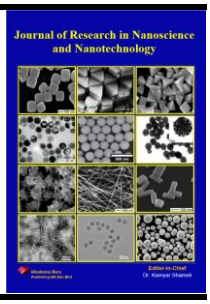
Penerbit Akademia Baru

Journal of Research in Nanoscience and Nanotechnology

Journal homepage:
http://akademiabaru.com/submit/index.php/jrnn/index
ISSN: 2773-6180



The Possibility of Employing Cysteine and Gold Nanoparticles for Skin Sensitization Analysis: Current Status

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https://doi.org/10.37934/jrnn.4.1.112

ABSTRACT

Determination of the toxicity potential of the ingredients is the first step in hazard assessment of cosmetics and personal care products. Skin sensitization analysis is one of the tests needed for the safety evaluation of these products. More countries in the world are turning their backs on animal testing for cosmetics and personal care products. This has led to the search for alternatives in skin sensitization analysis. Skin sensitization is defined as an allergic response to a skin sensitizer upon contact with the skin. Skin sensitization is induced through the covalent binding of a skin sensitizer to skin proteins (haptenation process). OECD Test Guideline No. 442C has adopted direct peptide reactivity assay (DPRA) and amino acid derivative reactivity assay (ADRA) as validated *in chemico* alternatives in the testing of skin sensitization potential of chemicals. The most cited nucleophilic amino acid that complexes with skin sensitizers (hapten) through covalent bonds is cysteine. Gold nanoparticles are mostly used to fabricate electrodes for the detection of analytes due to their chemical stability, high chemical conductivity, and biocompatibility. Cysteine has the capability to interact with gold due the formation of a strong cysteine-gold nanoparticles thiolate bond. The status of the possibility of employing cysteine and gold nanoparticles for skin sensitization analysis is discussed in this review article.

Keywords:
Skin sensitization, nucleophilic, gold nanoparticles, impedance, haptenation.

Received: 12 August 2021 **Revised: 16 September 2021** **Accepted: 24 September 2021** **Published: 17 October 2021**

1. Introduction

Animal testing used to be the method of choice in the toxicity assessment of the ingredients for the cosmetic and personal care industry. However, researchers are aware of the ethical issue that may arise and acknowledge that the experiments should be made as humane as possible. Skin sensitization analysis is one of the toxicity tests required and used to be performed using animal subjects [1]. However, animal testing may not even be as accurate as desired as there is a possibility that animal testing might not correlate well to human reactions [2]. In a study, the data showed that animal studies failed to predict human outcomes in 50 to 99.7 % of cases [3]. This might be because different species seldom suffer from the same diseases found in humans.

In March 2013, the European Union banned the sale of cosmetic products that were tested on animals [4]. The Cosmetics Directive and its 7th amendment introduce an end to animal testing by imposing bans on testing finished cosmetic products and ingredients on animals (testing ban), and marketing finished cosmetic products which have been tested on animals or which contain ingredients that have been tested on animals (marketing ban). The 7th amendment of the European Council Directive 76/768/EEC prohibited the repeated–dose toxicity test using animals for skin sensitization studies [5].

Safety requirements for cosmetic products highly influences and restricts policies established by European Union countries to ensure safety of products. In 2018, the European Union has urged a diplomatic drive for a worldwide ban on testing cosmetics on animals before 2023. Thus, there is a possibility that in 2023, animal testing for toxicology tests in cosmetics will be put to a complete stop globally, and validated alternative non–animal testing methods approaches will fully replace animal testing. As such, researchers have actively developed alternative non–animal testing methods that will be widely accepted and validated for future use.

As mentioned above, one of the required toxicity tests for cosmetics and personal care products is the skin sensitization test. Direct peptide reactivity assay (DPRA) and amino acid derivative reactivity assay (ADRA) are validated *in chemico* alternatives in the testing of skin sensitization potential of chemicals [1]. This research we will review the possibility of employing cysteine and gold nanoparticles in a biosensor for skin sensitization analysis.

2. Skin Sensitization

Allergic contact dermatitis (ACD) occurs when an active local immune response is stimulated by cutaneous inflammation [6]. Skin sensitization represents an enhancement of immunological reactivity for a small allergenic molecule (a hapten). Kostner [7] reported that the small allergenic molecule penetrates the skin and binds to a carrier skin protein (peptide), by a covalent bond, to form a hapten–protein complex.

An illustrative diagram of the mechanism of skin sensitization is shown below in Figure 1. This schematic diagram represents the current theory of the processes involved during the sensitization process. The hapten is first absorbed into the epidermis, where it can bind to skin protein, thus forming an immunogen. These modified proteins may then be recognized and internalized by a Langerhans cell (LC). The LC then migrates from the epidermis into the dermis and finally to the draining lymph node, whilst maturing into a dendritic cell [8]. The LC processes the immunogen into peptide which are bound covalently with a hapten and display them on their surface. The peptide–hapten complexes are then recognized by the T–cell response of a naive $CD4^+$ T–cell residing in the paracortex of the lymph node. This recognition then stimulates the generation and proliferation of a population of memory T–cells. The elicitation phase happens when the skin responses to the hapten.

For those people who suffer from sensitive skin, the hapten can stimulate type IV hypersensitivity that responded immediately [9].

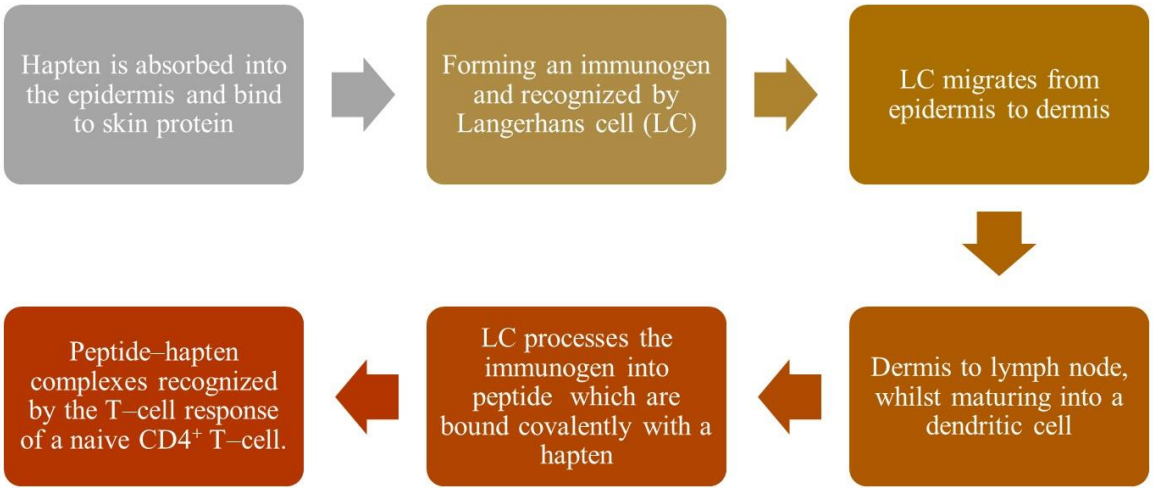

Figure 1. An illustrative diagram of the mechanisms of sensitization in delayed-type IV hypersensitivity.

Adverse outcome pathway (AOP) is a relatively new toxicological concept to regulatory sciences that describes the initial event of a toxicant with a biomolecule and continuing with intermediate key events (KE) that end with an adverse outcome. The AOP of skin sensitization is defined in four KEs (Figure 2). The molecular initiating event (MIE) is bound by covalent bonding to skin proteins (as a first key event), which leads to activation of keratinocytes (as a second key event), which is a key event at the cellular level [10]. Another key event at the cellular level is the activation of dendritic cells (as a third key event), which is caused by hapten–protein complexes as well as by signaling from activated keratinocytes. Dendritic cells subsequently mature and migrate out of the epidermis to the local lymph node where they display major histocompatibility complex molecules, which include part of the hapten–protein complex to naive T–lymphocytes (T–cells) (as a fourth key event) [8]. This induces differentiation and proliferation of skin sensitizer specific memory T–cells that leads to the key event resulting in the acquisition of skin sensitization [10]. The associated *in chemico* assays for skin sensitization that make use of the skin sensitization KEs are shown in Figure 2.

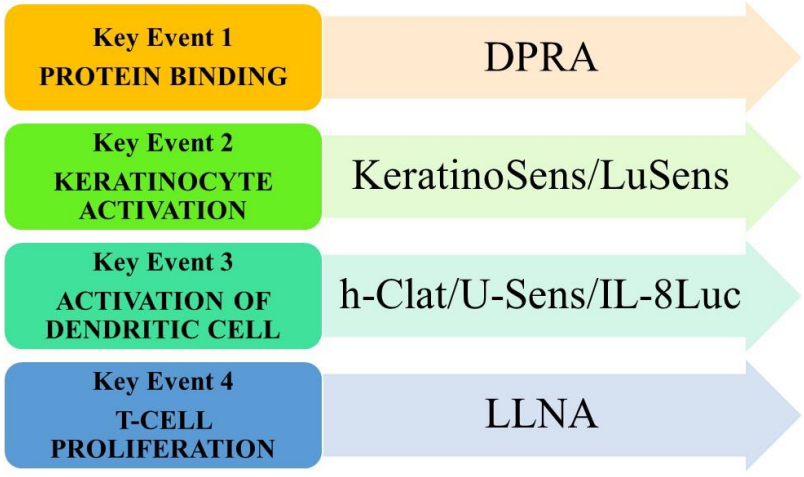

Figure 2. Adverse outcome pathway and associated assays for skin sensitization.

3. Approaches to Skin Sensitization Safety Assessment by Covalent Binding to Amino Acids

The MIE leading to skin sensitization is postulated in the AOP as the covalent binding of electrophilic chemical species (skin sensitizers) with selected nucleophilic molecular sites of action in skin proteins [11,12]. This characteristic is manipulated by some researchers of non–animal testing approaches for assessing skin sensitizer potential [13]. Some *in chemico* methods focus on manipulating the amino acids of the target skin protein as the nucleophilic molecular site of action and cysteine and lysine are often cited [14,13]. For example, the –SH group of cysteine is a strong nucleophile and has been employed frequently for assessing hapten (skin sensitizers) reactivity [12].

The Organisation for Economic Co-operation and Development (OECD) has adopted a few validated alternatives to animal testing Test Guidelines (TG) that address the first three key events of the skin sensitization AOP. The methods have been designed to detect skin sensitization hazards with quantitative measurement for potency prediction. TG No. 442C describes *in chemico* assays skin sensitization that addresses mechanisms under the first key event of the adverse outcome pathway [15]. TG No. 442C comprises test methods to be used for supporting the difference between skin sensitizers and non-sensitizers following the United Nations Globally Harmonized System of Classification and Labelling of Chemicals [15,13]. The test methods described are Direct Peptide Reactivity Assay (DPRA) and Amino acid Derivative Reactivity Assay (ADRA). The DPRA has been evaluated by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) followed by independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC) and has subsequently been validated as an alternative *in chemico* skin sensitization assay as part of an integrated testing strategy for the predictive identification of skin sensitization hazard [13,16]. The ADRA validation study was coordinated by the Japanese Centre for the Validation of Alternative Methods [4,15] followed by an independent peer-review [15].

Since protein reactivity represents only one key event of the skin sensitization adverse outcome pathway [15, 17], the data generated by DPRA and ADRA to address this specific key event may not be sufficient as a stand-alone method to conclude the skin sensitization potential. Data generated by DPRA and ADRA are proposed to support the prediction of skin sensitization potential of chemicals when used within the Integrated Approaches to Testing and Assessment (IATA), with complementary data from human, *in vitro* assays, *in silico* modeling, and chemical analogues [17,18]. '2 out of 3' prediction model from IATA achieved an accuracy of 90 % and 79 % when compared to stand-alone human and *in vitro* assays, respectively [19].

The DPRA and ADRA are based on similar principle which proposes to address protein reactivity by quantifying the reactivity of skin sensitizers towards model synthetic peptides containing either cysteine or lysine [15]. The DPRA was conducted by measuring cysteine or lysine percent peptide depletion values to categorize the chemicals in one of four classes of reactivity (high, moderate, low and no reactivity) for discrimination between skin sensitizers and non-sensitizers [15]. The analysis was based on the remaining concentration of cysteine or lysine containing phenylalanine following 24 hours incubation with skin sensitizers at 22.5 to 30 °C. Relative peptide concentration is measured using high-performance liquid chromatography (HPLC) with gradient elution and UV detection at 220 nm [4,27].

In contrast, ADRA quantifies the residual concentrations of the cysteine derivative N-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) or α-N-(2-(1-naphthyl)acetyl)-l-lysine (NAL) followed by incubation at 25±1ºC in the presence of a skin sensitizer. Both these derivatives include a naphthalene ring that is introduced to their N-terminal to facilitate UV detection [20]. The relative concentrations of NAC or NAL are measured using HPLC with gradient elution and UV detection at 281 nm. Percent depletion values are then calculated for both NAC or NAL and compared to a prediction model.

DPRA suffers from low detection sensitivity and as such a high concentration of skin sensitizers is required during analysis [4]. This limitation leads to the precipitation of skin sensitizers during the peptide depletion process. To address this issue, Fujifilm has developed the ADRA test method. The ADRA test method allows testing of poorly soluble skin sensitizers. As such, the ADRA test method could complement DPRA data test method [21].

While the DPRA assay identifies skin sensitizers with approximately 80% accuracy [4,19], the low selectivity and sensitivity of the HPLC/UV-based DPRA poses challenges to accurately identify the sensitization potential of certain chemicals. To enhance the reactivity of peptide, nuclear magnetic resonance spectroscopy (NMR)-based method was proposed by Chittiboyina [22] to improve the DPRA assay results. The reactivity and classification of the potential electrophiles was studied using thiol as a nucleophile by depending on the depletion of the electrophile signal. Moreover, NMR spectroscopy could serve as an ideal tool for accurately estimating the reactivity of electrophilic (skin sensitizer) candidates, the rate of reaction, and the quantification of NMR signal of interest. Andres [32], Zhang [24] and Wei [16] reported an evolution of the DPRA assay using Mass Spectrometry HPLC/MS-MS-based DPRA. Andres [23] was able to obtain a more specific detection of the peptide to complex mixtures by incubation of references chemicals and quality controls at low, medium, and high concentrations. This detection method provided a full spectrum of UV absorption on the peptide content of the reactional medium. Zhang [24] demonstrated that HPLC/MS-MS-based DPRA can address the issue of false-positive predictions of the DPRA method for hydrophobic substances, chemicals with UV peaks overlapping with those of the peptides, and compounds that non-covalently interact with the peptides. Wei [16] attempted to improve the DPRA assay throughput, accuracy, and sensitivity by using an automated 384-well plate-based RapidFire solid-phase extraction (SPE) system coupled with mass spectrometry (SPE-MS/MS-based DPRA). The automated SPE-MS/MS-based DPRA improved throughput from 16 min to 10 s per sample, and substrate peptides usage was reduced from 100 mM to 5 mM.

Meanwhile, *Cho, et al*., [25] developed a new spectrophotometric assay method (Spectro-DPRA) to determine the reactivity of chemicals toward two chemical groups (the thiol group of a cysteine containing peptide (cysteine peptide) detected using DTNB and the amino group of a lysine-containing peptide (lysine peptide) detected using fluorescamine). Using a depletion cut-off value for each peptide, a new prediction of sensitization potential model with a high degree of sensitivity (80.0%), specificity (86.7%), and accuracy (82.5%) by the log partition coefficient n-octanol/water (Log Pow) value was obtained.

Apart from *in chemico* assays for skin sensitization, researchers from the Institute National de la Recherche Agronomique (INRA) collaborated with Louis Vuitton Moet Hennessy (LVMH) to fabricate a skin sensitizer biosensor based on the principle of the electrophilic assay (cysteine, lysine, and histidine) and Surface Plasmon Resonance (SPR) [26]. The SPR biosensor was used to calculate the interaction between a ligand (nucleophilic amino acid) and an immobilized analyte (skin sensitizer/allergen) [27] using the direct binding of protein residues. The result was observed directly through the changes in the refractive index at the surface of the biosensor [28]. Cysteine was found to be more the most reactive towards the skin sensitizers. The order of reactivity from highest to lowest was cysteine, lysine and histidine. Weak allergens could quickly dissociate from the ligand, whereas strong and extreme allergens remained bound to the amino acids. However, the disadvantages of SPR include long response time, requiring a high volume of the sample, high regeneration time, and costly detection technique [29].

Due to the limitation of SPR biosensor technology, Noh and Abd Aziz [30] investigated electrochemical impedance spectroscopy (EIS) as a potential technique to study the interaction of skin sensitizers with nucleophilic amino acids based on peptide binding reactivity. Cysteine was

employed as the nucleophilic amino acid of choice in this work. EIS was carried out to measure the changes in charge transfer resistance of skin sensitizers ($\Delta R_{CT}^{sensitizer}$) as a result of different binding rates of affinity of skin sensitizers to cysteine. Investigation on the effect of potency of skin sensitizers on $\Delta R_{CT}^{sensitizer}$ readings suggested that $\Delta R_{CT}^{sensitizer}$ readings were directly proportional to the strength of the skin sensitizers with strong/extreme skin sensitizers displaying higher $\Delta R_{CT}^{sensitizer}$ readings compared to moderate and weak/non skin sensitizers [24,31].

4. Employing Gold Nanoparticles for Affinity Impedance based Biosensor for Skin Sensitization

Biosensors are usually portable and low–cost tools for rapid detection of substances. Generally, a biosensor is described as a three-element system consisting of a bioreceptor, a transducer, and a signal processing unit. When the analyte interacts with the bioreceptor, a quantifiable signal is generated. The interaction between the analyte and the capture agent is translated into a readable signal by a transducer by methods such as acoustic, optical, electrochemical, electronic, and photoelectrochemical mechanisms [32]. An affinity biosensor is a device in which the biorecognition molecules selectively bind to the analyte molecules leading to the formation of a complex, causing a physicochemical change that is detected by a transducer [33,34]. The receptor molecules of the affinity sensors can include compounds such as low-molecular weight biospecific ligands, proteins, antibodies, nucleic acids, and hormone receptors. The affinity-based biosensor works by detecting a particular biomolecule by binding the desired target (the affinity step) and then detecting a change in the surface properties (the readout step) (Figure 3).

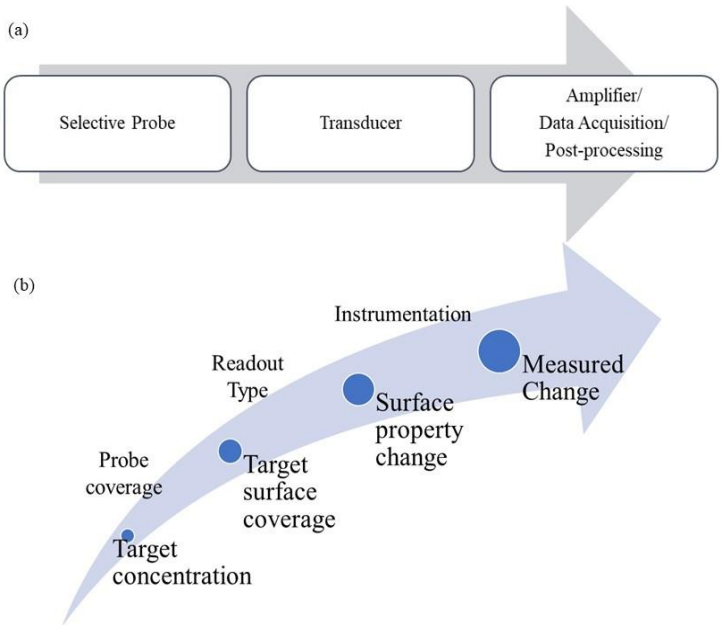

Figure 3. Generalized affinity biosensor showing (a) the physical arrangement, and (b) the steps involved.

Gold nanoparticles have been widely used in the fabrication of a biosensor due to their chemical stability, high chemical conductivity, and biocompatibility [35]. Gold nanoparticles play an important role in improving the specificity and sensitivity of electrochemical biosensors, such as modifying the sensing surface to enhance conductivity, increasing the immobilization of biomolecules and catalyzing the electrochemical reactions. In addition, gold nanoparticles are also used as

electrochemical indicators [36,37]. Shao et al. [38] reported a simple, disposable, and highly sensitive electrochemical sensor based on a screen-printed carbon electrode (SPCE) modified using gold nanoparticles/polyaniline-multi-walled carbon nanotubes composite for simultaneous detection of zinc (Zn(II)), lead (Pb(II)), and copper (Cu(II)). The proposed sensor exhibited excellent repeatability, reproducibility, stability, and selectivity. Electrochemical biosensor for the detection of As(III) based on the immobilization of *Alcaligenis faecalis* bacteria on gold nanoparticle (AuNPs–SPCE) has also been reported [39]. Other than that, Buffon and Stradiotto [40] reported the development of a molecularly imprinted polymer used for the electrochemical detection of ferulic acid. The device was prepared through electropolymerization of molecularly imprinted poly(phenol) film on screen-printed electrode modified with reduced graphene oxide and gold nanoparticles. The sensor displayed good repeatability and stability over time, as well as excellent selectivity when applied for the detection of ferulic acid molecule. Thus, due to its many advantages, gold nanoparticles have been widely used by researchers in the fabrication of electrochemical biosensors.

As such, researchers have also employed gold nanoparticles in the fabrication of affinity-based biosensors. For example, gold nanoparticles surfaces modified with oligopeptide exhibit several special characteristics, such as cell–repulsive surfaces, micropatterns of cell adhesion and non–adhesion regions for control over cell microenvironments [41]. Gold nanoparticles having thiol end can be conjugated with amino acids to give out positive amino groups. In some research works, a cysteine based self-assembled monolayer was used on top of gold nanoparticles surfaces as cysteine has the capability to interact with gold due the formation of a strong cysteine-gold nanoparticles thiolate bond [42-44]. Thiol bonds make a very stable bond in a variety of temperatures, solvents, and potentials. Affinity biosensor based on the gold nanoparticles and cysteine for the detection of skin sensitizers with associated signal processing by impedance technique has been reported by Noh and Abd Aziz [30].

The benefits of EIS include high sensitivity, low cost, label–free strategy, and simplicity [43-47]. EIS measures current–voltage ratio events without disturbing the properties of the analyte [48]. Electrochemical impedance measurements monitor changes in charge transfer resistance (ΔR_{CT}) when the affinity binding occurs between the nucleophilic amino acid and skin sensitizers on the surface of the electrode. The mass transports of the product and reactant are important to analyze the rate of electron transfer at the interface. The R_{CT} is a suitable signal to detect the interfacial properties of the interaction of the gold nanoparticles–cysteine ligand with the skin sensitizers [49, 50]. The increase or decrease in the value of R_{CT} is associated with the blocking behavior of the assembled layer on the electrode surface. The understanding of the connection between target binding and R_{CT} would enable improved affinity-based impedance biosensor design and sensitivity [46,47].

5. Analysis of Haptenation of Cysteine with Skin Sensitizers using Electrochemical Impedance Spectroscopy (EIS) technique

A hapten is a small molecule (low molecular weight) that must be bound to a carrier molecule to be recognized by the immune system [29]. Any skin sensitizer that can make a stable hapten–peptide conjugate with endogeneous peptides in the skin can induce skin sensitization [2,30]. Approximately 40% of skin sensitizers have at least an electrophilic center that is amenable to nucleophilic attack. Haptenation of a protein can occur by multiple mechanisms (primarily electrophilic attack) and is dependent on many factors such as chemical properties, bioavailability, and site of exposure [24].

Synthetic peptides of either cysteine, lysine or histidine have been studied as a model peptide for skin sensitization study. Cysteine was found to be more the most reactive towards the skin sensitizers [26].

In Noh and Abd Aziz's work of a potential skin sensitizer affinity biosensor based on impedance technique [30], ΔR_{CT} stipulated the degree of electrostatic interaction between $Fe(CN)_6^{3-/4-}$ and the skin sensitizers on a screen printed carbon electrode modified with gold nanoparticles and cysteine. The redox probe of $Fe(CN)_6^{3-/4-}$ was used to record the modified electrode's ability to transfer electrons. When skin sensitizers interact with gold nanoparticles–cysteine modified electrode surface, changes in the R_{CT} property of the surface can result solely from the presence of the skin sensitizer molecules. Investigation on the effect of the potency of skin sensitizers on R_{CT} readings suggested that the R_{CT} readings were directly proportional to the strength of the skin sensitizers with strong/extreme skin sensitizers displaying higher R_{CT} readings compared to moderate and weak/non skin sensitizers. Skin sensitizers employed in the study were the ones recognized in the globally harmonized system of classification and labeling of chemicals that can elicit allergic response upon skin contact [51].

High affinity binding of cysteine with extreme/strong skin sensitizers [52] was reflected in the increase in R_{CT} readings. In contrast, low affinity binding of cysteine with moderate skin sensitizers resulted in a decrease in R_{CT} readings. Low/non skin sensitizer resulted in the lowest R_{CT} readings. 96% of the prediction of skin sensitizers obtained from affinity biosensor based on electrochemical impedance method employed in the research work matched with the data obtained using local lymph nodes assay (LLNA), suggesting that the biosensor has the potential to be employed as a screening tool for the identification of skin sensitizers [53].

6. Conclusion

The possibility of employing cysteine and gold nanoparticles for skin sensitization analysis is discussed in this review. Skin sensitization is one of the important toxicity tests for the safety evaluation of the ingredients of cosmetic and personal care products. OECD TG No. 442C (DPRA or ADRA) has been adopted as an alternative non-animal testing method using *in chemico* approach based on the first key event of covalent protein binding with skin sensitizers. A biosensor manipulating the first key event of covalent protein binding will result in faster detection of skin sensitizers compared to *in chemico* approaches. This review suggests the potential of classifying skin sensitizers using gold nanoparticles and cysteine modified screen printed carbon electrodes analyzed using impedance technique.

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