. Mean values, standard deviation and frequency distribution of the laboratory data and biosensor data were calculated. Blood samples were 10 and 100 times diluted for the measurements with biosensor.

Fig. 7 illustrates frequency distribution of laboratory data and biosensor data of urine sample of normal people. Fig. 8 shows frequency distribution of laboratory data and biosensor data of urine sample of sick people. Frequency distribution data indicates that average value of urea

concentration in urine of 25 samples ranges 5-40.5. Frequency distribution of urea concentration in blood of 25 samples ranges 5-30.5 and is shown in Fig. 9.

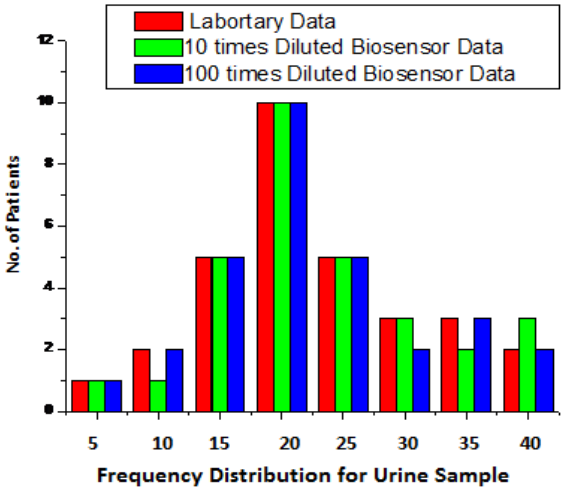

Fig. 7: Correlation for Frequency Distribution vs Laboratory data, 10 times Diluted Biosensor data, and 100 times Diluted Biosensor Data for URINE Sample.

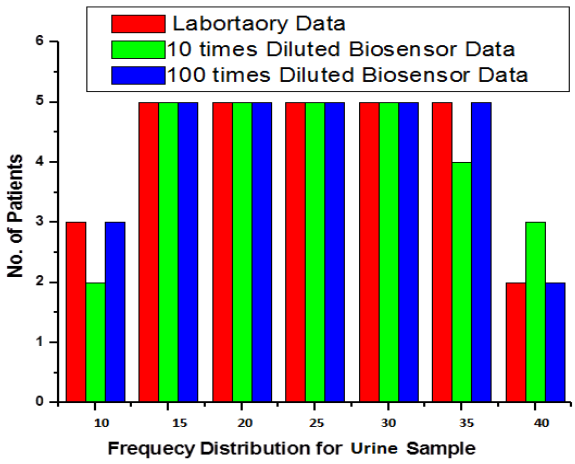

Fig. 8: Frequency Distribution vs Laboratory data, 10 and 100 times Diluted Biosensor data for Urine Sample (sick people).

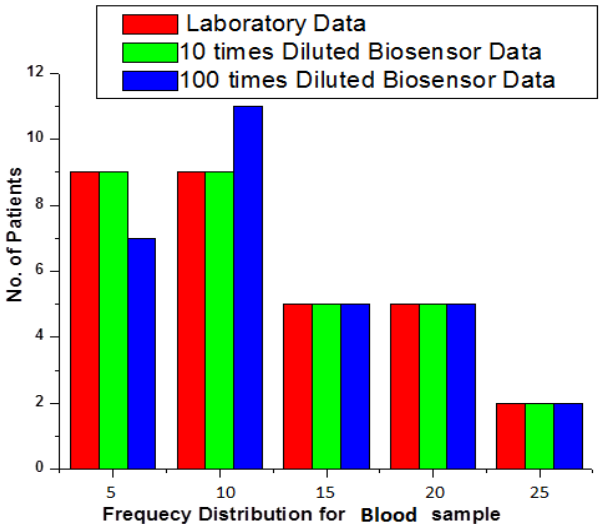

Fig. 9: Correlation for Frequency Distribution vs Laboratory data, 10 times Diluted Biosensor data, and 100 times Diluted Biosensor Data for BLOOD Sample.

Table 1 depicts average values and standard deviation values of laboratory data and biosensor data of blood and urine sample for normal people. Biosensor data was diluted 10 times and 100 times before the measurements. Table 2 demonstrates average values and standard deviation values of laboratory data and biosensor data of blood and urine sample for sick people. Biosensor data was diluted 10 times and 100 times before the measurements. This data indicates that average values and standard deviation values of biosensor data are consistent with the laboratory data.

The results of present study are shown in Table 3 where the value of urea ranges 5.4 -20 (mmol/dl) in blood of 25 normal people and ranges 15.5-40.6 (mmol/dl) in urine samples of normal people. In comparison reported or standard values of urea 1.4-13.2, 1.7-8.3 and 8-21 in blood samples of 76, 30, 20 volunteers from referenced countries [26-29] is also given in Table 3. Similary, in the reported work the urea level ranges 23-47 against urine samples of 3 volunteers. The comparison of test results depicts that present values are in line with the reported results. Table 4 shows the results of present work in terms of values of urea ranges from 2.1 -35.5 (mmol/dl) in blood samples of 25 sick people and ranges 7-57.1 in urine samples of 25 sick people. In comparison to the reported research results, it is evident that present work shows promising results as urea concentration was determined against test samples of 25 healthy and 25 sick people.

Table 1: Statistical information of the data analyzed for urea in blood and urine of healthy people.

Statistical Parameter	Blood			Urine		
	Laboratory Data	Biosensor data		Laboratory data	Biosensor data	
		10% dilution	100% dilution		10% dilution	100% dilution
Maximum	20.7	21.0	20.6	40.0	40.3	39.9
Minimum	5.4	5.8	5.3	15.0	15.8	14.9
Mean	13.5	13.4	12.9	27.5	28.0	27.4
Standard deviation	8.23	8.22	8.33	11.65	11.66	11.70

Table 2: Statistical information of the data analyzed for urea in blood and urine of sick people.

Statistical Parameter	Blood			Urine		
	Laboratory data	Biosensor data		Laboratory data	Biosensor data	
		10% dilution	100% dilution		10% dilution	100% dilution
Maximum	35.1	35.9	35.0	57.1	57.9	57.6
Minimum	2.1	2.4	2.0	7.0	7.8	6.9
Mean	18.6	19.1	18.5	32.0	32.5	32.3
Standard deviation	11.23	11.22	11.33	14.65	14.66	14.70

Table 3: Blood and urine samples of normal people's standard values comparison with earlier reported values.

Country	**Samples**	**Standard values (mmol/dl)**	**Ref**
		Blood	
UK	76	1.4 -13.2	[26]
India	30	1.7 – 8.3	[27]
India	20	8-21	[28]
Sweden	25	2.4 -35	Present research work
		Urine	
Indonesia	3	23-47	[29]
Sweden	25	7 -57.1	Present research work

Table 4: Blood and urine samples of sick people's standard values comparison with earlier reported values.

Country	Samples	Standard values (mmol/dl)	Ref
		Blood	
UK	76	1.4 -13.2	[26]
India	30	1.7 – 8.3	[27]
India	20	8-21	[28]
Sweden	25	5.4 -20.5	Present research work
		Urine	
Indonesia	3	23-47	[29]
Sweden	25	15 -40	Present research work

Performance of the biosensor is affected by pH and thickness of hybrid nanocomposite layer (Fe_3O_4-CH or Co_3O_4-CH). The performance was high at pH 7.3 and uniform thin layer of hybrid nanocomposite. The character of biosensor was 45 mv per decade, range of urea concentration was 0.1 - 80 mM. The response time of urea biosensor was 280 sec. Biosensor efficiency for measure of 50 samples showing 92-99% accuracy.

3.2 Magnetic Study

The magnetic measurement of Co_3O_4 and Fe_3O_4 nanoparticles prepared at 200°C were carried out at room temperature. The magnetization curve for the Co_3O_4 nanoparticles, as shown in Fig. 10, displayed higher ferromagnetic properties with saturation magnetization value of 0.37 emu g^{-1} at the applied field of 15 kOe. While the magnetization curve for the Fe_3O_4 nanoparticles displayed higher ferromagnetic properties with saturation magnetization value of 0.17 emu g^{-1} at the applied field of 10 kOe as shown in Fig. 10. The measurements were also carried out on a bulk sample in order to prove the ferromagnetic behavior of the nanoparticles [30]. The ferromagnetic behavior of the bulk Co_3O_4 and Fe_3O_4 nanoparticles can be described as follows: a normal spinel structure with antiferromagnetic replacement between ions where the ions occupy the tetrahedral and octahedral sites [31].

The experimental results show zero net magnetization to the complete compensation of sublattice magnetizations. Therefore, this change from an antiferromagnetic state for bulk Co_3O_4 and Fe_3O_4 to a weakly ferromagnetic state for the Co_3O_4 and Fe_3O_4 nanoparticles were denoted due to the uncompensated surface spins and/ or finite size effects [32-33].

Hence the magnetic properties of Co_3O_4 and Fe_3O_4 nanoparticles are strongly dependent on size and the shape of their particles, crystallinity and magnetization direction. In addition, the value of coercivity for both of the nano sized magnetic materials was found to be in excellent agreement with the results reported in earlier literature [32-33]. These unique properties of these nanomaterials also make them very promising candidates for a variety of applications in biomedical as well as recording technology [34].

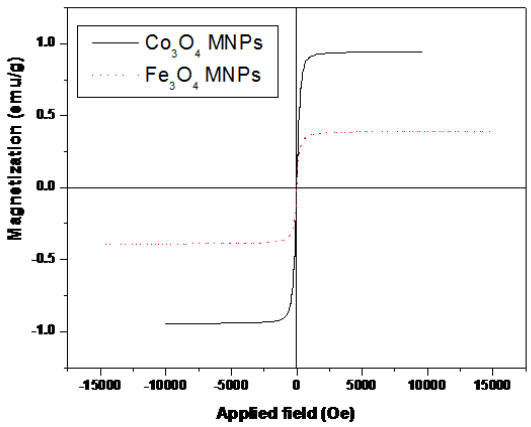

Fig. 10: M-H loops for Co_3O_4 and Fe_3O_4 magnetic nano particles measured at room temperature.

Co_3O_4 MNPs has been shown in black color line and Fe_3O_4 MNPs have been marked by doted red color lines.

4. Conclusion

For clinical diagnosis of urea in kidney and liver diseases, efforts have been made to devise sensitive nanobiosensors for regular monitoring of urea in blood and urine. With the advent of nano biotechnology it became reality to regulate devices at molecular level and introduce

new exciting materials for the enhancement of the working activity of the urea biosensor. Such electrochemical biosensors could be advantageous for diagnosing and monitoring infectious diseases, monitoring pharmacokinetics of drugs, detecting cancer. It is only a short span of time before such procedures are used for routine diagnostic applications. As available chemical analysis methods for urea are simpler and widely available but nanobiosensor is an attempt for highly accurate urea analysis due to its sensitivity. Magnetic study reveals that nano particles of both Fe_3O_4 and Co_3O_4 exhibit coercivity of a few oersteds thereby showing that they have soft magnetic character. Moreover, the value of this parameter is in good agreement to earlier reported value for this structure.

Acknowledgment

We are thankful to Higher Education Commission of Pakistan and Department of Science and Technology, Linköping University, Sweden for their help. The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project No. RGP-VPP-293.

References

[1] B.R. Egins, Chemical Sensors and Biosensors, John Wiley & Sons, LTD. Singapore (2002).
[2] E. Karakus, S. Pekyardımc, E. Kılıc, Urea biosensor based on PVC membrane containing palmitic acid, Blood Substitutes, and Biotechnology. **33**, 329 (2005).
[3] C. Eggenstein, M. Borchardt, C. Diekmann, B. Grundig, C. Dumschat, K. Cammann, M. Knoll, F. Spener,Bioelectron **14**, 33 (1999).
[4] M. J. R. Leema, S. M. V. Fernandes, A. O. S. S. Rangel,J. Agric Food Chem **52**, 6887 (2004).
[5] G. Hof, M. D. Vervoorn, J. Lenaers, S. Tamminga, J. Dairy Sci. **80**, 3333 (1997).
[6] A. V. Rebriiev, N. F. Starodub,Electroanalysis **16**, 1891 (2004).
[7] G. G. Guilbault, R. K. Smith, J. G. Montalvo,J.Anal. Chem. **41**, 600 (1969).
[8] D. Liu, K. Ge, K. Cheng, L. Nie, S.Yao,Anal.Chim.Acta **307**, 61 (1995).
[9] M. Mascini,Sens. Actuators B **29,** 121 (1995).
[10] X. Xie, A. A. Suleiman, G. G. Guilbault,Talanta **38**, 1197 (1991).
[11] S. B. Adeloju, S. J. Shaw, G. G. Wallace,Anal. Chim.Acta **281**, 611 (1993).
[12] S. B. Adeloju, S. J. Shaw; G. G. Wallace,Anal.Chim. Acta **281**, 621 (1993).
[13] M. F. Esteve, S. Alegret,J. Chem. Educ. **71**, A67 (1994).
[14] D. Liu, M. E. Meyerhoff, H. D. Goldberg, R. B.Brown, Anal.Chim.Acta, **274**, 37 (1993).
[15] K. Chen, D. Liu, L.Nie, S. Yao,Talanta **41**, 2195 (1994).
[16] A.S. Jdanova, S. Poyard, A. P. Soldatkin, N. Jaffrezic-Renault, C. Martelet, Anal. Chim. Acta **321**, 35 (1996).
[17] H. Sangodkar, S. Sukeerthi, R. S. Srinivasa, R. Lal, A. Q. Contractor,Anal.Chem. **68**, 779 (1996).
[18] R. E. Adams, P. W. Carr,Anal. Chem. **50**, 944 (1978).
[19] S. B. Adelju, S. J. Shaw, G. G. Wallace,Analytica Chimica Acta, **323**, 107 (1996).
[20] W. O. Ho, S. Krause, C. J. McNeil, J. A. Pritchard, R. D. Armstrong, D. Athey, K. Rawson, Anal. Chem. 71 (1999).
[21] J. L. Bennington (Ed.). Saunders Dictionary and Encyclopaedia of Laboratory Medicine and Technology. W.B. Saunders Co., Philadelphiia, U.S.A. (1984).
[22] S. L. Traub Ed. Basic Skills in Interpreting Laboratory Data. 2nd ed. Bethesda, MD: American Society of Health-System Pharmacists (1996).
[23] Traub SL (1996) Ed. *Basic Skills in Interpreting Laboratory Data.* 2nd ed. Bethesda, MD: American Society of Health-System Pharmacists.
[24] C. A. Fyfe, G. C. Gobbi, J. Klinowski, J. M. Thomas, S. Ramdas, Nature **296**, 530 (1982).
[25] J. Wang, Analytical Electrochemistry. 2nd ed. VCH Publisher, Inc, 133 (2001).
[26] K. Lesley, R.S.J. Bowkerl, P.J. Briggs, Gallagher, R.C.R. Dorothy, Postgrad Med J. **68**, 174 (1992).
[27] A. G. Elie, S. Brahim, A.M. Wilson, Biosensor based On conducting electroactivepolymer, Handbook of conducting polymer, Third addition CRC Press, Taylor and Francis (2007).
[28] J. Bobacka, A. Ivaska, A. Lewenstam, Electroanalysis **15**(5-6) 366 (2003).
[29] A. Mulyasuryani, A. Roosdiana, A. Srihardyastutie,. Indo. J. Chem.**10**(2), 162 (2010).
[30] Saeed F, Jalil S, Parisa Z (2013) Synthesis, characterization, and investigation ofoptical and magnetic properties of cobalt oxide Co_3O_4 nanoparticles. *J. Nanostructure in Chem*. **3**, 69.
[31] Y Ichiyanagi, Y Kimishima, S Yamada, J. Magn. Magn. Mater. 272–27. (2004)
[32] SA Makhlouf, J. Magn. Magn. Mater. **246**, 184 (2002)
[33] F Mohandes, F Davar, M Salavati-Niasari J Magn Magn Mater. **322**, 872-87. (2010)
[34] M. H. R. Khan, Farhad Alam, M. N. I. Khan, M.A. Hakim, D.K. Saha, A. K. M. Akther Hossain, (ICME2009) 26- 28 December 2009, Dhaka, Bangladesh.

*Corresponding author: akbarali_78614@yahoo.com, atifhull@gmail.com