A Comparative Study on the Feasibility of Graphene Based Avidin Conjugate for Potential Curcumin Detection

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Abstract. In the area of interdisciplinary research, modern and creative methods of interpreting substances are still of considerable importance. Curcuma longa’s active component commonly known as diferuloylmethane is the most sort after food preservative, medicinal spice, additive and coloring agent, which harbors a wide range of the biological spectrum. The major problem observed with this wonder drug is the low bioavailability of the drug in the gut, hence leading to further lower concentrations of the therapeutic reaching the tissues and plasma. Constituting only a minor part of its parent rhizome, curcumin is not the only compound to be offered by turmeric, but for certain ailments, the amount of curcumin required is more than others, and this higher concentration of curcumin cannot be met with a daily consumption of curcumin from the normal food diet. This very fact has led to the introduction of commercialization of supplements of curcumin as tablets and capsules. This artificial rise in curcumin levels by supplementation could have an opposite impact on the human body, as some reports have indicated. Therefore, a potential alternative, quick, economical way of sensing curcumin was proposed in the present research. The effect of protein decorated electrode in the same was studied. Before experimental implantation, the experimental feasibility of the same through docking was checked. In silico study on the possible interaction of the protein with curcumin, a protein with GO and rGO respectively using discovery studio 4 software was conducted. Experimental feasibility deals with the sensing of curcumin using electrochemical work station using avidin conjugated GO (Av-GO) and rGO (Av-rGO). The Av-GO modified electrode showed a lowest detection of 3.9nM while the Av-rGO modified electrode showed a lower detection limit of 4.9nM. These values have been the lowest using avidin that has been reported for curcumin. The concentration detected correlates to the quantity that is present in the gut physiology, hence substantiating the potential use in real time sensing of curcumin.

Keywords: Avidin, curcumin, Graphene oxide, reduced Graphene oxide, cyclic voltammetry

1. Introduction
Sensing has found its own place in the field of research. With many interdisciplinary studies coming up, this study was proposed with an intention to find a better sensing method for curcumin. This approach was found to be novel for the target drug. Curcuma longa, commonly known as turmeric or haldi or diferuloylmethane is the most sort after food preservative, medicinal spice, additive and coloring agent in Asia. This remarkable spice has various medicinal properties of traditional origin. This is a hydrophobicpolyphenol derived for Curcuma longa, a rhizome from the Zingiberene family. The main bioactive moiety of this wonder drug is a chromophore that harbors wide range of biological
spectrum [1]. Its many therapeutic properties have created such a sensation that it has bagged fancy titles like ‘curry against Alzheimer’s’, ‘from kitchen to clinic’, multi- anti spice’ and ‘curecumin’. It is known to be a remedy for most of the known ailments of the world. It works as an antioxidant, anti-inflammatory, anti-proliferating, antibiotic, anti-viral, and antifungal and many more [2].

Non-bioavailability and absorption in the intestines are essential problems for all dietary clients. Therefore, the consumer demand for curcumin supplements is strong. Artificial increase in curcumin concentrations with supplementation may, as a result of few studies, have an opposite effect on the human body. With the increased curcumin concentration, the efficiency, but also toxicity, will be increased. Curcumin was activated at 50-200 μM and human osteoblast cell lines were affected by apoptotic modifications below 25 μM and death of necrotic cells [3]. One of the major absorption factors pieridine used in curcumin supplement should be consumed cautiously as it is an inhibitor for a few drugs. So, people consuming additional medications along with these supplements know the consequences of the same [4]. Concentrations up to 5 μg/ml have shown both mitochondrial and DNA chromosomal damage [5]. Similarly, further studies have shown lower concentration [6] of curcumin harboring anti-oxidant properties but higher dosages give it a reactive oxygen species (ROS) property, which can increase cell malignancy [7]. Curcumin also causes anemia in people with sub-optimal iron intake [8]. Glutathione-s - transfers enzymes, P450, UDP-glucuronosyltransferase, etc., are hindered by curcumin that will increase toxicity by un-metabolized plasma drugs [9]. Any of the harmful effects of high dosages of curcumin include gastric discomforts, diarrhea, skin rash, nausea, constipation, anti-platelet properties, gall bladder contractions, etc. This may be attributed to an elevated dehydrogenase lactate and alkaline phosphatase serum. Patients with gallstones or thinners in their blood should also be vigilant about their consumption of curcumin [10].

Discovery Studio is comprehensive suite software. It is a suite for small and macromolecule interaction studies. It is a product from Accelrys. This is a United States-based software company having representations in Asia and Europe [11]. This company aims at providing software for materials science, bioscience, chemical science, pharmaceutical, aerospace, biotechnology and energy. It was solely owned by Dassault Systems and later renamed as BIOVIA. Discovery studio is used for modelling and analyzing molecular sequences, structures, etc. It is of great relevance to researchers in the field of life science [12]. It has features like data analysis, viewing, editing, etc. When compared with HOT analysis, this software gives 100 times more possible interaction data on a molecule and its legend. It also provides many graphical representation modes and rich viewer sets. Windows and Linux are the standard operating systems that support this software. Tools for viewing and editing could be accessed from the flexible panels, menus and bars. These panels are grouped into large sets based on the working area, such as receptor-ligand, pharmacophore interactions, etc., [13]. While hovering over these tools, an additional note pops up next to the tool to provide basic information about that function.

Generally, cyclic voltammetry (CV) has lower sensitivity. This mode has high sensitivity in high concentrations. This would be in terms of current per mole. It has a capacitance layer that is large enough not to give true current for an analyte at lower concentrations. This drawback is covered in other modes like differential pulse voltammetry (DPV) or square-wave voltammetry (SWV), etc. The major working principle behind DPV or SWV is that the non-faradic current is found to be negligible because of the current difference between the two points in the pulse and the short sampling time [14]. These modes subtract the double layer capacitance produced between the matrix of the analyte and the supporting electrolyte, but CV does not have this ability to correct this excessive layer of capacitance. In certain exception cases it has been noticed that CV has higher sensitivity in comparison to its better counterparts. This might be due to the analyte compound changes the capacitive layer usually presents at the electrode. There are various factors that govern the sensitivity of mode towards the analyte. A few are nature of the target analyte, size of the target analyte, Polarity of the target analyte, Analysis time and Viscosity of the supporting electrolyte.

With lighter analytes, DPV and LSV fit well with a less viscous aqueous medium. The explanation is that the sampling time and the double layer is very fast in these systems, but if the analyte has a
polar property, it has unusually high powers of contact with the supporting electrolyte that may be viscous in nature. The capacitance layer slowly and in large scale in DPV or SWV mode is established in these scenarios. In certain cases, CV performs best.

It would apply potentials continuously for a long time, which would deplete the capacitance double layer. When we analyse our target analyte curcumin, we find that compared to the sensing materials, we are optimizing, curcumin is much larger in size. It ranges to a few microns in size. It is a hydrophobic solid material, who’s enolic and phenolic groups are polar in nature, hence making the compound itself polar. The time we are opting for an assay is also short, as we would like to have a rapid sampling technique for curcumin. So, we could infer that CV mode might work for curcumin sensing as an exclusive mode for detection [15], and as we proceeded with our experiments, we did find our results favoring a highly sensitive detection of curcumin.

The sensing components used for detection of curcumin are CRTO, glassy carbon electrode modified by carbon nanotubes, carbon-paste electrode modified by dysprosium nanowire, Carbon-Screen Printed Electrodes, poly-Acid chrome blue K (poly-ACBK) film, Graphene, Electrochemically Reduced Graphene oxide, etc. The binding efficiency of curcumin with nanoparticles, polymers, carbons, etc., is the reasons behind the selection of these sensing materials to begin with. After intense study, we have chosen a set of materials for sensing curcumin. Use of proteins in biosensor is in trend nowadays. Our target molecule gets biotinylated easily for various drug delivery applications, and biotin avidin reaction is well known in the field of diagnostics. But biotin is an expensive vitamin with very strict storage conditions and short shelf life. Use of biotin could increase the cost of fabricating our sensing system. Hence, we tried to propose a set of experiments to study if avidin could directly bind to curcumin and act as a detector molecule for the same, but we could not find any literature evidence for the above-mentioned aim, as to why we should not use avidin alone apart from as being complex with biotin for sensing. So, a set of Insilco studies were carried out for the first time to study this interaction.

Sensing has found its own place in the field of research. With many interdisciplinary studies coming up, this research study was proposed with the intention of sensing curcumin using electrochemical workstation using avidin conjugated GO (Av-GO) and rGO (Av- rGO), but before we could proceed to the experimental implementation, we had to check for the experimental feasibility of the same through docking in silico study on possible interaction of protein with curcumin, protein with GO and rGO respectively using discovery studio4 software were conducted. The aim of our present study is to investigate the experimental binding feasibility of avidin to GO and rGO for potential sensing application. In the field of life science, it is well known that Biotin-avidin has the strongest non-specific binding. Many sensing assays are devised in immunology using this interaction as a major binding scenario. We also have literature evidence of biotin binding with curcumin for detection of human breast cancer cell lines. Hence, we thought of replacing the biotin candidate with avidin for sensing curcumin. Avidin seems to be a cheaper alternative to biotin. There is no evidence as to why it has not been used so far.

Effect of GO on proteins has been a major part of research in the field of life sciences. GO has been used to immobilize proteins and enzymes for different applications like imaging, drug delivery, biosensors, etc. The stabilization of the protein increases the overall protein conjugates’ shelf life. So, knowing fundamentals about the same is also important for protein-based application. GO or rGO sheets have oxygen harboring surface groups. This gives this sheet large surface for binding and functionalization. The chemicals commonly used for functionalization of protein are bovine serum albumin (cationized BSA), N-hydroxysuccinimide (NHS), polyethylene glycol (PEG), glycans, etc. This functionalization will help in stabilization of protein and activity. The body’s systemic circulation has various proteins of diverse functionalities, structures and compositions. When GO comes in contact with these proteins it undergoes immediate interaction under specific conditions. The native properties of GO like the surface charge, energy, affinity to water etc., dominates the nature of the interaction to take place between the proteins. The association between proteins and GO is generally confined to the surface. When GO is used for devices they are anchored on to a substrate
and the combined effect of the substrate and GO will be studied mostly in air, but for biosensor applications GO needs to operate in liquid medium. Again, the surface properties of GO would control the interaction between the protein and this carbon matrix.

But binding of a protein to GO or rGO sheets would require functionalization of the sheets first. Majorly, Dimethyl formamide (DMF) is used to couple proteins to sheets or using basic physisorption like sonication. But sonication provides weak Van der Waals attraction between the protein and GO sheets which might detach easily. But, recently carbodiimide chemistry involving 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) are used to couple proteins to nanomaterials. EDC is a crosslinking agent of zero length. It is used where carboxyl or phosphate ligands of the target or primary amines are involved in the coupling reaction. This chemical has been used extensively for formation of amide bonds, attaching nucleic acids, haptenes, etc. EDC’s water solubility is the major advantage of this compound. This property gives easy and accessible bio-conjugation and easier purification via dialysis, filtration, etc., but the reaction time should be fast as the active ester is very unstable and hydrolyses rapidly. To overcome this shortcoming NHS is used to stabilize the unstable amine-reactive ester intermediate to stable amine reactive NHS ester. The pH and the amount of EDC should be kept under check as excess of EDC would lead to agglomeration of nanoparticles. This is caused due to lack of repulsive forces between the particles. Concentration is generally kept as low as 0.1mg/ml of GO, 0.1mM of EDC and 0.05mM of NHS. All times of biomolecules could be attached to any nanoparticle using this coupling agent. The -COOH moiety of the GO/rGO sheets would couple with the amide groups of avidin. Hence, we were very much satisfied that avidin would anchor well on GO/rGO sheets to study experimental sensing of curcumin.

This chapter deals with sensing of curcumin using electrochemical workstation using avidin conjugated GO (Av-GO) and rGO (Av-rGO). We also preferred this conjugate to see if it is possible to perform the sensing assay in conditions similar to the body’s physiology. From our studies, we have found that no experiments so far have been done with curcumin in electrolytes of neutral pH using Av-GO or Av-rGO. PBS with no additional electrolytes was used as supporting electrolyte. Cyclic voltammetry has been used as the detection mode. Biotin avidin has always been used in sensing systems because of their high affinity to each other. Biotinylating of curcumin is also read in many drug delivery-related research works, but biotin is a very temperature-sensitive vitamin and is very expensive. Since we aim at having a cost-effective technique for highly sensitive detection of curcumin, we wanted to see if avidin and curcumin could form a sensing system without biotin. Silco studies prove that this reaction is possible, so we came up with Av-GO and Av-rGO conjugate because we found the literature being silent on the exclusive utilization of this combination for quantification. Hence, the present article deals with comparative evaluation of sensing abilities of Av-GO and Av-rGO for curcumin in PBS using cyclic voltammetry.

2. Material and Methods
The synthesis of graphene oxide and reduced graphene oxide were done as per our previously reported protocol published in 2018 [44]. The additional materials used in the present study are Avidin (Sigma Aldrich), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS).

The synthesis of avidin conjugated GO (Av-GO) and avidin conjugated rGO (AV-rGO) was done using three different protocols. The protocol which yielded the best results were optimized and taken for further study. These three protocols were done for GO and rGO separately. The steps used in each protocol are as follows: Protocol 1-8mg of GO or rGO was dispersed in 40ml of water. It was solicited for 30 minutes. EDC of 1.5mg was mixed in 20 ml water. This solution was added drop wise for 14 min under 60 rpm stirring to the GO/rGO solution. NHS of 0.46mg of solution in 10ml water was prepared. Later added drop wise for 7 min to the GO/rGO solution then stirred for 30 min. The pH was checked to be 7. The resulting solution was divided into four parts and each part was added with 1000µl, 750µl, 500µl and 250µl of avidin, stirred for 1hr at room temperature. Then it was centrifuged twice at 10,000rpm for 10 min. The supernatant was replaced each time and dried at 40°C. Protocol 2-8mg of GO or rGO was dispersed in 40 ml of water. It was solicited for 30 minutes. 40 mg of EDC
and 60 mg of NHS were added directly to the GO/rGO solution. The resulting solution was divided into four parts and each part was added with 1000µl, 750µl, 500µl and 250µl of avidin and stirred for 1 hr at room temperature. Then, it was centrifuged twice at 10,000 rpm for 10 min. The supernatant was replaced each time and dried at 40°C. Protocol 3-8mg of GO or rGO was dispersed in 40ml of water. It was sonicated for 30 minutes. The resulting solution was divided into four parts and each part was added with 1000µl, 750µl, 500µl and 250µl of avidin, stirred for 1 hr at room temperature. Then, it was centrifuged twice at 10,000 rpm for 10 min. The supernatant was replaced each time and dried at 40°C.

The protein data was taken from Protein Database (PDB). The protein used in our study was avidin. It can be found PDB with file number 3Fdc. Avidin consists of two chains, named A and B with a chain break threshold of 7. GO and rGO data, which was neither found in PubChem or ChemSpider, hence the structures of GO and rGO were drawn using Chemdraw and loaded into the software. The software generated a 3D structure for the same, which was used to check its interaction with avidin. Figure 1 represents the Chemdraw structure of rGO. The curcumin formation was obtained for PubChem. The file number for curcumin is CID: 969516. The 2D and 3D conformation of curcumin has been depicted.

2.1. *Insilico studies*

The Insilico studies were performed for (1) curcumin with avidin. The score around 50 and above shows experimental feasibility. The colors of the amino acid represented in the two chains of avidin are depicted in detail in Figure 2. Figure 3 shows the 3D image of curcumin binding with avidin. The red color long chemical structure on the left corner of the image represents curcumin.

![Figure 1: 3D image of Curcumin + Avidin binding](image1.png)

![Figure 2: 3D image of Biotin + Avidin binding](image2.png)

Strong sigma bonds are formed between the A chains histidine 50 and the benzene ring of curcumin. Similarly, electrostatic bonds are formed between A chains threonine 67 and B chains threonine 52 with the oxygen attached to the benzene ring of curcumin. Weak Van der Waals attraction was seen between curcumin and A chains leucine 49, proline 48, asparagine 69 and B chains glycine 65.
histidine 50, threonine 30, phenylalanine 29, valine 23 and glutamine 28. The binding score was found to be 60, which proves possible binding between curcumin and avidin is feasible. Figure 2 shows the 3D image of biotin binding with avidin. The red color long chemical structure in the left corner of the image represents biotin.

Figure 2 depicts the interaction between an already benchmarked nonspecific interaction, i.e., biotin and avidin. Electrostatic bonds are formed between B chains threonine 52 and A chains threonine 30 and threonine 67 of avidin with biotin. The distance of the bonds was around 4.4Å, 4.4Å and 3.5Å for threonine 52, threonine 30 and threonine 67, respectively. Water bonds were also speculated during simulation. Weak van der Waals attraction was seen between biotin and B chains phenylalanine 29. The binding score was found to be 85, which proves binding between biotin and avidin is feasible. This data is very much in sync with previously reported data on nonspecific binding between biotin avidin. This binding is extensively used nowadays to form immunological assays for sensing specific targets. When we compare the binding scores of biotin avidin with curcumin and avidin there is a difference of 25. So, we would like to infer although the binding will not be as specific as biotin and avidin but interaction is feasible, and sulfate form shows score more than biotin avidin, which makes us curious if it would be experimentally as proficient as it claims to be theoretically. Figure 3 shows the 3D image of GO and rGO binding with avidin (left to right) respectively. The red color flat chemical structure in the corner of the image represents GO and rGO from left to right respectively.

As the carbon skeleton of both the materials is the same with a variation in the number of oxygen atoms, which was similar for both the materials. The only difference is that GO has more number of oxygen groups in comparison to rGO. Electrostatic bonds are formed between B chains threonine 55 and A chains lysine 71 and GO or rGO oxygen groups. The distance of the bonds was around 3.4Å and 5.4Å threonine 55 and a chains lysine 71 respectively. Pi bonds are formed between B chains arginine 26 and the carbon skeleton of GO or rGO. Weak van der Waals interaction is seen between A chains histidine 50, proline 48 and GO or rGO. The binding score was found to be 106.66 for GO and 43.88 for rGO. The reduction in binding score in rGO might be due to lack of oxygen groups in former than in GO. More oxygen groups provide more binding sites for the protein to bind and have proper anchorage. Hence, we infer that avidin can possibly bind to GO and rGO providing experimental feasibility, but based on our data, the binding would be more efficient in GO than in rGO. The binding score between avidin and GO is higher than the benchmark molecule also, hence showing very high experimental feasibility. Figure 4 shows the 3D image of GO and rGO binding with avidin and curcumin (left to right) respectively. The red color flat chemical structure in the corner of the image represents GO and rGO from left to right respectively. The green long chemical structure is curcumin. The binding score was found to be 60.43. This score is similar to the score for curcumin and avidin. Therefore, we could speculate that avidin curcumin binding has more theoretical binding significance.
The 2D interactions obtained for this complex was the same as obtained for GO or rGO with avidin, hence we would like to speculate each material would bind independently to avidin in their own fashion. Even the amino acids involved are different for GO and rGO and curcumin. So, a sandwich layer could be possibly formed with these three compounds if two are left to react at a time and then exposed to the third.

![Graphene oxide + avidin + Curcumin and rGraphene oxide + avidin + Curcumin binding respectively(left to right)](image)

**Figure 4:** Graphene oxide + avidin + Curcumin and rGraphene oxide + avidin + Curcumin binding respectively(left to right)

Hence, we were able to conclude that there is experimental feasibility among curcumin, avidin and GO. Discovery studio 4 has provided us with a general visualization of the possible interaction between our major reacting candidates. Binding score between avidin and curcumin is above 50. So, we infer experimental feasibility. The binding score between avidin and GO is higher than the benchmark molecule, hence showing very high experimental feasibility. Due to lesser oxygen species in rGO, it shows lesser binding compatibility with the protein. With GO or rGO as an anchor material, avidin can be used to bind to curcumin theoretically. Hence, we can try having a sensing system using avidin as a detector molecule for curcumin. Therefore, a sensing system is possible with the same theoretically. We have checked its experimental feasibility by running wet lab reactions.

2.2. Characterization

Avidin was commercially purchased and physically it appeared to be white in hue with a molecular weight of 66,000 Da. It is a glycoprotein of tetrameric structure with four subunits. Each subunit has 128 amino acids of identical origin with three carbohydrate groups. It can be strongly dissociated under certain conditions with a characteristic UV-Vis wavelength of 338nm. This protein is very much economical and stable than biotin and can be stored up to 2-8°C for about six years. This wonder protein is stable in salt solutions, ionic pH conditions, temperature and water up to 20mg/ml. When it comes to temperature, it is stable up to 85°C without biotin. Since our experimental conditions are around room temperature, hence we would like to study the use of avidin alone as detector molecule with GO/rGO for curcumin. Monomeric form of avidin can exist to act as label only when it has been immobilized onto some matrix, especially agarose. Since agarose is not a preferred conducting material for electrochemical reactions, therefore, GO/rGO were used as anchoring matrix for the same.
Three different protocols (P1, P2, P3) were used to attach avid into GO or rGO sheets. This was done to see which protocol would yield the most amino defunctionalized sheets and hence the best possible way to synthesize protein adhered GO/rGO sheets.

Figure 5: FTIR image of Av-GO and Av-rGO using three protocols respectively

Figure 5 shows the FTIR data on the functionalization in GO sheets noticed on the three protocols. For GO sheets, P1 and P2 exhibit similar functionalizations. Stake-shaped band was seen in the middle of the spectrum around 1710 cm⁻¹ representing C=O stretch. As with primary amides show two spikes while secondary amides show one spike only. Since we notice only single stake for P3, we suggest the protein has secondary structures, which coincides with literature data. We see a small additional shouldering for P2 and P1 that shows the presence of primary amides. This is a predominant characteristic or avidin, as reported previously by other researchers. They have said this is due to the extended chain and 3-sheet structure.

Figure 6: A-C SEM images of Avidin, avidin-GO (Av-GO) and Avidin-rGO (Av-rGO) respectively
Figure 6 A exhibits an image of commercial avidin. This glycoprotein seems to have a large size of few microns. When decorated on GO or rGO, the SEM images seemed respectively. The protein seems to be evenly decorated on the sheet matrix of GO or rGO. Proteins agglomerate usually on hydrophobic surfaces. It has been previously reported that GO and rGO sheets manifest permeable behavior of wettability in air and aqueous medium. It has been stated earlier by researchers that in aqueous or high humid medium, water gets confined between the layers of GO and rGO and the underlying substrate onto which it is coated. In GO sheets, site-specific reaction takes place between the stable water layers and the sheets. This forms the basis of the protein binding to GO/rGO sheets. Hydrophilicity of the GO/rGO sheets is the major factor that affects the binding of avidin on to it.

Figure 7: A&B AFM images of Avidin-GO (Av-GO) and Avidin-rGO (Av-rGO) respectively.

Figure 7A & Figure 7B shows the AFM images of avidin coated on the surface of GO and rGO respectively. It is seen that a single protein molecule of 120 nm height places itself in the GO sheet, while in agglomeration of protein up to 800nm is noticed on the surface of rGO sheets. The agglomeration degree on GO/rGO sheets will always depend on mobility of the molecules on those sheets. It is a known fact that avidin or any protein molecule diffuses slowly on hydrophobic surfaces. This is due to strong binding between the hydrophobic surface and protein. On the contrary, hydrophilic surfaces have good molecule mobility in terms of protein adhesion on the surface. And GO is more hydrophilic than rGO. Hence, we could suggest that on GO sheets, large clusters of low density avidin are deposited while the more hydrophobic rGO has more avidin clusters of high density. This very much coincides with the results that we have received. Avidin initially forms a layer on the GO/rGO sheets by absorption. Later it denatures. Once the denature layer covers the surface, the further mobility of the protein is restricted by strong protein-protein interaction, and hence leading to the formation of big clusters of avidin. So, the strong interactions formed at hydrophobic surface forms more denatured initial layers of protein and later large single cluster of avidin due to restricted molecule mobility.

2.3. Appraising the surface of Av-GO modified electrode and Av-rGO modified electrode

The proposed sensing mechanism in the present study is depicted in Figure 8. Av-GO or Av-rGO material would be sonicated with ethanol and nafion and cast down to glassy carbon electrode (GCE). Then using cyclic voltammetry mode, it will be used to sense curcumin in PBS solution. The Voltagram results obtained were studied and analyzed to calibrate the detection limit and so the sensitivity and later the selectivity. 15 ml PBS was placed in 20ml cell of CH Analyzer. Three electrode systems were placed in the cell (glassy carbon working electrode, Ag/AgCl reference
electrode and Platinum counter electrode). The voltage sweep was kept from range -0.9 to +0.9 mV with a scan rate of 50 mV/sec.

**Figure 8:** Proposed mechanism of sensing in chapter 6

When modified with Av-GO and Av-rGO, the redox and the background current gets increased. This very much exhibits the enhancement of the surface properties and electroactive nature of the modified electrodes. For Av-GO modified electrode oxidation peak was observed at +0.59 V and reduction peak was observed at -0.52 V. These readings were taken in PBS for a very minute concentration of our target, i.e., 10^{-10} M/L. This has been depicted in Figure 9A. The material was found to be unstable at successive runs. The signal produced started to break after three runs. This might be due to the denaturation of the protein at the electrode, which hinders the further flow of current.

**Figure 9:** (A) Cyclic voltogram of Av-GO modified electrode with and without curcumin. (B) Cyclic voltogram Av-GO modified electrode at different concentrations.
For Av-rGO modified electrode, redox peaks were seen at +0.23V and at -0.6V for the 10-12 M/L concentration of curcumin. When compared between the above two sensing materials, Av-GO produces predominant oxidation peak than its other counterpart. Although rGO is eight times more conductive than GO, the cluster of protein deposited is more in rGO than in GO. This is due to the sheet’s nature, as discussed in the AFM studies in the previous section. The signal quality was found to be better in Av-GO than in Av-rGO. This might be due to the fact that Av-rGO has high density clusters of avidin spread across the rGO sheets that might get denatures due to application of continuous voltage and so hinders the flow of electrons, but in Av-GO low density, large but few clusters of avidin are deposited, so there is still some carbon skeleton left to transfer electrons and produce predominant peaks at least for few runs before it denatures. Henceforth, we would like to speculate that with Av-GO and Av-rGO, modified electrodes are sensitive to curcumin for a few runs for a single casting at the electrode, but the lowest concentration that we could detect using Av-GO was lesser than that of Av-rGO.

Figure 10: Cyclic voltagram of Av-rGO modified electrode at different concentrations.

2.4. Effect of concentration and scan rate
The concentration-based studies were taken for Av-GO modified electrode. Figure 9B depicts this information. The concentration was varied for 10-10 to 10-7 M/L. The electrode was coated only once. As the concentration was increased gradually, the current was found to be decreasing. This might be due to the polymerization of curcumin and denaturation of avidin at the surface of the electrode. Such a low amount of curcumin with continuous usage of a single coated electrode for avidin without conjugation with biotin has never been reported before. The possible interaction taking place at the surface has already been discussed in the silico studies of chapter 5 section 5.3. Strong sigma bonds are formed between the a-chains histidine 50 and the benzene ring of curcumin. Similarly, electrostatic bonds are formed between a chains threonine67 and B chains threonine52 with the oxygen attached to the benzene ring of curcumin. Weak van der Waals attraction was seen between curcumin and a-chains leucine 49, proline48, asparagene69 and Bchains glycine65, histidine50, threonine30, phenylalanine29, valine23 and glutamine 28. Figures 11A and B illustrate dependence of scan rate and oxidation current and the linear variation with square root of the scan rate. For a constant concentration of 1X10-10 M/L of curcumin, two peaks of oxidation shifted in a random fashion of current, hence the mechanism of reaction at the electrode surface was unclear for us. The number of runs that obtained a decent peak without recasting was found to be around N =3.

Av-rGO decorated electrode was also checked for its response with different concentration of curcumin (Figure 10). This varied from 10-12 to 10-7 M/L. Decrease in current was noticed with increase in concentration of curcumin. The deposition of curcumin and denaturation of avidin might be the reason, but the variation in the current increase was much lesser than Av-GO modified
electrode. The signal produced was also not much more defined than Av-GO decorated electrode system.

![Figure 11](image1.png)

**Figure 11:** (A) Cyclic voltammetry response of Av-GO modified electrode at different scan. (D) Linear variation of scan rate with current for Av-GO modified electrode.

As curcumin lodges itself onto Av-RGO, the conductivity might get hindered as graphene is known to accumulate charge better and act as a supercapacitor. Electrostatic bonds are formed between B chains threonine55 and A chains lysine71 and GO or rGO oxygen groups. The distance of the bonds was around 3.4 Å and 5.4 Å threonine55 and A chains lysine 71, respectively. Pi bonds are formed between B chains arginine26 and the carbon skeleton of GO or rGO. Weak van der Waals interaction is seen between A chains histidine50, proline48 and GO or rGO. The binding score was found to be 106.66 for GO and 43.88 for rGO. The reduction in binding score in rGO might be due to lack of oxygen groups in the former than in GO. More oxygen groups provide more binding sites for the protein to bind and have proper anchorage. The oxidation current also varies almost linearly with linearly increasing scan rates. The concentration for which this reaction was done was 1X10-12 M/L. Figure 12 on the left represents the variation in current in terms of varying scan rates and Figure 12 rights represents linear variation of scan rate with current. The reaction was found to be diffusion based with regression coefficient of R2 = 0.99 and number of runs N=3.

![Figure 12](image2.png)

**Figure 12:** (Left to right) Cyclic voltammetry response of Av-rGO modified electrode at a different scan and linear variation of scan rate with the current. Sensitivity and detection limit.
Concentration variation of curcumin (10-12 to 10-7 M/L for Av-rGO and 10-10 to 10-7 M/L for Av-GO) with the current produced or specific concentration was plotted as a graph. The scan rate was optimized to 50mV/s. This graph was used to calculate the detection limit of each modified electrode. The statistical analysis of the above graph yielded two linear ranges for each modified electrode. For Av-GO modified electrode, the linear ranges ranged from 0.1nM to 10nM (R²=0.98) and 10nm to 100nM (R²=1). Figure 13A depicts the same. The linear equation for each range was as follows:

\[
\begin{align*}
I_{ab} (\mu M) &= -0.004 C_{\text{curcumin}} (\text{nM/L}) + 0.006 \quad (10^{-3} \text{nM to } 0.1 \text{nM}) \\
I_{bc} (\mu M) &= -2.9 \times 10^{-5} C_{\text{curcumin}} (\text{nM/L}) + 0.006 \quad (0.1 \text{nM to } 10 \text{nM})
\end{align*}
\]

Figure 13: A&B Cyclic voltammetry responses for different concentrations of curcumin (10-10 to 10-7 M/L) at the Av-GO modified electrode and (10-12 to 10-7 M/L) at the Av-rGO modified electrode

Similarly, for Av-rGO decorated electrode, the linear ranges ranged from 10-3nM tonM (R²=0.817) and 0.1nM to 10nM (R²=0.994). The linear equation for each range was as follows:

\[
\begin{align*}
I_{ab} (\mu M) &= -0.004 C_{\text{curcumin}} (\text{nM/L}) + 0.006 \quad (10^{-3} \text{nM to } 0.1 \text{nM}) \\
I_{bc} (\mu M) &= -2.9 \times 10^{-5} C_{\text{curcumin}} (\text{nM/L}) + 0.006 \quad (0.1 \text{nM to } 10 \text{nM})
\end{align*}
\]

The decrease was seen to be drastic for Av-GO electrode till 10nM and 10-3nM for Av-rGO modified electrode. Above this concentration, the decrease was very much gradual. We would like to infer that for a very low concentration, the species of curcumin taking part in the redox reaction are so few that after conducting the initial reaction, there are not much species left free in the electrolyte to continue the reaction, and it is also a known fact that curcumin polymerizes at the electrode and avidin denatures, hence when the concentration is increased for the same electrode without recoating, the electrode sites that are free to react with curcumin (since we already have some curcumin species attached to the electrode from the previous reaction) to generate and produce current, but lesser than the former run. This leads to further accumulation of the target at the electrode. So, this justifies the drastic fall till a particular concentration in both the electrodes. Above this particular concentration, the decrease is gradual, as the number of species that react with the electrode is much more in the electrolyte. The cap was adjusted according to three sigma laws. The lowest detection of 3.9nM was seen in the Av-GO adjusted electron detection, while the Av-rGO modified electron detection maximum was 4.9nM. The two values lie between both electrodes' initial linear range. These values were the lowest for curcumin-reported avidine.

2.5. Analytical application
Fetal bovine serum was used to check the analytical application of our sensing system. Curcumin of known concentrations of 1nM, 3nM and 5nM were added to serum and percentage of recovery was calibrated. The materials were very unstable and do not stick to the electrode, as the environment of the supporting electrolyte changes. Therefore, we could stipulate that the current system could not be used to assay processed plasma from the body.
2.6. Reusability studies and selectivity

10^{-7} \text{M/L} \text{ solution of curcumin was used to study the reusability of the electrodes. The study was done for seven cycles and current generated was noted. The materials started to show breaking of the signals, as the materials started to denature with continuous application of voltage. This throws lights on the inefficiency of our avidin-modified electrodes. Therefore, our modified electrodes are neither reproducible nor have good long-term storage properties. Interference studies need to be conducted to study the effect of possible interference species present in the plasma. This would provide the selectivity of our electrodes to curcumin. The avidin-modified electrodes again failed to stay adhered to the electrode after the addition of interference species. }

3. Conclusion

The Av-GO and Av-rGO were synthesized using standard protocols. These materials were characterized using preliminary studies like FTIR, SEM and AFM. From these studies, we concluded that we have successfully formed Av-GO and Av-rGO, but since our work basically focuses on sensing of curcumin using these materials, we limited our study to preliminary analysis of the sensing materials to just confirm that the synthesized materials. Material science aspect of it was not looked into in detail because of the same reason; hence we next checked the interaction between Av-GO and Av-rGO with curcumin, as the next part of our characterization studies. For Av-GO, modified electrode oxidation peak was observed at +0.59 V and reduction peak was observed at -0.52 V. Unfortunately, after three successive runs, the material started to break, exhibiting interrupted signal in CV, which might be due to the denaturation of the protein at the electrode. As the concentration was increased, the current gradually decreased. Such a low amount of curcumin with continuous usage of a single coated electrode for avidin without conjugation with biotin has never been reported before. The two peaks of oxidation for a given concentration shifted randomly in terms of current. So, the mechanism of reaction is unclear. The number of runs that obtained a decent peak without recasting was found to be around N = 3. Decrease in current was noted with an increase in concentration of curcumin for Av-rGO modified electrode. The deposition of curcumin and denaturation of avidin might be the reason, but the variation in the current increase was much lesser than Av-GO modified electrode. rGO exhibited low binding score for avidin as reported in this paper. The reduction in binding score in rGO might be due to lack of oxygen groups in former than in GO. More oxygen groups provide more binding sites for the protein to bind and have proper anchorage. The oxidation current was found to be varying linearly with increased scan rate. Henceforth, we could conclude the following about the evaluated materials, (1) Both Av-GO and Av-rGO modified electrodes show good prospects of non-enzymatic properties, (2) The detection limit reported by us is the lowest till date with avidin only without biotin, (3) Unfortunately, this modified electrode lacks stability, selectivity, reproducibility and long-term storage and (4) A system of this kind paves way for futuristic application of proteins for biosensor.

Declaration of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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