This full text paper was peer-reviewed at the direction of IEEE Instrumentation and Measurement Society prior to the acceptance and publication.

Spectrophotometer measurements to characterize conformational state of the proteins: p53 analysis

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***Abstract*— The development of sensing techniques for the analysis of protein samples represents an area of great interest for biotechnology, pharmacology and diagnostics. Traditional biochemical assays available present limitations in term time and cost efficiency. On the contrary, optical based techniques, such as spectrophotometry, might offer the interesting possibility of a non-invasive and continuous monitoring. The present work proposes a spectrophotometer-based method of identifying different conformational states of p53, a redox sensitive protein involved in several pathophysiological processes. Samples containing three different structural states of p53 (Wild type p53, Denatured p53 and Oxidized p53) has been investigated using spectrophotometer in a measuring wavelength range from 185nm to 1400nm, in order to detect the differences in light absorption. The unfolding state of p53 redox products has been further studied performing a label-based silver stripping voltammetry. Results from the spectrophotometric analysis showed different absorbance peaks at distinct wavelength for each conformation, suggesting the possibility to use this technique to discriminate the different p53 redox products tested. Further, these results appeared to be well supported from the binding-affinity label-based test. Overall the study allowed to state the possibility to identify p53 different conformational states through this simple and non-invasive method, thus reducing the complexity of the procedures involved in conventional methods.**

Keywords*— *Spectrophotometry, p53 protein, absorbance spectrum, potentiostat, voltammetry, infrared spectra, protein detection.

I. INTRODUCTION

The development of non-invasive techniques for the investigation of protein conformational states, represents an active research area of relevant interest. As highlighted in recent literature [1], the combination of the newest achievements in material science, engineering, mathematics and bioinformatics with specific principles of intrinsic redox activity of protein molecule has acquired more and more importance as analytical method for the protein analysis of samples from different biological sources. In light of this, bio-engineering is trying to bring a strong improvement addressing the needs of basic and applied research related to protein structures and functions. Indeed, the strong correlation existing between protein conformational state and their biological functions [2], makes the study of the relationship between their structure and chemical modifications a possibility to get new insight in the understanding of the complexity of their activities. Additionally, the possibility to develop techniques able to discriminate a protein conformational state from another, might represent an important achievement to improve the sensitivity and detect more precisely disease related biomarkers.

In this light, p53 protein represents an interesting redox sensitive protein involved in different pathophysiological processes, ranging from cancer to neurodegenerative diseases. It is located at the crossroads of complex networks of stress response pathways. Various intercellular or extracellular stresses elicit cellular responses directly or indirectly through activation of p53-redox modulation [15]–[17]. Many studies demonstrated that the interplay among p53 and Reactive Oxygen or Nitrogen Species (ROS/RNS) is crucial for the cellular fate, giving rise to different p53 redox products.

Further, the possible correlation among the most common genetic alterations in cancer, with the transition from wild type conformation to a mutant one, makes the possibility to recognize these different conformations, an extremely promising starting point for developing new therapeutic approaches [3]. Further, several studies demonstrated that conformational alterations in p53 could occur also in absence of mutations [4] and in the field of neurodegenerative diseases conformationally altered p53 has been correlated with possible diseases onset [5]. Considering all this premise, the ability to successfully and easily discriminate different conformations of p53, possibly correlated with specific loss or gain of function, is of significant interest. Currently, biochemical assays (e.g. ELISA) provide only information related to a quantification of the protein, but not to the conformational state of this protein.

Aiming to combine sensitivity with non-invasiveness and ease of methodology, spectrophotometry represents one of the most promising, widely used, analytical procedures in biochemistry. This method is based on the two laws of light absorption by solutions, namely Lambert's Law and Beer's Law which states: “the amount of energy absorbed or transmitted by a solution is proportional to the solution’s molar absorptivity and the concentration of solute [22].” Beers Lambert law is mathematically expressed as:

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$$A = eLc$$

Where, A is the absorption, e the molar attenuation coefficient, L the path length and c the concentration of solution

The application of this concept can be found in some example of the literature applied to the field of cells and protein analysis. More specifically, in the work performed by J. Zhou, et al [24] demonstrates how thanks to the different absorption at different wavelength it is possible to discriminate between different conformational states of BSA protein pH-dependent. Further, a similar concept has been applied in [23] to measure the different conformational states of protein and cells by using infrared spectroscopy.

Thus, considering the results obtained with these other proteins, the novelty of this work relies on applying this technique for the first time to help in the detection of different p53 conformational states, thanks to the detection of absorbance change caused by different protein folding. Therefore, an experiment was designed using spectrophotometer to detect three different p53 conformational states: wild type, oxidative and denatured state, obtained following a protocol extensively described in the literature [25] – [27]. In order to strengthen our hypothesis, the same samples have been tested using a specific conformational antibody, which recognizes an epitope exposed in Denatured p53, while hidden in the wild type conformation.

II. MATERIALS AND METHODS

A. Sample preparation

In order to investigate the absorption of different conformational states of p53 protein, p53 wild type recombinant protein was exposed to different oxidant stressors to generate different redox-p53 products: i) metal chelator agent that distrains Zinc atom and induce the opening of the protein [19]; ii) Fenton reaction, mainly mediated by the OH· derived from the decomposition of H_2O_2 in the presence of redox metals (Fe^{2+} and Cu^{+}) [20] generates a burst of oxygen radicals involved in protein oxidation. Thus, p53 recombinant protein was incubated 1 hour at 37°C with the appropriate buffer: i) 200 µM EDTA and 5 mM DTT (denatured p53); ii) 30 µM $FeSO_4$ and 10 mM H_2O_2 (oxidized p53). In the label-based approach, anti-p53 conformational specific antibody, PAb 240 was used (PAb240, Neomarkers-Lab Vision, Fremont, CA, USA). This antibody recognizes an epitope masked when the protein is in its native conformation and accessible only when the tertiary structure is unfolded. PBS buffer solution alone was used as reference.

B. Sample testing through label-basedVoltammetry

Before proceeding with the spectrophotometric analysis, each sample has been tested following a protocol previously described in [7] based on a silver Anodic Stripping Voltammetry (ASV) technique, in order to evaluate the specific conformational state in each sample, thanks to the specific binding with the antibody PAb 240.

All electrodes were exposed to the same bio-functionalization steps as follow: i) p53 immobilization on the sensor surface via drop-casting plus storage for 2 h at 4 °C. Analyzed samples containing 8 µg/ml of the target proteins were prepared in Phosphate Buffer Solution (PBS) as background solution. ii) PAb 240 incubation for 2 h at room temperature (RT). iii) Incubation with AP-labelled detection antibody for 1 h 30 min at RT. iv) Incubation at RT in dark conditions for 30 min with the *Anodic Stripping Voltammetry* (ASV) solution containing oxidation of ionic Ag ($AgNO_3$) to metallic Ag, thanks to the reaction developing in presence of ascorbic acid (AA-p), as described in [8].

Once completing the functionalization, label-based electrochemical analysis was performed in PBS as supporting electrolyte after Ag deposition where immunocomplexes were recognized.

All the measurements have been performed using the portable potentiostat Palmsens 3EIS (Palmsens, Compact Electrochemical Interfaces). An initial constant potential of -0.12 V was applied for 5 seconds before running a *Linear Sweep Voltammetry* (LSV) procedure in the range -0.12 to +0.7 V, with a scan-rate of 40 mV/s, to cause Ag stripping. Each experiment was performed in triplicate, thus to evaluate the reproducibility and repeatability of the techniques, through a statistical analysis of the results. Peaks observed in LSVs were analyzed using Nova 1.11 statistical software and compared after removing the capacitive current contribution.

C. Spectrophotometer testing

The spectrophotometry measurement was acquired at ambient conditions with Shimadzu UV-2600 system. The equipment has a measuring wavelength ranging from 185nm – 1400nm with a wavelength accuracy of ±0.3 nm, it used 50 W halogen lamp and deuterium lamp with have a noise level of 0.00003Abs RMS (500nm) and used UV Probe application for operation of equipment.

The experiment is designed using spectrophotometer to detect p53 protein conformation changes by identifying absorbance change as each state of protein is assumed to have a different absorbance for different states of protein, figure 1 shows the experimental setup of spectrophotometer experiment used to measure the absorbance of solutions prepared with alterations in target protein. Quartz cuvette of 1ml capacity for holding solutions with varied conformational states of target protein. MATLAB software for entering data to obtain a visual graphical trend of absorbance spectrum.

Quartz cuvette containing 1ml of PBS buffer solution is set in spectrophotometer to note the absorbance of reference

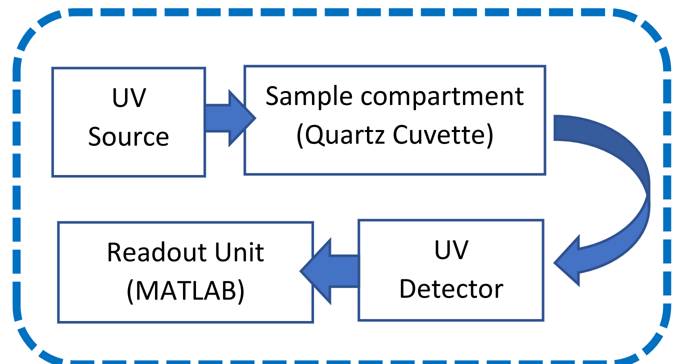

Figure 1: Experimental setup

solution and to set the baseline for the experiment. The procedure is repeated with wide type, denatured type and oxidized states of p53 solution along with PBS as a reference solution to attain and record their absorbance respectively the experiment was repeated thrice to measure the repeatability of the results.

The absorbance data obtained from each solution prepared is then collected on which a MATLAB algorithm is applied to acquire a graphical absorbance spectrum for interpretation and obtain meaningful information related to absorbance of varied p53 protein conformational states.

III. Results

D. *Sample carachterization through label-based Voltammetry*

Results obtained from label-based analysis of different redox p53-products confirm a higher specificity of PAb 240 for the open isoform (denatured p53), as expected from the literature [25] – [27], while no affinity for the wild type conformation has been found (Fig.2). Interestingly, PAb 240 recognizes denatured, oxidized and nitrated p53 respectively with decreasing affinity, suggesting that the origin of oxidant species (RNS or ROS) and the rate of oxidant for the reaction could give rise to different redox-isoforms of p53.

E. *Sample carachterization through Spectrophotometery*

The absorbance spectrum obtained from the spectrophotometer for the different conformational states of p53 clearly distinguish the structural states of p53 protein.

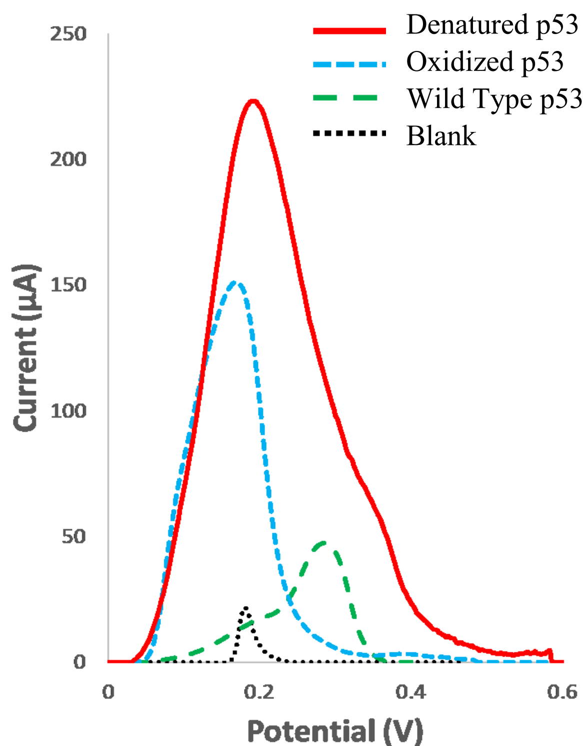

Figure 2: Label Based Voltammetry evaluation of different conformations of p53 protein using PAb240.

Table 1: Absorbance wavelength of p53 Wild type and p53 Denatured type and oxidized p53

Solution	**Absorbance peak 1**	**Absorbance peak 2**	**Molecular orientation of p53**
Wild type p53	205nm	----	
Denatured p53	213nm	240nm	
Oxidized p53	230nm	274nm	

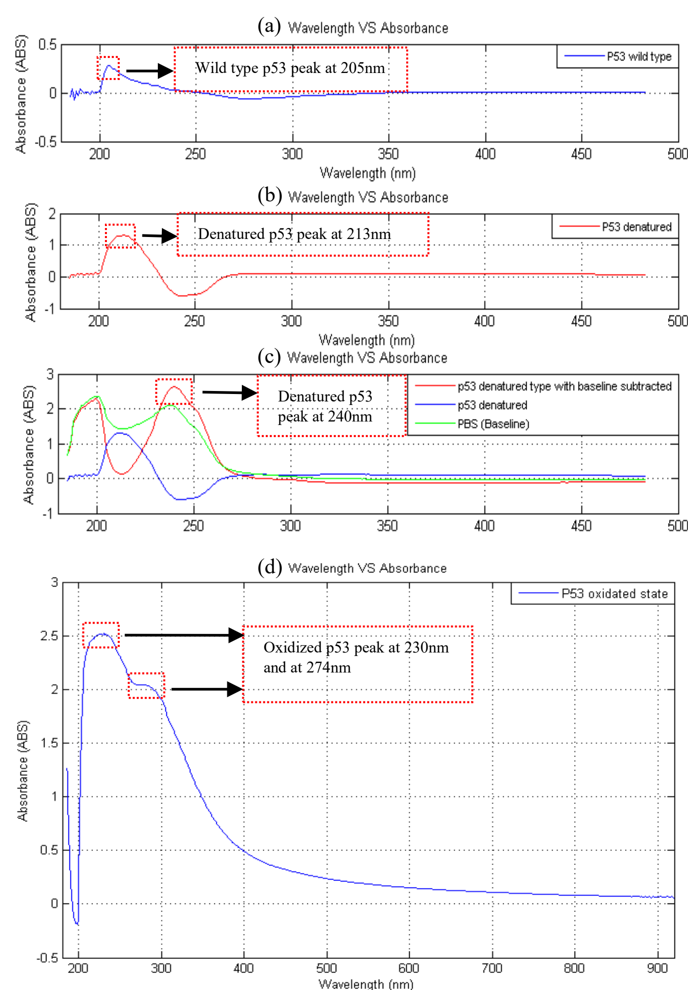

Figure 3: a) Absorbance graph of Wild type p53 b) Absorbance graph of Denatured p53 c) Absorbance of denatured p53 with baseline subtracted d) Absorbance graph of oxidized p53

Table 1 presents the peak absorbance of different solution and from Figure 3 we can narrow down the concentrated

regions of absorbance. Wild type p53 protein is found to be lie in the region of 205nm – 206nm whereas denatured type p53 protein shows major absorbance within the region of 212nm – 214nm with maximum peak at 213nm. Furthermore, denatured p53 showed another negative peak around 235-245 nm. On the other hand, when the spectrum of PBS solution was subtracted by denatured p53, a positive absorbance was measured in this wavelength region with a peak at 240nm. Moreover, the absorbance region for oxidized p53 protein was found to be expressing in two regions: i) 225nm – 235nm with a peak absorbance at 230nm and ii) 270nm – 278nm with a peak absorbance at 274nm.

The experiment was successfully able to distinguish the three states of p53 protein, suggesting that by using spectrophotometry it is possible to identify the different molecular orientations of the protein.

Hence it is comprehensible from the absorbance data as shown in table 1, figure 2 and absorbance graphs obtained by the technique of spectrophotometry as appreciated from figure 3 that the absorbance spectrum varies with variation in protein structure.

IV. Discussion

For the detection of p53 protein transitions from wild type to oxidized state and then to denatured type, the spectrophotometry technique was employed, the results of which are also supported using voltammetry approach.

This specific conformationally altered antibody used in the voltammetry experiments recognizes the amino acids harbored in the DNA binding domain, that are masked when the protein is in the wild type conformation, and exposed when it is denatured. Therefore, the different affinity of this antibody in recognizing the different p53 redox products suggest the possibility to distinguish different p53 isoform during the transition from the wild-type to the unfolding structure.

The graph obtained using voltammetry or label-based approach as indicated from figure 2 shows a significant difference in peak height for each of the different p53 redox products. PAb240 appears to be more specific to the completely *Denatured p53*, in agreement with what observed in the literature [21], suggesting that denaturation process of p53 was successful and the target epitope was correctly exposed. Further, the peak height related to the oxidized p53, intermediate between wild and unfolded type, suggests that the specific conformation obtained after protein oxidation is not completely denatured, and thus recognized less specifically from the PAb240 then *Denatured p53.*

In light of these different decreasing affinity between the specific conformationally altered antibody and different p53 products, the result obtained from spectrophotometer allows to appreciate that each peculiar state of protein shows different absorbance spectrum. The peak absorbance for wild type is noted at 205nm as appreciated from fig 3a. For denatured p53 protein positive absorbance height is obtained at 213nm and a negative peak was found at 240nm (fig3b and fig 3c). Also, the absorbance for oxidized form of target protein was measured for which optimum absorbance was obtained at 230nm and 274nm as shown in fig 3d. In the light of above observations, it can be concluded that different conformational states of protein can be identified by using spectrophotometer.

V. Conclusion

The dispersions curves obtained from the label-based voltammetry of p53 protein showed distinguishable peaks for all the conformational states of protein. Oxidize p53 has intermediate affinity to the specific conformationally altered antibody respect to the Wild type and Denatured one, thus it is possible to speculate that the epitope masked in wild type p53 is still not completely exposed in Oxidized p53. The absorbance peaks obtained from spectrophotometry were appreciatively distinct and thereby proving the concept of using spectroscopy as an independent technique to identify the different conformational states of protein. The peak wavelengths for all three states of protein i.e. Wild type p53, denatured p53 and oxidized p53 are 205nm, 213nm & 240nm, 230nm & 274nm respectively.

The positive results obtained from the spectrophotometry technique has therefore proven to be a novel technological advancement in improving health care by enabling the identification of different conformational state of proteins, thus in turning different molecular orientations, hence assisting clinicians to detect disease in its initial stages.

References

[1] E. V Suprun, V. V Shumyantseva, and A. I. Archakov, “Protein Electrochemistry: Application in Medicine. A Review,” *Electrochim. Acta*, vol. 140, pp. 72–82, 2014

[2] Z. Svobodova, M. Reza Mohamadi, B. Jankovicova, H. Esselmann, R. Verpillot, M. Otto, M. Taverna, J. Wiltfang, J.-L. Viovy, and Z. Bilkova, “Development of a magnetic immunosorbent for on-chip preconcentration of amyloid beta isoforms: Representatives of Alzheimer’s disease biomarkers.,” Biomicrofluidics, vol. 6, no. 2, pp. 24126–2412612, Jun. 2012.

[3] C. Humpel, “Identifying and validating biomarkers for Alzheimer’s disease,” Trends Biotechnol., vol. 29, no. 1, pp. 26–32, Jan. 2011.

[4] A.J. Levine p53, the cellular gatekeeper for growth and division Cell, 88 (1997), pp. 323-331

[5] D. Uberti, C. Lanni, T. Carsana, S. Francisconi, C. Missale, M. Racchi, S. Govoni, and M. Memo, “Identification of a mutant-like conformation of p53 in fibroblasts from sporadic Alzheimer’s disease patients.,” Neurobiol. Aging, vol. 27, no. 9, pp. 1193–1201, Sep. 2006.

[6] C. Lanni, M. Racchi, G. Mazzini, A. Ranzenigo, R. Polotti, E. Sinforiani, L. Olivari, M. Barcikowska, M. Styczynska, J. Kuznicki, A. Szybinska, S. Govoni, M. Memo, and D. Uberti, “Conformationally altered p53: a novel Alzheimer’s disease marker?,” Mol. Psychiatry, vol. 13, no. 6, pp. 641–647, Jun. 2008.

[7] L. Buizza, G. Cenini, C. Lanni, G. Ferrari-Toninelli, C. Prandelli, S. Govoni, E. Buoso, M. Racchi, M. Barcikowska, M. Styczynska, A. Szybinska, D. A. Butterfield, M. Memo, and D. Uberti, “Conformational altered p53 as an early marker of oxidative stress in Alzheimer’s disease.,” PLoS One, vol. 7, no. 1, p. e29789, 2012.

[8] A. P. Dhawan, W. J. Heetderks, M. Pavel, S. Acharya, M. Akay, A. Mairal, B. Wheeler, C. C. Dacso, T. Sunder, N. Lovell, M. Gerber, M. Shah, S. G. Senthilvel, M. D. Wang, and B. Bhargava, “Current and Future Challenges in Point-of-Care Technologies: A Paradigm-Shift in Affordable Global Healthcare With Personalized and Preventive Medicine,” Translational Engineering in Health and Medicine, IEEE Journal of, vol. 3. pp. 1–10, 2015.

[9] D. Polese, A. Convertino, L. Maiolo, A. Ferrone, L. Pazzini, M. Marrani, F. Maita, A. Pecora, G. Fortunato, and G. Fiaschi, “Investigation on nanostructured biosensor for Biotin detection,” SENSORS, 2014 IEEE. pp. 1627–1630, 2014.

[10] S. Feng, L. E. Roseng, and T. Dong, “Quantitative detection of Escherichia coli and measurement of urinary tract infection diagnosis possibility by use of a portable, handheld sensor,” Medical Measurements and Applications (MeMeA), 2015 IEEE International Symposium on. pp. 586–589, 2015.

[11] M. M. S. Silva, a. C. M. S. Dias, M. T. Cordeiro, E. Marques, M. O. F. Goulart, and R. F. Dutra, “A thiophene-modified screen printed electrode for detection of dengue virus NS1 protein,” Talanta, vol. 128, pp. 505–510, 2014.

[12] R. Elshafey, A. C. Tavares, M. Siaj, and M. Zourob, “Electrochemical impedance immunosensor based on gold nanoparticles-protein G for the detection of cancer marker epidermal growth factor receptor in human plasma and brain tissue.,” Biosens. Bioelectron., vol. 50, pp. 143–9, 2013.

[13] Y. H. Yun, B. K. Lee, J. S. Choi, S. Kim, B. Yoo, Y. S. Kim, K. Park, and Y. W. Cho, “A glucose sensor fabricated by piezoelectric inkjet printing of conducting polymers and bienzymes.,” Anal. Sci., vol. 27, no. 4, p. 375, 2011.

[14] A. M. Pernia, M. J. Prieto, I. C. Orille, J. A. Martin-Ramos, and A. Costa-Garcia, “Development of Optimized Screen-Printed Immunosensors,” Instrumentation and Measurement, IEEE Transactions on, vol. 58, no. 7. pp. 2181–2188, 2009.

[15] R. A. S. Couto, J. L. F. C. Lima, and M. B. Quinaz, “Recent developments, characteristics and potential applications of screen-printed electrodes in pharmaceutical and biological analysis,” Talanta, vol. 146, pp. 801–814, Jan. 2016.

[16] V. Escamilla-Gómez, D. Hernández-Santos, M. B. González-García, J. M. PingarrónCarrazón, and A. Costa-García, “Simultaneous detection of free and total prostate specific antigen on a screen-printed electrochemical dual sensor,” Biosens. Bioelectron., vol. 24, pp. 2678–2683, 2009.

[17] Y.-F. Liang, C.-Y. Huang, and B.-D. Liu, “A Voltammetry Potentiostat Design for Large Dynamic Range Current Measurement,” Intelligent Computation and Bio-Medical Instrumentation (ICBMI), 2011 International Conference on. pp. 260–263, 2011.

[18] G. Martínez-Paredes, M. B. González-García, and A. Costa-García, “Genosensor for detection of four pneumoniae bacteria using gold nanostructured screen-printed carbon electrodes as transducers,” Sensors Actuators, B Chem., vol. 149, pp. 329–335, 2010.

[19] P. Kara, A. de la Escosura-Muñiz, M. Maltez-da Costa, M. Guix, M. Ozsoz, and A. Merkoçi, “Aptamers based electrochemical biosensor for protein detection using carbon nanotubes platforms,” Biosens. Bioelectron., vol. 26, pp. 1715–1718, 2010.

[20] B. Jeong, R. Akter, O. H. Han, and C. K. Rhee, “Increased Electrocatalyzed Performance through Dendrimer- Encapsulated Gold Nanoparticles and Carbon Nanotube-Assisted Multiple Bienzymatic Labels: Highly Sensitive Electrochemical Immunosensor for Protein Detection,” 2013.

[21] C. Meplan, M. J. Richard, and P. Hainaut, “Redox signalling and transition metals in the control of the p53 pathway.,” Biochem. Pharmacol., vol. 59, no. 1, pp. 25–33, Jan. 2000.

[22] http://www.chem.ucla.edu/~harding/IGOC/B/beers_law.html

[23] S. D. Gupta, G. Kelp, N. Arju, S. Emelianov and G. Shvets, "Metasurface-enhanced infrared spectroscopy: From protein detection to ceils differentiation," *2017 Conference on Lasers and Electro-Optics Europe & European Quantum Electronics Conference (CLEO/Europe-EQEC)*, Munich, 2017.

[24] J. Zhou, X. Rao, J. Tian, J. Wang and T. Li, "Study of protein conformation change induced by pH condition using terahertz spectroscopy," *2017 10th UK-Europe-China Workshop on Millimetre Waves and Terahertz Technologies (UCMMT)*, Liverpool, 2017.

[25] Hainaut P and Milner J, A structural role for metal ions in the “wild-type” conformation of the tumor suppressor protein p53. Cancer Res 53: 1739–1742, 1993.

[26] Méplan C, Richard MJ, Hainaut P. Redox signalling and transition metals in the control of the p53 pathway. Biochem Pharmacol. 2000 Jan 1;59(1):25-33.

[27] Méplan C, Richard MJ, Hainaut P. Metalloregulation of the tumor suppressor protein p53: zinc mediates the renaturation of p53 after exposure to metal chelators in vitro and in intact cells. Oncogene (2000) 19,