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The Electrochemical Detection of Interleukin-8, Cancer Biomarker, Based on a Gold Nanoparticle Platform and its Political Implications

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Chapter 1

Introduction

a. Purpose of the study

Cancer is a disease that affects the lives of millions worldwide as no cure exists. According to the American Cancer Society, it was projected that in 2010 alone, there would be a total of 1,529,560 new cases of cancer and 569,490 deaths from cancer.[1] Of these new cases, nearly 36,540 of them will be caused from squamous cell carcinoma of the oral cavity and pharynx, causing about 8,000 deaths.[1] Although no cure for cancer is currently available, there is a strong connection between survival rate and early detection. According to a 2008 study on lung cancer, it was found that 84% of cases are diagnosed at an advanced stage.[2] In the same year, 1.5 million people worldwide were diagnosed, leaving 1.3 million people dead.[2] Patients diagnosed at an early stage, however, have an 86% overall five-year survival.[2] For this reason, early detection strategies must be employed if mortality rates are to decrease.

The goal of this research is to develop an extremely sensitive and accurate method for early detection cancer biomarker proteins and monitoring response to therapy. Ideally, the method will lead to reduced costs of treatment, stress among patients and families, and provide devices for early screening and on-the-spot diagnosis. Interleukin-8 is a cytokine involved in the inflammatory response.[3] When found at elevated concentrations in the human blood serum, IL-8 serves as a biomarker for many cancers, including head and neck squamous cell carcinoma (HNSCC). HNSCC poses an extreme difficulty in monitoring a biomarker for early detection, thus leading to diagnoses at advanced stages.[4]

This pell honors senior thesis (chapter 2) demonstrates recent advances in electrochemical detection of cancer biomarker IL-8 in a clinically relevant calf serum sample. Two immunosensor approaches were employed, resulting in a detection range of 1.0 fg mL^{-1} to 2000 pg mL^{-1}. The approach taken used nanostructured electrodes with a glutathione protected gold nanoparticle (GSH-AuNP) platform along with 1.0 μm superparamagnetic beads. Antibody-antigen chemistry was used on the electrode along with the beads for an ulatrsensitive immunoassay. The beads bound horseradish peroxidase (HRP) to react with injected hydrogen peroxide (H_2O_2). The change in current that results from this reaction correlates to the concentration of IL-8 antigen present, creating the calibration curve and detection range.

The initial studies involved optimization of the concentrations of the capture (Ab1) and detection (Ab2) antibodies in the sandwich immunosensor protocol. This critically important step in immnosensor development minimizes non-specific binding events (NSB) that often control the detection limits. The optimum analytical conditions for IL-8 detection was were

then used to produce the calibration curves, giving us the detection range. Our most sensitive method usinf massively labeled 1 mm superparamagnetic beads gaive an IL-8 detection limit of 1 fg mL-1 which is 30,000 fold lower detection limit than the current ELISA method used in hospitals.

Chapter 3 addresses the economic and political implications of early detection of cancer. The importance of the cancer research being conducted in the laboratory is that it will be implemented into the lives of those infected with cancer. The lines of communication are a key aspect to making the hands-on research worthwhile. The information and conclusions reached must get into the hands of policymakers and doctors in order to make early detection in hospitals possible. This chapter takes a look at the importance of communication and what is being done about it currently. It also integrates the current costs of treating head and neck cancer versus treating a case of cancer that has been detected early.

Finally, chapter 4 discusses improvements that can be made for cancer. Education and advocacy are crucial components in improving cancer rates and statistics. In order for one to accurately know the health factors that can increase or decrease cancer risks, education is vital. Examples of educated versus non-educated smokers are compared, along with examples of improvements made from advocacy. The chapter moves forward to look at what is needed in legislation to implement needed laws. The final stage of this chapter focuses on the idea of an “aging society” and how it will affect the future of cancer research and early detection.

b. Interleukin 8

Interleukin 8 is a cytokine that is released by monocytes and other cell types as a response to an inflammatory stimulus.[5] Specifically, IL-8 activates leukocytes, which have a role as novel mediators of inflammation. IL-8 was originally found in cultures of human blood monocytes, and it was later found to be biologically active in tumor cells.[6] Interleukin 8 has been found to be correlated with the presence of head and neck squamous cell carcinoma.[7] The three dimensional structure of interleukin 8 derived from the Brookhaven Protein Data Bank is shown in Figure 1.[8]

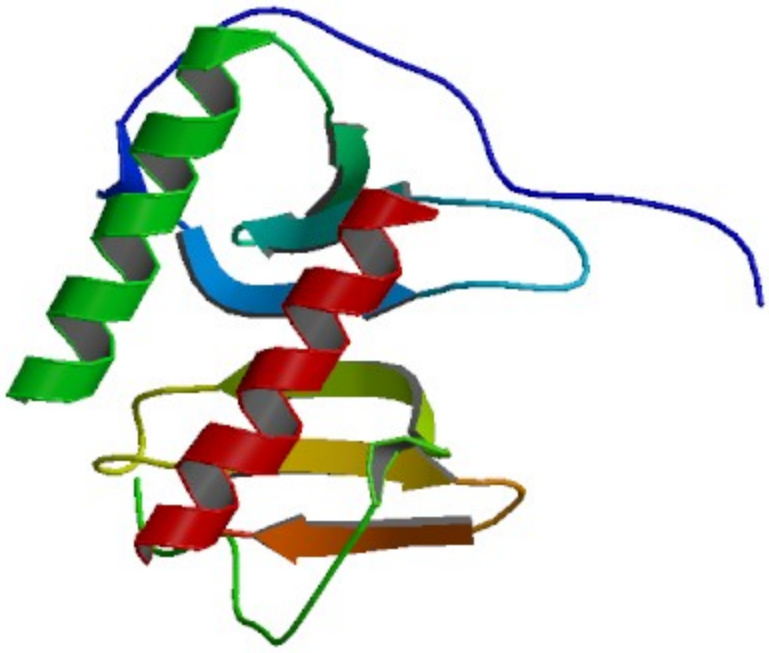

Figure 1: Three dimensional structure of interleukin 8 in solution according to the Brookhaven Protein Data Bank.

c. GSH-AuNP platform

Our immnunosensor protocol utilizes glutathione protected gold nanoparticles (GSH- AuNP) assembled on a pyrolytic graphite (PG) electrode. The GSH-AuNP were synthesized using a mixture of Methanol, $HAuCl_4$, glutathione, $NaBH_4$ and HEPES buffer following an established procedure. Poly(diallyldimethylammonium chloride) (PDDA) is a positively charged organic molecule, while GSH-AuNP are negatively charged. Because of the opposite charges, the GSH-AuNP can be adsorbed onto the monolayer of PDDA (3). This is done by adding 20 mL of 3mg/mL PDDA solution containing 0.5 M NaCl for 20 min. The electrode is then placed with 10 mL of 130 mM GSH-AuNP for 20 minutes. This causes an electrostatic force between the PDDA and GSH-AuNP and creates the platform in which the capture antibody can bind.

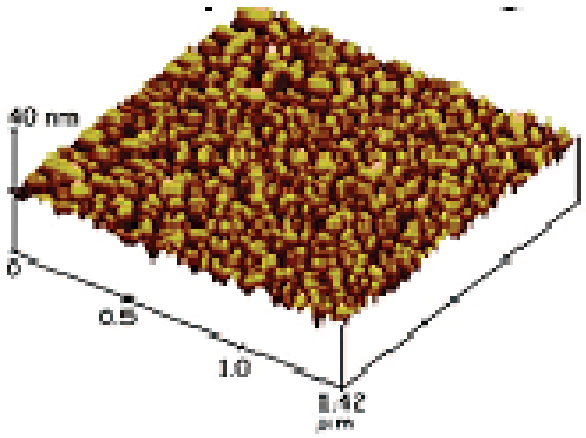

Figure 2: Atomic force microscopy image of GSH-AuNP platform atop the PG electrode. This picture demonstrates even distribution along the electrode tip of nanoparticles approximately 5 nm in size.

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Chapter 2

Electrochemical Detection of IL-8 Biomarker Using Nanotechnology

a. Abstract

Herein we report on an ultrasensitive immunosensor based on glutathione protected gold nanoparticle (GSH-AuNP) for the electrochemical detection of interleukin 8 (IL-8), cancer biomarker in calf serum and proof of concept IL-8 detection in HNSCC cells. GSH-AuNP were bioconjugated to the primary antibodies (Ab_1) and used to capture human IL-8 in a sandwich electrochemical immunoassay coupled to horseradish peroxidase enzyme labels. Using the optimized concentrations of the primary (Ab_1) and secondary antibodies (Ab_2), two sensor approaches were used to measure ultra low ($\leq$ 500 fg mL^{-1}) and elevated levels of IL-

8. Biotinylated Ab_2 bound to streptavidin HRP with 14-16 labels per antigen was used to measure high IL-8 concentration with a DL of 10 pg mL^{-1} (1.0 pM) in 10 μL calf serum. The second approach greatly amplified the signal using 1 μm magnetic beads coated with over 500,000 HRP labels providing the highest sensitivity of (1061.8 nA mL (fg IL-8)$^{-1}$ cm^{-2} and the best detection limit of 1 fg mL^{-1} (100 aM) for IL-8 in 10 μL calf serum. This represents a 10,000-fold and 30,000-fold decrease in the DL over the Ab-$HRP_{(14-16)}$ system and the industry standard ELISA for IL-8 respectively. The immuonsensors were also used to accurately measure IL-8 in HNSCC cell lines with excellent correlation to the standard enzyme linked immunosorbent assay (ELISA). These GSH-AuNP based immuonsensors show great promise for the fabrication of ultrasensitive biosensor microarrays for point-of-care cancer diagnosis.

b. Introduction

The measurement of biomarker proteins for early detection and monitoring of cancer poses an incredible challenge. However, the development of an extremely sensitive and accurate system that requires only simple maintenance is critically important and will lead to reduced costs of treatment, stress among patients and families, and will provide devices for early cancer screening and on-the-spot diagnosis.[1,2,3] Such devices will also offer a fundamental understanding of disease progression and allow monitoring of patients response to therapy.[4]

Interleukin-8 (IL-8) is a cytokine involved in the inflammatory response. It is a biomarker protein found at elevated levels in the presence of many different types of cancers including head and neck squamous cell carcinoma (HNSCC).[5,6,7,8,9] Approximately, 37,000 patients are diagnosed with HNSCC each year in the United States and about 8,000 results in death.[10]

This high number of deaths is due to the difficulty in monitoring a measurable biomarker protein for early detection, usually resulting in diagnosis at advanced stages.[10] The average concentration of IL-8 found in a healthy individual is $\leq$ 13 pg mL^{-1}, compared to the elevated levels of $\geq$ 20 pg mL^{-1} in patients with HNSCC.[11] In order to give a reliable diagnosis and accurately monitor the HNSCC, changes in both normal and elevated levels of IL-8 need to be measured.

A single biomarker found at an elevated level, however, does not give complete accuracy for a diagnosis. For example, PSA, the most widely used serum biomarker for prostate cancer, has a positive predictive value of ~75%.[12] Recent studies have shown that ~100% predictive success can be achieved by measuring 5 to 10 biomarkers of a particular cancer.[13,14,15,16] Multi-protein arrays are necessary for point-of-care detection. The ultrasensitive immunosensor development for IL-8 serves as the starting point to the development of electrochemical immunosensor arrays for many different biomarker proteins.

Conventional immunoassay methods, including enzyme-linked immunosorbent assay (ELISA),[17,18] fluorescence immunoassay, [19,20] surface Plasmon resonance (SPR),[21,22] magnetic bead-based electrochemilumincence(ECL),[23] chemiluminescence,[24,25,26] liquid chromatograpy-mass spectrometry (LC-MS)[27,28,29] and immuno-polymerase chain reaction (PCR) assay[30] allow reliable protein detection. However, these approaches are yet to meet all requirements for point-of-care diagnosis which require the sensor to be rapid, operationally simple, low cost and highly sensitive to address both levels of the biomarkers in normal and cancer patient serum. Recent methods for sensitive protein detection including nanowire nanotransistor,[31] arrays based on electrochemical[32,33] and optical[34] detection have been

reported. These methods are however at an early stage of development and have not been applied to measure IL-8 in real samples.

Our group is focused on using nanostructured electrodes coupled to multi-label signal amplification strategies to achieve highly sensitive electrochemical immunosensors. Previously, we have reported on a non amplified AuNP immunosensor for the detection of IL-6 with a DL of 10 pg mL^{-1} in calf serum.[35] More recently, we reported a DL of 0.5 pg mL^{-1} for PSA in serum using ~1 μm magnetic beads containing ~7500 HRPs per nanoparticle.[36] Alternatively, we have used vertically aligned SWNT immunosensors coupled to multi-labeled HRP-multiwall carbon nanotubes (MWNT)-HRP-Ab_2 bioconjugate to obtain a DL of 0.5 pg mL^{-1} for IL-6[37] and 4 pg mL^{-1} for PSA[38] in serum respectively. In another strategy we used 0.5 μm multi-labeled polymeric beads, polybeads–HRP-Ab_2 to achieve a DL of 10 pg mL^{-1} for MMP-3[39] in calf serum.

Herein, we report on an electrochemical immunosensor for detection of both very low and elevated levels of IL-8. The highly sensitive immunosensor is achieved by the use of ~5 nm glutathione gold nanoparticle (GSH-AuNP) platform, coupled with ~1.0 μm magnetic beads conjugated to the detection IL-8 antibody (Ab_2) and hundreds of thousands of horseradish peroxidase enzyme (HRP) labels (Ab_2-MB-HRP) via avidin-biotin interaction. This approach provides the highest number of HRP labels per binding event allowing extremely sensitive monitoring of any changes in serum concentration. The immunosensor is assembled on an electrode with a pyrolytic graphite tip, starting with the platform of GSH-AuNP. The capture antibody (Ab_1) is bound to the platform, followed by the IL-8 antigen. The magnetic bead conjugate is added to bind Ab_2 to the antigen. The signal produced through amperometry is proportional to the concentration of IL-8 antigen. At high IL-8 levels

we used Ab_2 conjugate with 14-16 labels per antigen providing a DL of 10 pg mL^{-1} in serum while for ultrahigh sensitivity, the multi-labeled Ab_2-MB-HRP bioconjugate method gave a remarkable detection limit of 1.0 fg mL^{-1}. This DL is 30,000-fold lower than the conventional hospital ELISA method. This ultralow DLs show the AuNP immunosensor offer great potential for early cancer detection and point-of-care cancer screening. Most importantly, the device is amenable to future immunosensor array fabrication.

c. Experimental Section

Chemicals and Materials. Monoclonal antihuman interleukin-8 (IL-8) antibody, biotinylated antihuman IL-8 antibody, recombinant IL-8 (carrier-free) in calf serum, and streptavidin-horseradish peroxidase (HRP) were from R&D Systems, Inc. (Minneapolis, MN). Biotinylated HRP was from Invitrogen. HRP (MW 44 000 Da), lyophilized 99% bovine serum albumin (BSA), and Tween-20 were from Sigma Aldrich. Methanol, (99% - spectrophotometric grade), 99.99% Acetic acid (glacial), 99.99% Sodium borohydride (granules), 99.9% Gold (III) chloride trihydrate, and L-Glutathione (reduced) used in the synthesis of the glutathione protected gold nanoparticle platform were from Sigma Aldrich. Poly(diallyldimethyl ammonium chloride) (PDDA), 20 wt. % in water was also from Sigma Aldrich. Immunoreagents were dissolved in pH 7.2 phosphate saline (PBS) buffer (0.137 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM NaH_2PO_4). 1-(3-(dimethylamino)-propyl)-3-ethylcarbodiimidehydrochloride (EDC) and *N*-hydroxysulfosuccinimide (NHSS) from Aldrich were dissolved in water immediately before use.

Cell Lines and Conditions

Immunosensor Fabrication. A gold nanoparticle platform was assembled on the pyrolytic graphite (PG) tip of an electrode using a monolayer of Poly(diallyldimethyl ammonium

chloride) (PDDA). Glutathione protected gold nanoparticles (GSH-AuNP) were synthesized using a reaction mixture containing acetic acid, methanol, and sodium borohydride, followed by the addition of gold chloride and glutathione to optimally obtain 5 nm gold particles coated with glutathione. The GSH-AuNPs were then adsorbed on the electrode surface by the electrostatic layer-by-layer self assembly using PDDA polyion "glue". Capture antibody (Ab_1) was attached to the GSH-AuNP platform using 30 μL freshly prepared mixture of 400 mM EDC and 100 mM NHSS in water, washing after 10 minutes, then incubating overnight, for 9 hours, with 20 μL of 10 μg mL^{-1} primary anti-IL-8-antibody (Ab_1) in pH 7.2 PBS buffer. Then the immunosensor was washed with 0.05% Tween-20 in PBS buffer for 3 minutes, replacing with new buffer after 1.5 minutes, and then washed with PBS buffer for 3 minutes, for a total of 4 washes. A blocking step involved a 1 h incubation with 20 μL of 1% BSA, followed by another wash with 0.05% Tween-20 in PBS buffer then with PBS buffer for 3 minutes each. Washing steps were optimized in previous experiments to minimize non-specific binding (NSB) to achieve the optimum sensitivity.[39]

For standardization, the immunosensor was incubated with 10 μL of calf serum containing human IL-8 for 1 h 15 min, followed by washing with 0.05% Tween-20 in PBS buffer and PBS buffer for 3 minutes each. Next, 10 μL of 0.05 μg mL^{-1} biotinylated detection antibody (Ab_2) in 1% BSA was incubated for 1 h 15 min, followed by washing with 0.05% Tween-20 in PBS buffer and PBS buffer for 3 minutes each. For the amplified system assay, the blocking step required an increase to 5% BSA. For moderate sensitivity, the immunosensor was incubated with 10 μL of streptavidin-HRP for 30 min, followed by washing with 0.05% Tween-20 in PBS buffer and PBS buffer for 3 minutes each. For a more

sensitive detection, the Ab_2 and HRP incubations were replaced by a multilabel Ab_2-Magnetic Bead-HRP bioconjugate (Ab_2-MB-HRP) (described below).

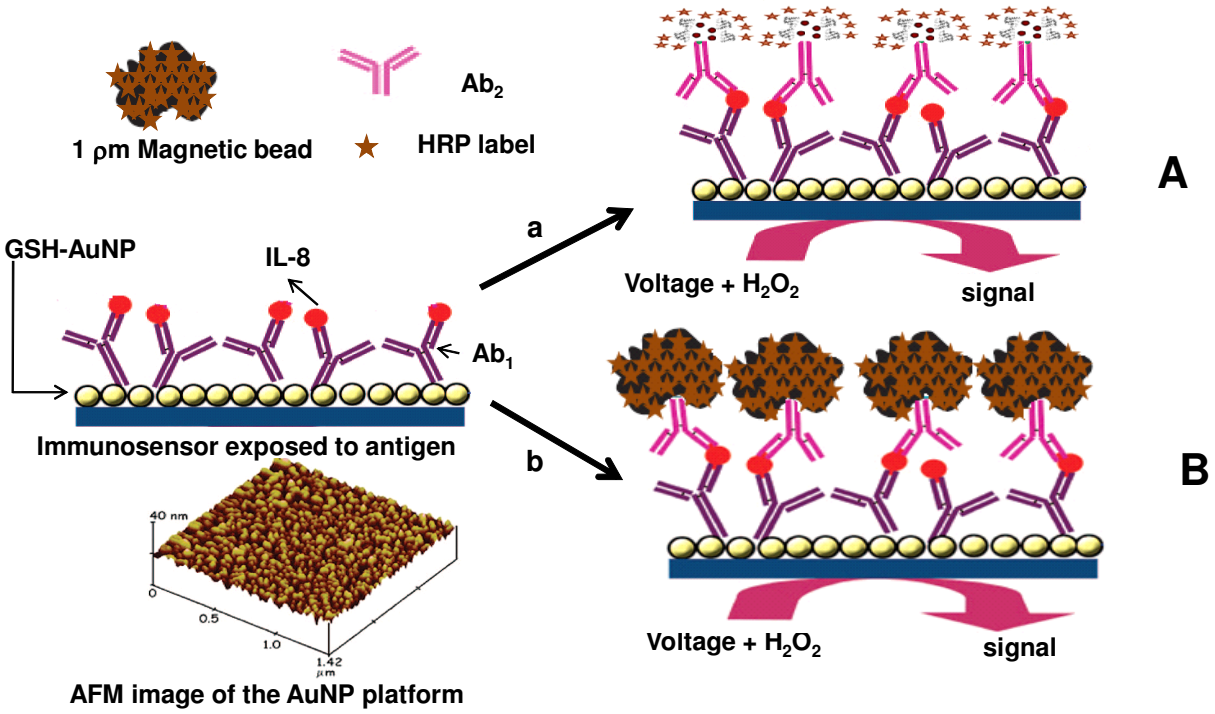
The sensor was then placed in an electrochemical cell containing 10 mL of pH 7.2 PBS buffer with 1 mM hydroquinone as a mediator. Amperometry was used by rotating the disk at 2000 rpm, and then injecting 0.4 mM H_2O_2 to generate the electrochemical signal. This same immunoassay was used to detect conditioned media from cell cultures previously described.[37] These samples were also analyzed using a standard human IL-8 Elisa kit.

Synthesis of Ab_2-Magnetic Bead-HRP Bioconjugate. Streptavidin coated magnetic beads were conjugated to multiple HRP labels and Ab_2 in order to detect the small concentration of IL-8 antigen. 200 μL (1 mg) of the beads were dispersed in 800 μl of PBS buffer. The magnetic beads were washed two more times with 1000 μl of PBS buffer. The washing step was done using MCB 1200 Biomagnetic separation platform (Sigris research, CA) while stirring at 1.0 rotation per second for 1 minute each. Using the MCB magnetic platform, the beads were separated from the supernatant, which was removed. After the 3 washes, the streptavidin modified magnetic beads were dispersed in a 50 μL of PBS buffer containing 1 mg mL^{-1} biotinylated HRP and 0.5 μg mL^{-1} biotinylated Ab_2 at a concentration ratio of 2000:1 respectively. The magnetic beads were incubated while spinning the homogeneous mixture at 0.5 rotations per second on the MCB platform for 30 minutes. Then, the magnetic beads conjugate (Ab_2-MB-HRP) were magnetically separated and the supernatant removed. This step was necessary to remove any free HRP and Ab_2. This was then followed by a quenching step where the beads were dispersed in 1000 μL PBS buffer with 1% BSA, 0.1% NaN_3 and 1mM Sodium EDTA for 30 minutes at 0.5 rotations per second. The supernatant was removed again using the MCB magnetic platform, and the beads were then washed with

PBS buffer 3 more times. Finally, the conjugate, Ab_2-MB-HRP was dispersed in 200 μL of PBS + 0.1%T-20, 5 mg mL^{-1}, stored in the refrigerator at 4°C, and then diluted with PBS + 0.1% Tween 20 before use. The magnetic beads conjugate were characterized using ABTS activity assay.

d. Results

Immunosensor Strategy. Scheme 1 show the electrochemical immunosensor steps, including a traditional labeled protocol (A) and a signal amplification strategy using multienzyme-antibody labels (B) on glutathione modified gold nanoparticles (AuNPs). Herein, we used Glutathione modified gold nanoparticles (GSH-AuNP) platform and multienzyme labeling strategy to enhance sensitivity and detection limits. AFM image of the immunosensor platform (Scheme 1, insert) show ~ 5 nm AuNPs consistent with previous reports[40] Our initial approach for elevated IL-8 antigen (Scheme 1A), we utilized a sensitive Ab_2-biotin-streptavidin-HRP (14-16) label for each assay. Minimization of non-specific binding (NSB) events is critical to achieving best sensitivity and detection limits.[41,42] BSA and Tween 20 were used to effectively block NSB. Furthermore, the capture antibody (Figure 1A) and detection antibody (Figure 1B) concentrations were optimized. Optimization experiments were done using 4.0 ng mL^{-1} of IL-8 in calf serum with all other components of the immunoassay kept constant. Figure 1A(a) shows results of the complete immunoassay with fixed detection antibody, Ab_2 concentrations at 1 μg ml^{-1} while varying the capture antibody, Ab_1 concentration. Control experiments, Figure 1A(b) to assess the level of NSB events involved all the immnosensensor steps with serum without the hIL-8 antigen. Results gave an optimum Ab_1 concentration at 10 μg mL^{-1} with the lowest signal response for control compared to the sample. Similarly, Ab_2 concentration was optimized using the best



Scheme 1. Illustration of detection principles of AuNP immunosensors. Picture (A) on the left shows the immunosensor after treating with a biotinylated Ab_2 followed by streptavidin modified HRP resulting in HRP-Ab_2 providing 14-16 label per binding event. Picture (B) on the right shows the immunosensor after treating with Ab2-MB-HRP to obtain amplification by providing 500,000 enzyme labels per binding event. On the bottom left is a tapping mode atomic force microscope image of a AuNP that serves as the immunosensor platform.

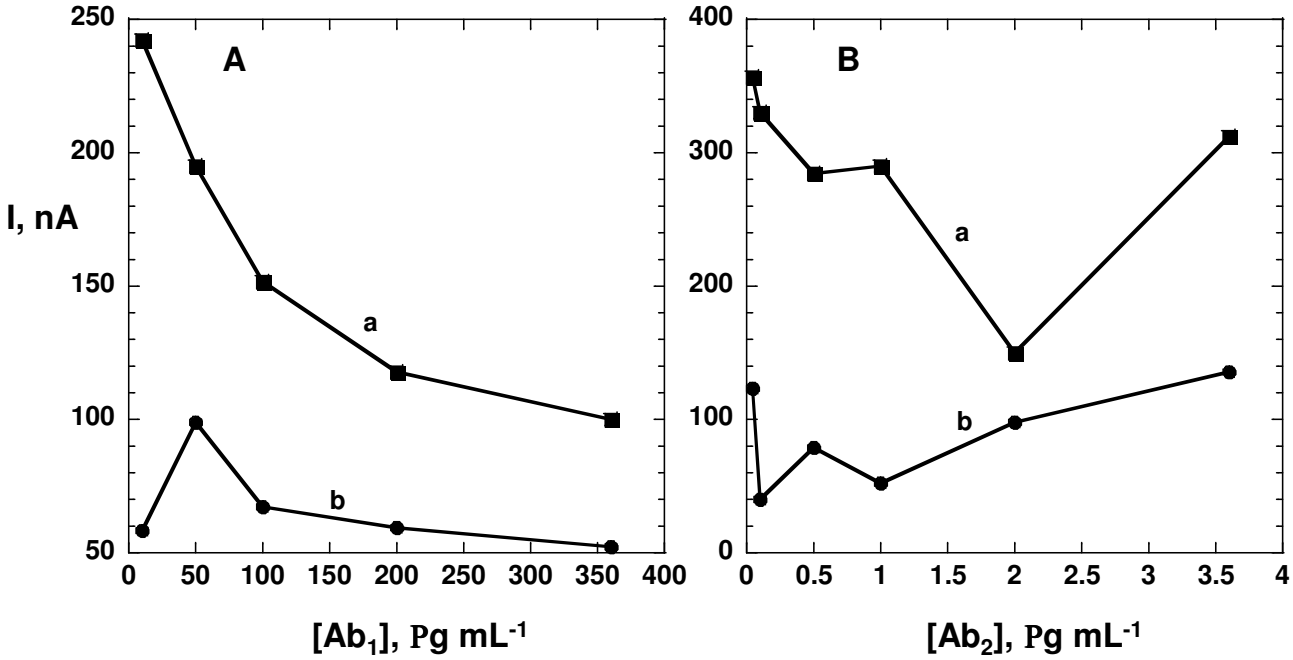


Figure 1. Optimization of; (A) Capture antibody, Ab_1 using [Ab_2] at 1 μg mL^{-1} and (B) detection antibody, Ab_2 using optimum [Ab_1] at 10 μg mL^{-1}. Streptavidin modified HRP label is used to generate the electrochemical signal after dilution at 1:200 ratio. (a) incubation with [hIL-8] at 4.0 ng mL^{-1} in new born calf serum for 1 h 15 min and (b) control, indicating full immunoassay with serum containing 0 pg mL^{-1} hIL-8.

conditions obtained in Figure 1A. Figure 1B shows the optimum Ab_2 concentration at 0.05 μg mL^{-1}. These optimal analytical conditions for the immunosensor were used to obtain a calibration curve with a sensitivity of 0.3395 nA mL (pg IL-8)$^{-1}$ cm^{-2} and detection limit for the Ab_2-$HRP_{(14-16)}$ system of 10 pg mL^{-1} human IL-8 antigen as shown in Figure 2. Figure 2A presents data obtained from the actual amperometric responses of 6 different concentrations of IL-8 that range from 10 pg mL^{-1} to 2000 pg mL^{-1}. The calibration curve (Figure 2B) shows there is a linear relationship between the concentration of IL-8 and the change in current as a result of the amperometric response. However, a more sensitive system is required in order to detect levels of IL-8 that fall below this detection limit.

The Ab_2-Magnetic Bead-HRP conjugate was used to significantly increase the sensitivity of our system. This amplified system allowed for ultrahigh sensitivity by greatly increasing the amount of HRP labels, which corresponds to the concentration of hIL-8 in a given sample through the amperometric response. Our strategy involved bioconjugating biotinylated HRP and Ab_2 to streptavidin coated magnetic bead with a reaction mixture having a 6800/1 HRP/Ab_2 mole ratio. The mutilabelled magnetic particles with hundred thousands of HRP labels were used in place of the conventional Ab_2-$HRP_{(14-16)}$ complex.

Streptavidin-biotin interaction (K_a = 10^{15}/M vs. 10^{7}-10^{11}/M for antibody-antigen interactions) [43] was used to simultaneously attach the Ab_2 and HRP to the 1 μm magnetic bead. To determine the amount of active HRP per unit weight of magnetic beads, the Ab_2-MB-HRP dispersion was reacted with HRP substrate 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)[44] and H_2O_2. The reaction produces a soluble product with characteristic optical absorbance at 405 nm. A linear increase in absorbance of the product at

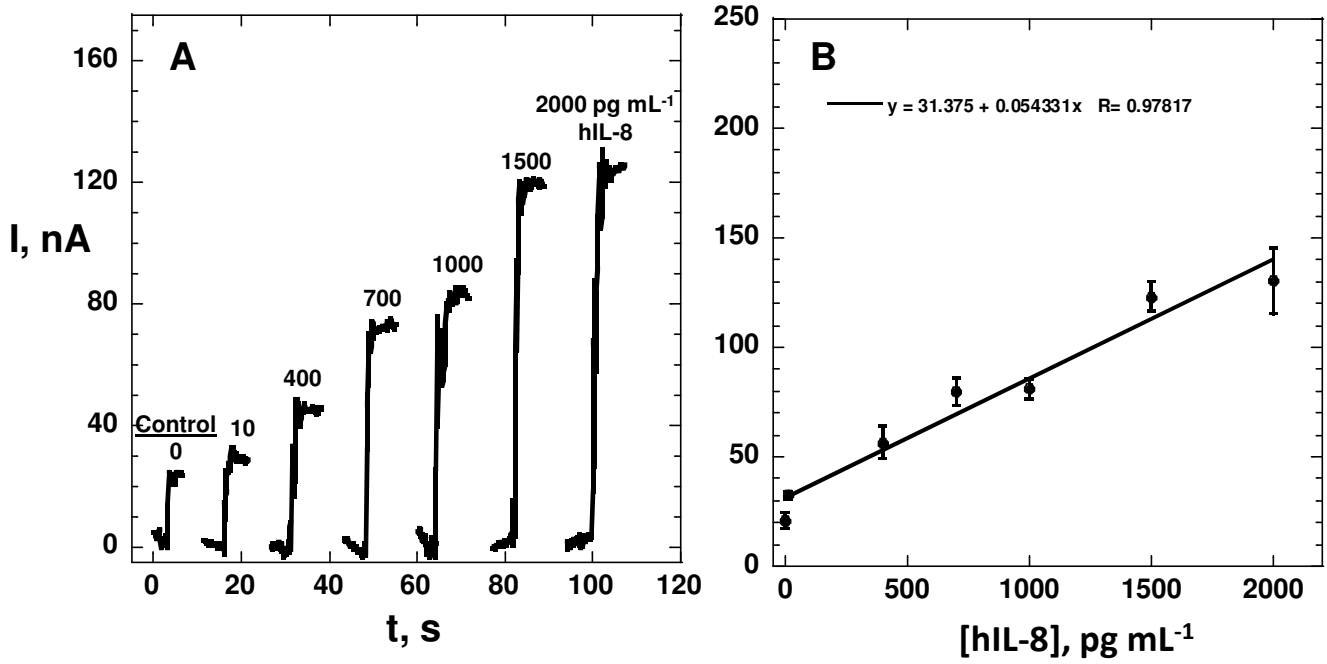


Figure 2. Amperometric response for AuNP immunosensor incubated with hIL-8 in 10µL undiluted newborn calf serum for 1.25 h then conventional anti-IL-8-biotin in 0.05% Tween-20 for 1.25 h followed by 30 min incubation with 10 µL streptavidin modified HRP at 1:200 dilution, (A) showing current response at -0.3 v and 2000 rpm after placing electrodes in buffer containing 1 mM Hydroquinone mediator, then injecting H_2O_2 to 0.4 mM to develop the signal. Control shown on the left, indicating full immunoassay with serum containing 0 pg mL^{-1} hIL-8. (B) Corresponding calibration curve of hIL-8. Errors bars in part B showing device-to-device Standard deviations (n = 3).

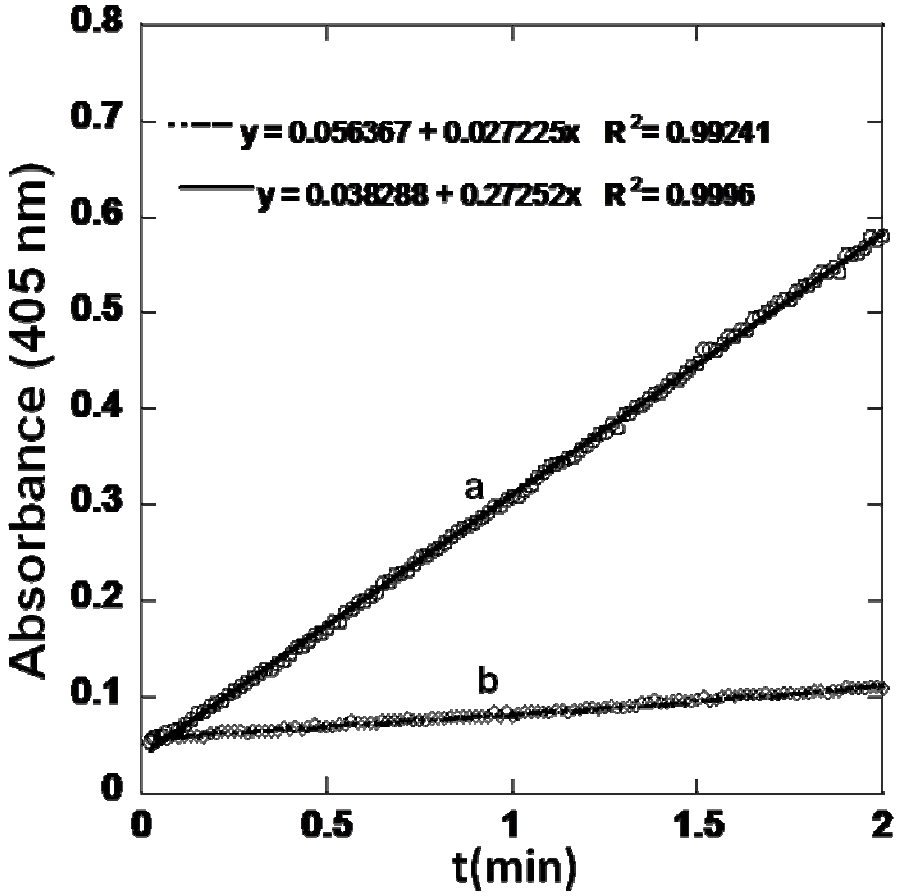


Figure 3. Results of enzyme activity assay of (a) HRP-Magnetic beads-Ab_2 bioconjugate and (b) control beads without HRP activated by H_2O_2 with ABTS as substrate to give colored product with absorbance at 405 nm.

405 nm (Figure 3) was found, and the slope was used to estimate[45] an HRP activity of 162.6 Units mL^{-1} of undiluted Ab_2-MB-HRP. This was compared to a standard curve constructed with underivatized HRP, after subtracting the background absorbance of an equivalent dispersion of underivatized magnetic beads.

The concentration of active HRP in the stock Ab_2-MB-HRP dispersion was determined in this way to be 175.9 μg mL^{-1}. Considering 1.0 mg of magnetic beads used to prepare the Ab_2-MB-HRP conjugate, we had 3.99 μmols HRP/mg beads or 19.98 μmol HRP/mL of dispersion. Using the manufacturer's specifications for the beads with 1 μm diameters and a density of 5 x 10^9 beads/mL, the number of active HRP was estimated at 502,688 per bead.

In order to reduce NSB in the amplified system, it was necessary to increase our blocking step from 1% to 5% BSA with the same amount of incubation time. Calibration data for the GSH-AuNP assay utilizing the Ab_2-MB-HRP bioconjugate with IL-8 in 10 μL dissolved calf serum is presented in Figure 4. In Figure 4A, the increasing change in current, represented by the actual amperometric response achieved with the addition of increasing concentrations of IL-8, ranges from 1 fg mL^{-1} to 500 fg mL^{-1}. Figure 4B presents a non-linear calibration curve for the amplified system with an extremely low detection limit of 1 fg mL^{-1}. This was achieved by using the Ab_2-MB-HRP bioconjugate, which significantly increased the sensitivity of the system as compared to the Ab_2-$HRP_{(14-16)}$ system. Results show that the amplified system can detect concentrations of IL-8 10,000-fold lower than that of the Ab_2-$HRP_{(14-16)}$ system.

IL-8 Secreted by Human Squamous Cells. As a proof of concept we then used the immunosensor to determine secreted levels of IL-8 in vitro cell preparations. Conditioned media from heterogeneous populations of 7 different cell lines were analyzed to test the

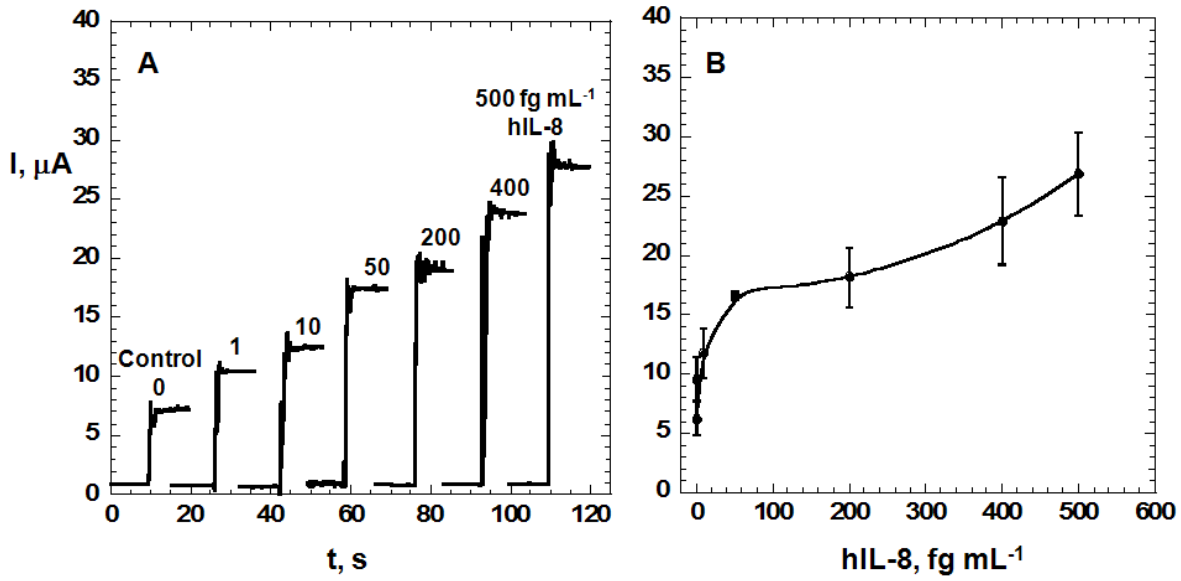


Figure 4. Amperometric response for AuNP immunosensor incubated with hIL-8 (concentration in fg mL^{-1}, labeled on curves) in 10 μL undiluted newborn calf serum for 1.25 h. ***(A)*** Current at -0.3 V and 2000 rpm using Ab_2-magnetic beads –HRP bioconjugate. Control shown on left with AuNP immunosensor with 0 pg mL^{-1} IL-8. ***(B)*** The corresponding calibration curve of hIL-8 immunosensor using Ab_2-Magnetic beads-HRP bioconjugate. Errors bars in part B represent device-to-device standard deviations.

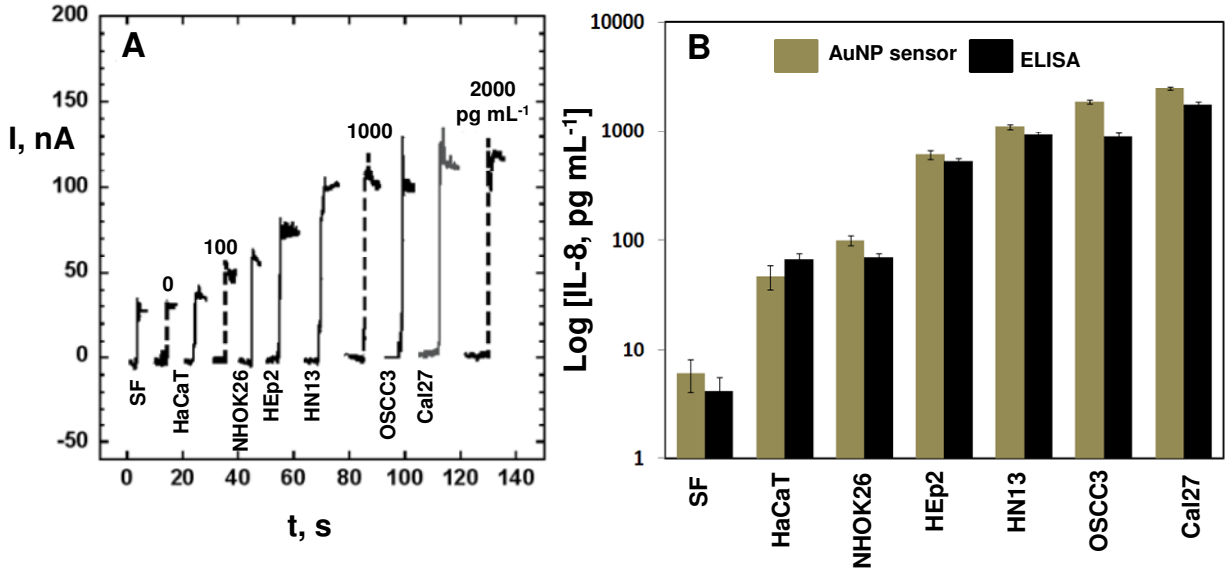


Figure 5. Amperometric response for AuNP immunosensor incubated with hIL-8 labeled on curves or conditioned media containing hIL-8 secreted by human squamous cells. Conditioned media samples SF, HaCat, NHOK26, HEp2, HN13, OSCC3 and Cal27 were analyzed using 10 µL of 0.05 mg mL^{-1} biotinylated secondary antibody (Ab_2) in 0.1 % BSA in pH 7.2 PBS buffer and 10 µL of streptavidin modified HRP. *(A)* Current at -0.3 V and 2000 rpm using hydroquinone mediator in PBS buffer, after injecting H_2O_2 to 0.4 mM; *(B)* AuNP sensor results for conditioned media shown with results from ELISA for the samples.

validity of our immunosensor approach towards hIL-8 detection in head and neck squamous cell carcinoma (HNSCC). Figure 5A shows amperometric signals for difference cell lines along with human hIL-8 standards in serum at comparable levels. Most of the cell lines (HEp2, HN13, OSCC3, Cal27) were found to contain high levels of IL-8, ranging from 612 to 1759 pg mL^{-1}, while HaCat and NHOK26 demonstrated low levels of the antigen , ranging from 46 to 99 pg mL^{-1}. Samples were also analyzed using the standard hospital ELISA method, and gave excellent correlations with our AuNP immunosensor (Figure 5B). These results suggest the validity of using this imunosensor for measurement of hIL-8 levels in samples of a variety of cancer cell types.

e. Discussion

Results described above show AuNP immunosensor coupled to multilabel amplification strategy can be used to accurately and reproducibly detect protein cancer biomarkers at extremely low, fentogram to thousand picograms per milliliter in clinical serum samples (Figure 2 & 4) and complex biological matrix (Figure 5). Two approaches were used to achieve such low detection limit including the Ab_2-$HRP_{(14-16)}$ and Ab_2-MB-HRP with 502,688 HRP labels providing the best sensitivity of 1061.8 nA mL (fg IL-8)$^{-1}$ cm^{-2} at low concentrations (1 – 50 fg mL^{-1}) and a remarkable low detection limit of 1.0 fg mL^{-1} (Figure 3). The extremely high HRP label loading on the magnetic bead bioconjugate is achieved by taking advantage of streptavidin-biotin interactions. This detection limit is 10,000-fold and 30,000-fold lower than the Ab_2 biotin-streptavidin-$HRP_{(14-16)}$ and the standard ELISA[46] techniques respectively. Furthermore, the sensitivity is 6-orders of magnitude better than the Ab_2-$HRP_{(14-16)}$. Moreover, these results offer 500-fold lower detection limit and 55000-fold better sensitivity than our recent results using SWNT forest immunosensor[37] reported for IL-

6, a cytokine similar to IL-8. This extremely low detection limits were achieved by effective minimization of NSB which often controls the detection limits using BSA and tween-20 and optimization of the capture and detection antibody concentrations respectively (Figure 1).

The two AuNP immunosensor approaches, Ab_2-$HRP_{(14-16)}$ and Ab_2-MB-HRP show good reproducibility as demonstrated by small device to device standard deviations (Figures 3, 4-5). Furthermore, good accuracy for hIL-8 was demonstrated by good correlation of AuNP immunosensor results with ELISA assay for a wide variety of conditioned media samples. Accurate determination of IL-8 in conditioned media which normally contains a range of other proteins demonstrated selectivity of our immunosensor.

f. Conclusions

We have demonstrated ultrasensitive, selective and reproducible electrochemical detection of IL-8 representative of extremely low, cancer free and high level cancer patients in a broad range of head and neck cancer cell lines. The streptavidin modified magnetic bead amplification strategy gave extremely high sensitivity of 1061.8 nA mL (fg IL-8)$^{-1}$ cm^{-2} and ultra-low detection limit of 1.0 fg mL^{-1} which is 30,000-fold lower than the conventional hospital ELISA method. These results show great promise for the fabrication of immosensor arrays for point-of-care cancer screening and monitoring of patients response to therapy.

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Chapter 3

Current Ways of Addressing Head/Neck Squamous Cell Carcinoma

a. Communication

Cancer in general is a disease that almost every family in the world has to overcome at some point in time. Increased awareness over the past few decades has shown a more accurate number of how many people really suffer from all different kinds of cancer. With head and neck squamous cell carcinoma affecting nearly 37,000 people a year,[1] awareness is absolutely essential. Unfortunately, in most cases, the diagnosis of this disease is delayed because it relies on the patient presenting his/her concerns to the doctor and physical examination with biopsy confirmation[2]. Thus, the majority of patients with head and neck cancer are not diagnosed until the cancer mass has become life-threatening.[3]

Due to such late diagnoses, the survival rate has not shown appreciable change in decades. Five-year survival data reveal overall disease specific survival rates of less than 60%, although those who do survive often endure major functional, cosmetic, and psychological burden due to dysfunction of the ability to speak, swallow, breathe, and chew. [3] Such devastating statistics has made it very much the will of a huge sector of Americans to change the course of cancer. [4]

Easily, the most important aspect of cancer research is the communication between the researchers and policymakers. While laboratory workers find promising and successful results of early detection, they must get these results into the hands of doctors and nurses who can accurately update treatment and early testing/screening. If these communication lines were not available, the research done would simply be useless since it would not be implemented in treating patients and treatment plans. Thus, effective communications and networks are the keys to making this vision a reality. In depth discussions must be had with purchasers, doctors and nurses, and others involved with cancer services within hospitals or the community across the region. [5]

The success of communications has been achieved by engaging the business, government, and scientific communities as partners in advocacy. [6] Once results are obtained and are ready to be made public, political action must be taken in order to implement the findings as soon as possible. By working together for legislative, regulatory, and funding changes, cancer advocates seek to achieve benefits, such as heightened screening, improved treatment, increased clinical trial enrollment, and rapid translation of research findings. [6]

For example, the communications between the scientific world and the political world proved successful in the 80's and 90's when the Mammography Quality Standards Act was passed for expansion of the Breast and Cervical Cancer Early Detection Program, and there were extensive increases in federal funding for the National Cancer Institute. [6] The near constant updates of the scientific research must be told to the policy makers in order to immediately put the results into action. The policy makers can also decide where increased funding gets distributed to in order to be the most effective. As shown, these lines of communication have proved important and useful in the past, and will continue to be the key to early screening and detection.

b. Costs

Squamous cell carcinoma of the oral cavity and pharynx accounts for over 37,000 cases per year in the United States with about 8,000 deaths per year. [7]Thus, it has become the sixth most common cancer in the world. This fact along with its late diagnosis, treating a case of this cancer can cost an upwards of $250,000. [8] Care and treatment for cancer has nearly doubled over the past twenty years, going from $96.1 billion in 1990 to $189.8 billion is 2004.[8]

As discussed earlier, the purpose of this research is to be able to detect whether a person is predisposed and/or at a higher risk of getting head and neck cancer. The National Cancer Institute (NCI) and others are now funding major initiatives to identify cancer at its earliest and most curable stage, for example, by basic research in identifying serum biomarkers indicating that an early cancer is developing. [9] Detecting cancer at such an early stage will

diminish the need for extremely expensive treatments. The cancer can and will be monitored closely by the patient's doctor, and thus it will never fully develop since they will be able to properly care for it before it becomes life-threatening. As a large scale result, costs for cancer will decrease as fewer people need extensive treatment.

By knowing what biomarkers are already present in a person's blood, one may be more cautious of his/her actions since 75% of these cancers are related to alcohol and tobacco use. [10] People will also be more aware of the need for screening more often so that the tumors are caught at an early stage. Screening for oral, head and neck cancer can be easily completed in less than 5 minutes, and people should know the importance of this process, especially those who are predisposed to this form of cancer. [10]

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Chapter 4

Improvements in Public Cancer Education

a. Education/Advocacy

"Knowledge is an important precursor to behavior change."[1] Education of cancer and the risks that can cause cancer is the first step to being able to cure cancer. Although scientists have made break-through discoveries about the risks that certain health choices have of getting cancer, those who are uneducated will not benefit. The importance of educating the public properly is key, and making sure that knowledge stays with them plays a crucial role.

In 2005 alone, nearly 1.7 million Americans were diagnosed with cancer. Of those, 1,500 would die per day. To make matters worse, it is estimated that 50%, if not more, of all cancer

would be preventable if individuals chose to live more healthy lifestyles.[1] Such choices include diet, exercise, smoking tobacco, and drinking alcohol.

The easiest way to inform the public about what they should know about modifiable cancer risks is by the media. "We should assume that cancer knowledge about topics receiving prominent coverage should be more easily retrieved from memory than those covered less frequently."[1] Health Information National Trends Survey (HINTS) conducted a survey of Americans asking if they reported paying attention to health information in the newspapers. Of 3,784 people surveyed, 51% and 58.8% recalled risks of food/diet and smoking, respectively. On the other hand, only 25%, 9% and 11% recalled information on exercise, avoiding sun/sunscreen, and alcohol, respectively.[1]

Such results show which cancer risks are most closely watched by Americans - smoking. In the past, studies have shown that news coverage has been associated with decreases in the amount of people who smoke.[1] The National Cancer Institute's (NCI) Office of Cancer Communications ran the same study in two different years to analyze cancer news coverage. The first was done in 1977and the second in 1980. In both years, cancer news focused on cancer causes, celebrities with cancer, and treatment. The coverage on prevention went up from 1% in 1977 to 3% in 1980, and the discussion of risks went up from 20% to 50% as well. However, modifiable risk factors were rarely discussed in either year.[1]

Among these modifiable cancer risks, smoking is the leading cause of preventable death in the United States. Smoking itself accounts for 30% of all cancer deaths.[2] The health risks of smoking became public in 1964 with the first surgeon general's report on smoking and health. A group of over 150 consultants and 7,000 scientific articles were examined over a

period of two years, resulting in the first public statement on the risks of smoking in relation to cancer. By 1970, labels were made for every pack of cigarettes warning people of the health risks.[3] Since this breakthrough, America has become much more aware of the dangers of smoking. However, "research suggests that knowledge perceptions of associated risks of tobacco use may not be evenly distributed in the population."[2]

According to a study done by Health Information National Trends Survey (HINTS) in 2003, the highest percentage of people who currently smoke was in the southeastern part of the United States and up into Wisconsin, Minnesota, and North Dakota. A corresponding study was done with the question, does smoking increase chances of cancer? The lowest percentage of people who believed the smoking made them at a higher risk for getting cancer were in nearly all the regions where smoking rates were highest. These graphs give some insight into where the media is failing to properly educate the public.[2]

Another study was done in 2005 complimenting the previous results. The cancer mortality rates of each state were calculated and separated into categories: white males, black males, white females, and black females. Within the top ten states listed in each category as having the highest mortality rates for cancer, at least five of the states with the highest smoking rates from the previous study were listed. This information shows that not only is smoking increased where education and media is decreased, but the mortality rates are also increased.[4]

The importance of educating the public on the risks that they can change themselves is extraordinarily important. Reducing one's own risks allows him or her to take control of life. Being aware of what effects certain choices have on the body is the first step to making such

decisions. It has been proven that "low levels of scientific literacy generally impair the public's ability to accurately understand and apply scientific concepts or issues to their own lives."[5] Thus, such concepts as modifiable preventions must be in the mainstream for the public to learn in order to change their lives. "Because what people learn is partly a function of available information, it is important to ascertain the extent to which the news media discuss cancer prevention."[4]

b. Legislation

Legislation is what makes the dream of laboratory workers a possible reality. They are the ones who create the laws regarding cancer screening and coverage. These laws prove the necessity for screening as a direct result of the lab data. Thus, the current communication being implemented in terms of cancer research needs to be directed particularly toward legislation so that the laws can be made in the most efficient time.

Coverage for cancer screening is not mandated in all states of the United States. A study was done in 2000 to "determine the prevalence and nature of state coverage mandates for cancer screenings."[6] In order to do so, researchers contacted the insurance departments of the 50 US states, Washington DC, and Puerto Rico. They asked for copies of the state codes that mandated coverage for a variety of cancers by private insurers.[6]

The results stated that "forty-three states and the District of Columbia currently mandate coverage of cancer screening. Breast cancer–screening coverage was most frequently mandated (n =44), followed by cervical (n =22), prostate (n =18), and colorectal cancer screening (n =1).Of 85 mandates in place, 57 have been passed since 1990. State mandates

for insurer coverage of cancer screening are common and increasing."[5] However, they are not mandatory in every state, and they do not cover every cancer.

In an ideal situation, all states would have cancer screening coverage so that every person can get screened for all types of cancer. This would directly result in fewer extreme cases of cancer, and thus spending would decrease. The amount of people paying for extensive treatment would also decrease, and, as a long term result, the US would be saving money on treatment programs. The research done throughout this thesis shows why screening for head and neck cancer in particular should be mandatory. The amount of money that would be saved for both the patient and the state would be tremendous, as no extensive care would be necessary.

In a long-term situation, it must be made mandatory that all patients are covered for early detection screening. This would ultimately lead to the cure for cancer. If every person had the ability to be screened for what is naturally in their body, along with the education necessary, they will be able to properly care for themselves and make wise decisions regarding their own health. Although these outcomes seem far off and quite costly as of now, the long-term savings would be huge, and cancer could slowly diminish.

c. Aging Society

The legislation that is current now makes it extremely difficult to decrease spending. In light of an aging society, the problem of treating cancer involves "rapid increase of US spending, greater use of screening services, and new treatments that come with a very high

price tag."[7] The present society is getting very used to new technology and relying on it completely. However, with this comes money, and that leads to an increase in spending.

"Cancer is getting much more expensive to treat. One well-known reason is that most "breakthroughs" come with a very high price. Several recently approved chemotherapy agents for cancer come with price tags that are 300-500% higher than the costs of traditional treatments."[6] These statistics show how the aging society is hurting the possibility of curing cancer. The higher the price, the fewer people would realistically be able to afford such treatments. Therefore, the amount of people suffering from extreme, life-threatening cases of cancer would increase, and the amount being cured would decrease.

This problem of an aging society directly portrays the need for education, advocacy, and proper legislation. Without these key factors, people will never be able to properly care for themselves because they will be uninformed, and they will also not be able to afford the new detection screening that will be made available. While it is true there will be a sudden increase in spending to provide all of this, the long-term money savings will be tremendous.

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