RESEARCH ARTICLE

Graphene-Based Nano Drug Delivery of Curcumin for the Treatment of Dengue Fever

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Received: 20th February, 2024; Revised: 27th March, 2024; Accepted: 15th August, 2024; Available Online: 25th September, 2024

ABSTRACT

Graphene, which is defined as a single-layered sp^2 hybridized carbon atoms placed in a honeycomb-like crystal lattice when converted to graphene oxide, has proven its worth by showing its tremendous capacity in controlling the release rate of drugs by entrapping in the numerous pockets present inside it through π-π stacking interaction when used as a nanocarrier. In this experiment graphene oxide was synthesized through traditional Hummer's method and was used as a nanocarrier to target nanosized drugs, which was prepared by loading curcumin drug into the PVP functionalized graphene oxide by using necessary procedures. After the successful confirmation of the stability, functionalization, and compatibility through various characterization procedures and effective drug loading into the systems, the release behavior of the formulations has been checked through dialysis bag diffusion technique using potassium phosphate buffer saline (pH 7.2, 4.0 and 9.2) for selection of the best medium for determination of the successful sustained-release behavior of the drugs through the dialysis membrane which concluded with satisfactory, sustained and constant drug release phenomenon in the acidic environment reflecting an idea of the fact its favorable release in acidic pH. It is a fact known that dengue fever causes systemic acidosis i.e., reduction of the pH of blood below 7. Thus, when used *in-vivo* there is a possibility that the formulation has easy access to its target and, performs the desired mechanism of action and can be used as a therapeutic delivery system against the Dengue virus.

Keywords: Graphene oxide, Nanocarrier, Dengue virus, Curcumin, Targeted drug delivery, Systemic acidosis.

International Journal of Drug Delivery Technology (2024); DOI: 10.25258/ijddt.14.3.40

How to cite this article: Pritam D, Amita R, Mahendra R. Graphene-Based Nano Drug Delivery of Curcumin for the Treatment of Dengue Fever. International Journal of Drug Delivery Technology. 2024;14(3):1539-1546.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Nanoparticles are one of the components that are used as a novel drug delivery to fight a number of diseases thereby reducing the intensity of adverse reactions that are induced by the active pharmaceutical ingredient. So far as the basic concept of nanoparticles are concerned, any substance whose particle size ranging between 1 to 100 nm can be inferred as a nanoparticle. For a drug to get delivered as a nano sized entity which mainly involves reduction of its size it the given range thus increasing the surface area and bioavailability followed by its incorporation within a nanocarrier combined with its administration inside a living individual with reduced adverse effects,[1,2-5]

The principal utility of the nanocarrier is to serve as a vehicle or a mode of transport of the drug entity into its site of action. Various materials like polymers viz. polyurethanes, polylactide, acrylamides, polyethylene glycol, eudragit, polyvinyl alcohol has been commonly used as nanocarriers in a wide range of research studies.[6] In this experiment graphene oxide (GO) is used as a nanocarrier for development of a potential drug deliverable strategy against Dengue viral infections. Graphene is a carbon allotrope, single layered carbon atoms sequenced like a hexagonal carbon grid whose basic structural element is similar to many other allotropes of carbon viz. graphite, diamond, charcoal and is composed of sp^2 hybridized carbon atoms.[7-10] Pan et al. explored the utility of graphene oxide (GO) as a molecular carrier for in vitro and in vivo drug delivery, including successive cancer therapy. In the application to drug delivery, graphene oxide (GO) has a wide range of potential applications in the biomedical field, ranging from fluorescence resonance energy transfer biosensor, detection and imaging of living cell, antibacterial materials, to biocompatible platforms for cell culture. The physic-chemical properties of graphene oxide (GO) show young's modulus of 100 gigapascal (GPa), fracture strength of 125 GPa, electrical conductivity of ~100 S/cm and thermal conductivity of ~5000 watt/meter-kelvin (w/m K), mobility of charged carriers amounting to ~200000 ($meter^2$/Vilt second (m^2/V s) and a specific surface area of 2630 m^2/gm.[7] Water soluble polymers like Polyethylene glycol (PEG),[11] Poly vinyl pyrrolidone (PVP),[12] Poly ethylimine (PEI),[13] Poly

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(N-isopropyl acrylamide) (PNIPAM),[14] Chitosan[15-16] are grafted on their surface to enhance the biocompatibility and also reduces the toxicity resulting in enhancing the solubility of the water insoluble drugs.[8] Grafting of small molecules into the outer surface of GO by the formation of a covalent and amino groups and can provide a novel drug delivery system. In earlier studies it has been proved that graphene oxide (GO) has a huge prospect to develop into a nanocarrier in research studies related to cancer.[1,16,17] It has also shown it's worth as a bio-detector of type 2 dengue virus (DENV-2) in some studies.[18,19] But in this experiment, it has been used as a nanocarrier for detecting its potential prospect in the delivery of curcumin (CUR), which has been used for its antiviral activity against dengue virus as shown in some studies.[20-21]

Dengue virus (DENV) (family *Flaviviridae*, genus *Flavivirus)*, usually transmits to human beings by *Aedes egypti* mosquitoes. Generally, in Dengue fever due to the infection caused by the Dengue virus possessing a pH of 5.5, the number of antibodies that is present in the body increases to a great extent which is very familiar for any infections in the body. These excessive additional antibodies tend to cross react with the plasmin and the platelets resulting in the lowering of the platelet count which is below 100,000 in case of dengue fever and below 50,000 in case of severe dengue fever whereas the average normal platelets count in an adult healthy human being is 150,000-450,000 /cm^3. Due to this reduction of the platelets count thrombocytopenia (decrease in platelet count) occurs leading to increase in the vascular permeability resulting in the leakage of plasma from the blood vessels designated as 'internal haemorrhage' gradually culminating in death. In Dengue fever the infection leads to reduced platelet count and increased vascular permeability thereby affecting coagulopathy and inducing plasma leakage followed by dissemination of intravascular coagulation leading to severe internal bleeding and hypovolemic shock culminating to death.[23] The main targets of the Dengue virus are the hepatocytes (Hep G_2 and Hep 3B) and the Kupffer cells of the liver [22], the sodium bicarbonate symport – antiport ion channels of the glomeruli and the renal tubules of the kidneys.[24,25] and the alveoli of the lungs.[26] Due to the impairment of the mechanism of action of the sodium bicarbonate symport – antiport ion channels cases have shown acidosis to take place i.e., the lowering of the pH of body below 7.4.

Studies conducted by Afzali et al**.** conclude that nanosized graphene oxide significantly increases the amount of Kupffer cells numerically and megakaryotic cells which are the precursors of platelets in NMRI strain mouse embryo livers.[22]

The main reason behind this study is to establish the possibility of graphene oxide to act as a nanocarrier for the formulation of a targeted drug delivery of curcumin against dengue fever with a dual effect of antiviral combined with increase in the number of platelets count *in-vitro.*

MATERIALS AND METHODS

In this experiment Hydrogen peroxide (30% aq.), potassium permanganate, sulfuric acid, and Sodium nitrate ($NaNO_3$) were procured from Himedia Laboratories (Mumbai, India); polyvinylpyrrolidone (Mw~40000) was procured from Calbiochem (Mumbai, India); graphite flakes and hydrochloric acid was bought from Sigma Aldrich Pvt. Ltd (Bengaluru, India).; 4-dimethylaminopyridine (DMAP), N, N', dicyclohexyl carbodiimide (DCC), were procured from SRL (Mumbai, India) and Di methyl sulphoxide (DMSO) was being procured from Finar Ltd (Ahmedabad, Gujarat). The API curcumin (CUR) powder was bought from Yarrowchem Ltd (Mumbai, India).

Estimation of Curcumin

To estimate the sample using FTIR spectrophotometer, it was analysed using Perkin Elmer UATR Spectrum II model to obtain the peaks.[27]

Development of Graphene Oxide

Generally, graphene oxide (GO) was synthesized by traditional Hummer's Method, where 1g of graphite was taken and mixed with 1 g sodium nitrate ($NaNO_3$) and 46 ml of concentrated sulfuric acid (H_2SO_4) which was stirred constantly for 4 h without heating at 200 rpm at normal room temperature. Then 6g of potassium permanganate ($KMnO_4$) was slowly added combined with stirring for the next 2 h. Then 92 ml of demineralized water was mixed and was maintained at C for 2 h. Then the heater was turned off. Then 200 ml of distilled water was added and was stirred for constantly for 1 hour. Then 20 ml of hydrogen peroxide (H_2O_2) was added combined with constant stirring for 1 hour, which was then washed with 5% hydrochloric acid (HCl) and then with water. After that the whole setup was centrifuged at 7000 rpm for separation of the GO from the reagents. Then GO was collected and is then left to dry at normal room temperature.[31]

Estimation of Graphene oxide

Estimation of GO was done by using FTIR spectrophotometer of model Perkin Elmer UATR Spectrum II[28] and Raman Spectrophotometer of model RIRM LP1519[18] which is a reliable technique for the proof of identification and successful functionalization of graphene oxide where we can able to estimate the yielding of graphene oxide. Here the peaks with respect to D band, G band and 2D band should enable us in successful determination of graphene oxide.

Functionalization of Graphene oxide

Functionalization was done using carbodiimide activated esterification reaction. At first 100 mg purified GO was taken and is mixed with 30 ml dimethyl sulphoxide (DMSO) which was subjected to sonication for 30 min forming a uniform brown coloured solution. Then the catalysts 4.6 g N, N'-Dicyclohexylcarbodiimide (DCC) and 0.34 g 4 Dimethyl amino pyridine (DMAP) were mixed with it in a flask. After that the whole setup was stirred with a mixture 10 ml of polyvinylpyrrolidone (PVP) and DMSO (conc. 100 mg/ml) continuously for 3 days at C. Then it was filtered by a 0.2 µm PTFE microporous membrane which is then rinsed thoroughly by dimethyl formamide (DMF) and acetone. Then to eliminate the unreacted solid PVP the whole setup was dissolved in hot water. After that it was filtered with the help of a nylon membrane of pore size 0.2 µm. Then it was left to dry in a vacuum oven to obtain the end product PVP functionalized GO (GO-PVP).[8]

Confirmation of Functionalization of Graphene oxide with PVP

Functionalization of GO was confirmed using thermo gravimetric analysis (TGA) (Perkin Elmer TGA 4000) which is the study of the rate of degradation of mass with respect to temperature.[17] In this type of study generally degradation occurs on the basis of bond stability and is performed through nitrogen purging performed at 30-600°C in a rate of 10 ˚C per min and also through Raman Spectrophotometer[17] and through Perkin Elmer UAVP Spectrum II FTIR[29] spectrophotometers through the study of peaks and also through Agilent Technologies Cary 60 UV-Visible spectrometer.[17]

Preparation of Sample

Here three formulations were prepared namely formulation A, formulation B¸ and formulation C respectively containing 7 mg CUR, 1 ml DMSO. The variation was made in the concentration of GO-PVP containing 15 mg in formulation A, 5 mg in formulation B and 10 mg in formulation C respectively. The respective formulations were prepared by stirring curcumin in DMSO in one beaker and GO-PVP in distilled water in a second beaker at C with the help of a magnetic stirrer for 24 hours where the drug was allowed to entrap to GO-PVP. Then they were subjected to centrifugation at 7000 rpm for 10 min at 5°C. The precipitates were separated and eliminated. Then the collected supernatant liquid portion is again centrifuged at 7000 RPM for 10 min. The precipitates were allowed to settle and were eliminated. The supernatant liquid portion is collected which are the formulations for the respective characterization experiments.

Compatibility Studies of CUR and GO-PVP

The CUR and GO-PVP compatibility studies have been carried out using a Perkin Elmer UATR Spectrum II FTIR spectrophotometer through the studies of the peaks have been achieved upon input of the three formulations.[27-29]

Estimation of Successful Drug Loading into GO-PVP Nanocarrier

The successful loading of drug moiety in the GO-PVP Nano carrier was determined by using Agilent Technologies Cary 60 UV-Visible spectrometer. Here 2 ml of the sample was pipetted out and was placed in the quartz cuvette. The maximum wavelength was set to 420 nm [30]. By studying the absorbance v/s wavelength curve the amount of drug that has been taken up by GO-PVP is to be determined.

Particle size and Zeta potential analysis

The particle size and zeta potential analysis of the respective formulations; formulation A, formulation B¸ and formulation C were carried out using Malvern Zen 3600 Dynamic Light Scattering Zetasizer.[17]

In-vitro Dialysis Bag diffusion Drug Release Studies

At first, three solvent medium was prepared using potassium phosphate buffer saline (PBS) (pH 7.2, 4.0 and 9.2) for each sample amounting to 100 ml each. Then, the Sigma dialysis tube of pore size 0.2 µm was collected cut into respective measurements and were dipped in distilled water for softening and broadening of the pores to a slight extent. Then, the tubes were filled with the formulations and both the ends were tied up tightly for eliminating any chance of leakage. After that the formulation filled tubes were dipped in the respective PBS solutions allowing the drug to release from the pores in acidic, basic and neutral mediums. 2 ml of the samples were pipetted out and were replaced with freshly prepared stock solution at different time intervals till 72 h.

RESULTS AND DISCUSSION

Estimation of Curcumin

For the confirmation of the procurement of CUR, the Fourier transform ınfra red data of the synthesized CUR was performed. The FTIR spectrum of CUR showing absorbance peaks at 3324.63, 2916.48, 1626.43, 1601.38, 1500-1250, 1000-1200 and 450-970 cm^{-1} for hydroxide Stretching, carbon-hydrogen Stretching, ketone stretching, Alkene, methylene Bending, alkyl Bending, carbonyl Alcohol and Aromatic Bending as shown in Figure 1. The above-mentioned peaks enable us to successfully estimate curcumin.[27]

Estimation of Graphene oxide and confirmation of successful functionalization

The conformational studies for the successful synthesis and its subsequent functionalization have been performed. The FTIR of GO showing absorbance peaks at 3226.06, 1720.11, 1624.15 and 1052.02 cm^{-1} for hydroxide stretching, Ketones, Aromatic Alkene and carbonyl Alcohol as shown in Figure 1[28] and for GO-PVP 3321.66, 2800-2950, 1550-1680, 1435.34, 1200-1300, 900-1150 cm^{-1} for hydroxide stretching, Ketones, Aromatic Alkenes, alkyl Bending, carbonyl Alcohol, Aromatic Bending as shown in Figure 1.[30] The above-mentioned peaks enable us to successfully estimate GO and polyvinylpyrrolidone functionalized graphene oxide (GO-PVP).

Through the Raman Spectroscopic studies, a reliable technique for the proof of identification and successful functionalization of graphene oxide we can able to estimate the yielding of graphene oxide. Here the peaks with respect to D band, G band and 2D band should enable us in successful determination of graphene oxide. In graphical representation it has been clearly found that the intensity of D band of GO occurred at 1355 cm^{-1} (I_D) resulted due to the sp^3 carbon stretching where intensity of G band was observed at 1620 cm^{-1} (**I_G**) due to the sp^2 hybridized carbon atoms. Randomness present in the GO sheets = I_D/ I_G = (1355/1620) = 0.836 as shown in Figure 2. Hence proved the confirmed synthesis of GO.[17] In the Raman Spectroscopic studies of GO-PVP, the D band was observed at 1362 cm^{-1} and that of G band was observed at 1572 cm^{-1}. In this case the degree of randomness

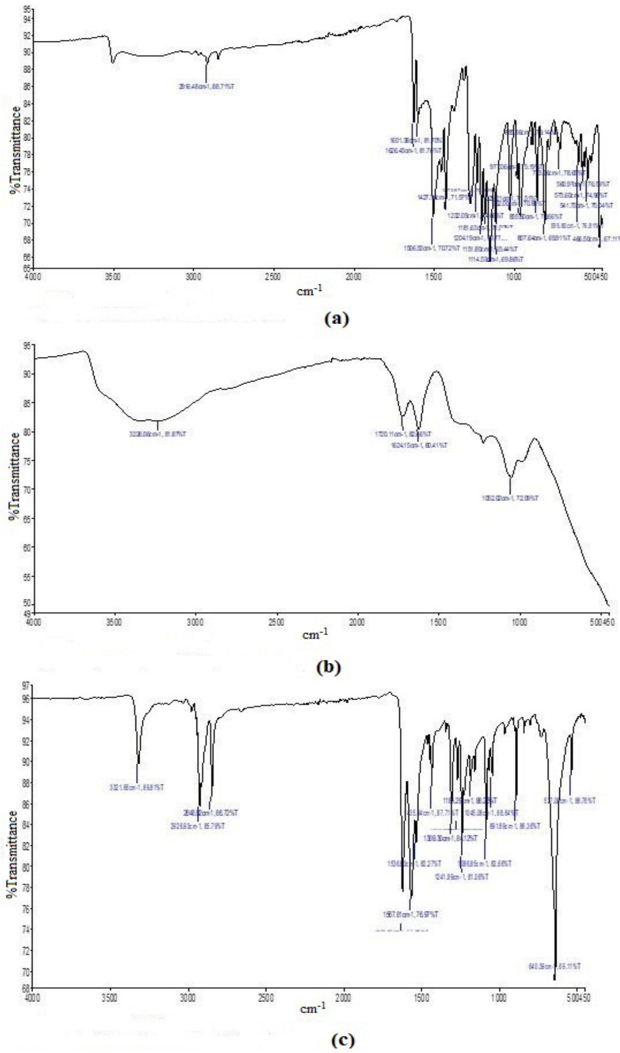

Figure 1: FTIR spectra of drug and polymers are given: (a) showing spectra of curcumin showing various peaks of transmittance at different wave numbers for different characteristic groups for the estimation of curcumin, (b) showing spectra of GO showing various peaks of transmittance at different wave numbers for different characteristic groups for the estimation of GO, (c) showing spectra of GO-PVP showing various peaks of transmittance at different wave numbers for different characteristic groups for the estimation of GO-PVP.

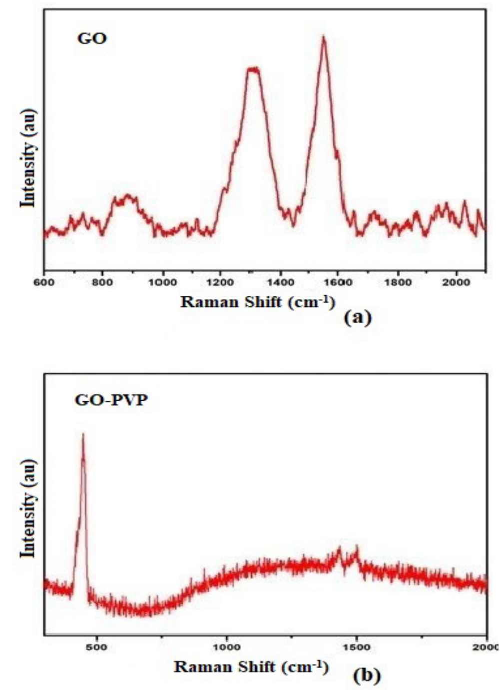

Figure 2: Raman spectra of drug and polymers are given: (a) showing spectra for plain Graphene oxide (GO) showing the appearance of peaks at 1355 cm^{-1} (I_D) in D band due to sp^3 carbon stretching and at 1620 cm^{-1} (I_G) in G band due to sp^2 hybridized carbon atoms, degree of randomness found to be 0.836 confirming the estimation of GO, (b) showing spectra for PVP functionalized Graphene oxide (GO-PVP) showing the appearance of peaks at 1362 cm^{-1} (I_D) in D band due to sp^3 carbon stretching and at 1572 cm^{-1} (I_G) in G band due to sp^2 hybridized carbon atoms, degree of randomness found to be 0.866 confirming the estimation of GO-PVP by conversion of sp^2 hybridized carbon atoms to sp^3 form followed by peak shifting.

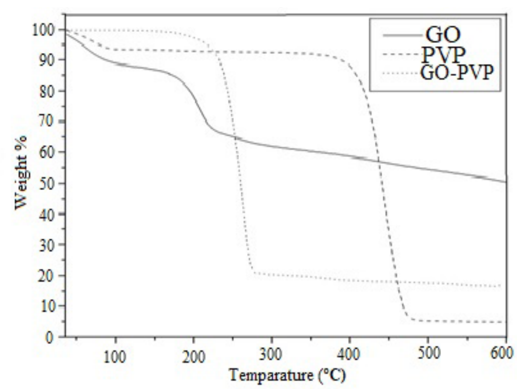

Figure 3: Thermo gravimetric analysis data for the comparison of degradation rates for GO, PVP and GO-PVP

was proved to be 0.866 as depicted in graphical representation as shown in Figure 3 [17]. Therefore, the increment of the degree of randomness from 0.836 to 0.866 resulted for the alteration of the sp^2 hybridized carbon atoms to sp^3 ones indicating the successful binding of GO and PVP the superior chaotic nature and the stability of GO-PVP.

While performing the thermo gravimetric analysis study of GO and GO-PVP, the study of the rate of degradation of mass with respect to temperature. In this type of study generally degradation occurs on the basis of bond stability and is performed through nitrogen purging. Through the TGA data of GO its instability was found which generally starts from 200°C through the pyrolysis of functional groups containing oxygen. The degradation occurring in the carbon structures was confirmed when more than 40% of loss was found at 600 °C. It has been found out that the thermal stability of GO increased after its functionalization with PVP which started from 360 °C with the degradation of PVP. In graphical representation it was seen that the rate of degradation of GO-PVP is intermediate to the degradation of GO and PVP as shown in Figure 3. Hence the successful functionalization

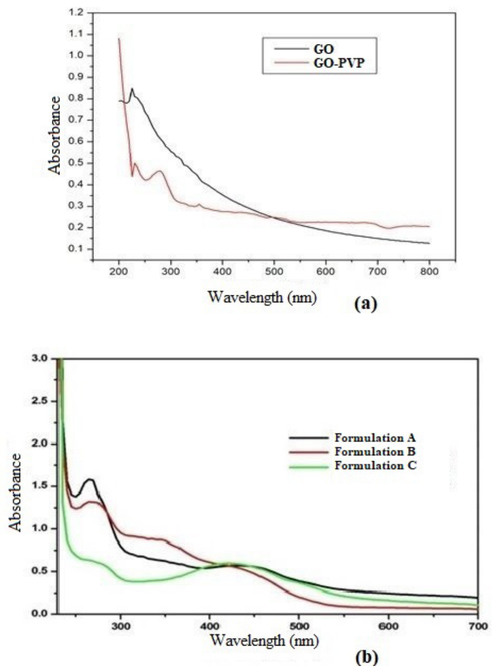

Figure 4: UV data for compatibility has been shown: (a) showing UV-Visible Spectroscopic data of GO and GO-PVP, (b) showing UV spectroscopic data for CUR-GO-PVP for formulation A, formulation B and formulation C showing successful CUR loading into GO-PVP.

has been proved and through the obtaining of peaks in 220 nm for PVP and 235 nm for GO by UV-visible spectroscopy as shown in Figure 4, the successful functionalization has been confirmed[17] and successful formulation of nano drug delivery system has been confirmed through particle size analysis data.

Confirmation of successful loading of Curcumin into GO-PVP (CUR-GO-PVP)

The successful binding of the CUR-GO-PVP in three different formulations have been analysed through the Fourier transform ınfra red studies. It is one of the most important studies regarding pre-formulation aspects since it affects the physicochemical parameter and bioavailability of the respective active pharmaceutical ingredient in the path of developing a safe and effective formulation. For the three formulations namely; formulation A, formulation B and formulation C the subsequent peaks have been observed at 3000-3500, 1500-2000, 1643.13, 1437.84, 1319.81, 1010.37 and 950.6 cm^{-1} due to hydroxide Stretching, carbon-hydrogen Stretching, ketone Stretching, alkyl Bending, carbonyl Alcohol and Aromatic Bending based upon the different optimized amounts of GO as shown in Figure 5. When compared with the FTIR spectra of curcumin as shown in Figure 2. and GO-PVP as shown in Figure 2 with that of the formulation A, formulation B and formulation C the successful compatibility between the drug and the polymer have been confirmed[27-29] and also verified when peaks were obtained between 200-300 nm for GO-PVP [17] and 400-430 nm for curcumin as shown in Figure 4.[30]

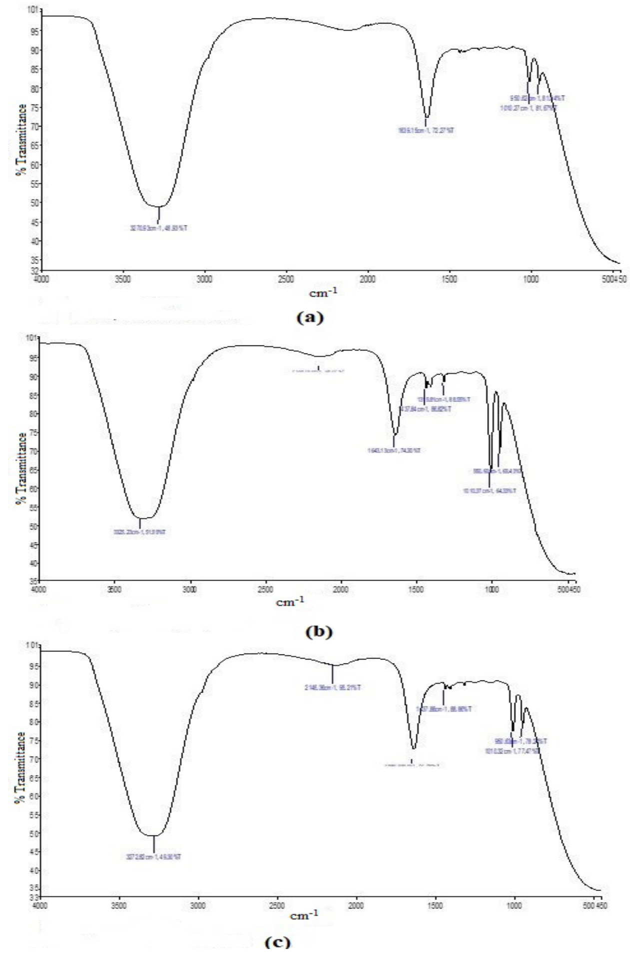

Figure 5: FTIR spectra of three formulations are given: (a) showing compatibility data for CUR-GO-PVP for formulation A with different peaks from the characteristic functional groups, (b) showing compatibility data for CUR-GO-PVP for formulation B with different peaks from the characteristic functional groups, (c) showing compatibility data for CUR-GO-PVP for formulation C with different peaks from the characteristic functional groups.

Particle size Determination

The particle size analysis determines successful synthesis of nanoparticles where the sizes of formulation A and formulation B as shown in Figure 6 are below 100 nm obeying the criteria for ideal sized nanoparticles and for formulation C as shown in Figure 6 the size of the particles is slightly above 100 nm showing the synthesis of large nanoparticles. For the zeta potential analysis, the data strengthens the production of nanoparticles as mentioned in the Zeta potential reports as shown in Figure 7.

In-vitro Dialysis Bag Diffusion Studies

In-vitro studies show the dialysis bag diffusion release performance of CUR from the CUR-GO-PVP system in potassium phosphate buffer saline (pH 7.2, 4.0 and 9.2) at 25°C for three days. The data of the in vitro dialysis bag drug diffusion and release kinetics were shown in three different media as shown in Figure 8 it is proved the poor release profile

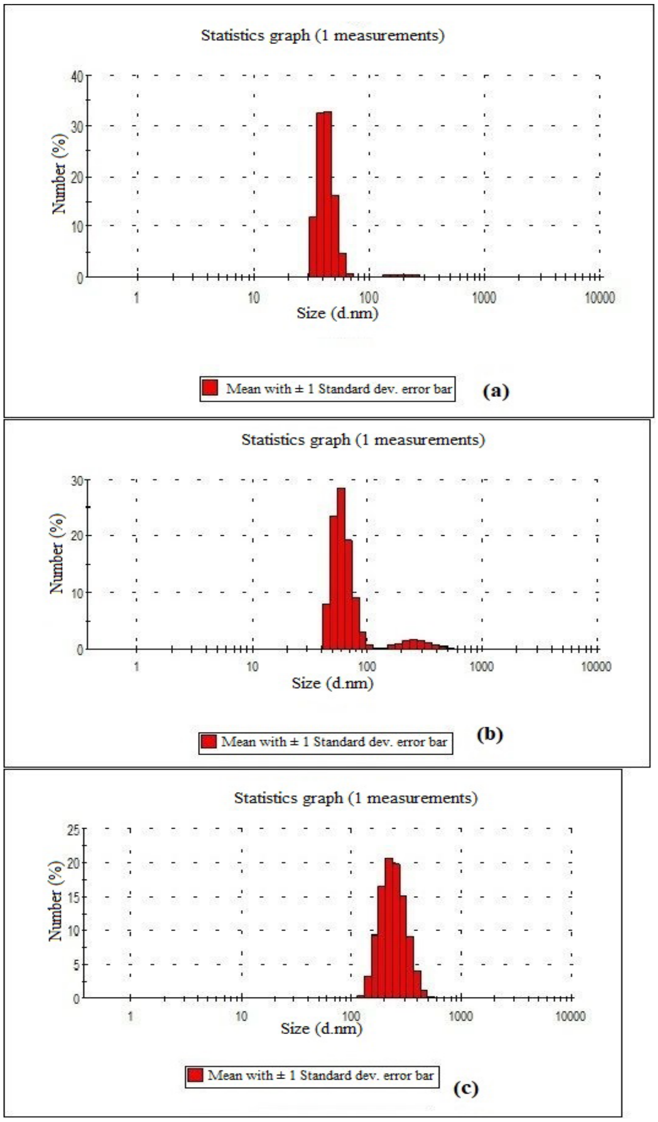

Figure 6: Particle size distribution analysis using Malvern Zetasizer for showing successful formation of nanoparticles for (a) formulation A, (b) formulation B and (c) formulation C.

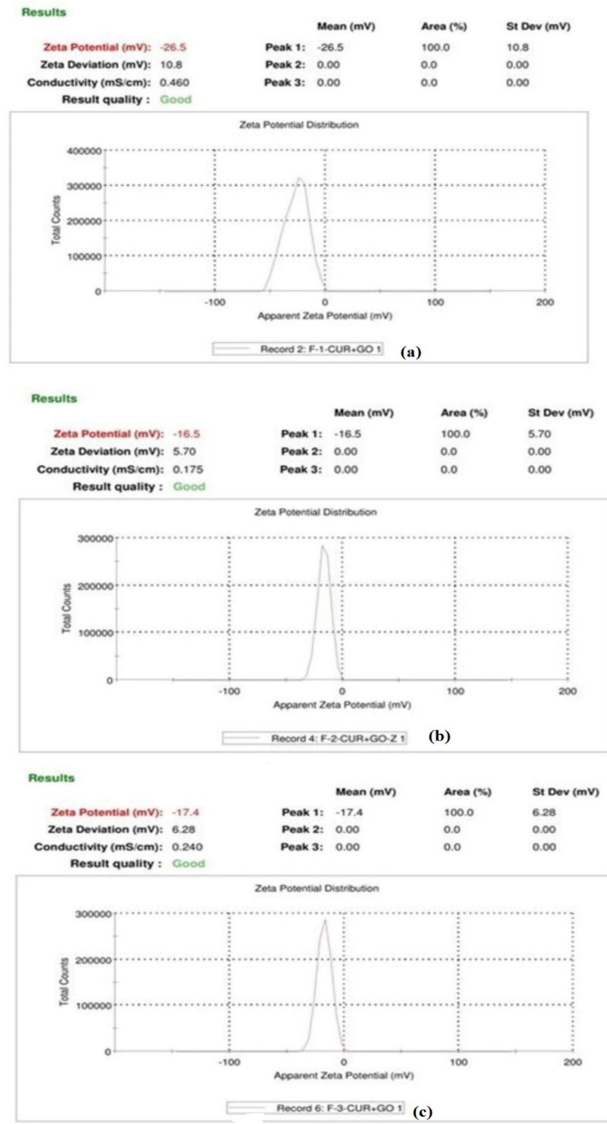

Figure 7: Zeta potential result during particle size distribution analysis using Malvern Zetasizer for showing successful formation of nanoparticles for (a) formulation A, (b) formulation B and (c) formulation C.

of the CUR-GO-PVP in pH 9.2 and pH 7.2. Favourable results were seen in the release kinetics in the pH 4.0 PBS in a gradual manner. In graphical representation it has been confirmed that a more sustained release profiles were seen. This proves enhanced functional capability of the system at acidic pH.

For an ideal novel drug delivery, the concentration of drug in the systemic circulation should remain constant throughout the therapeutic window. It generally deals with the constant and sustained and uniform release of drug throughout a definite period of time. For the three samples; formulation A, formulation B and formulation C it was found that the release rate is in a poor manner throughout the time intervals through the therapeutic window and various oscillations in concentrations at pH 9.2 PBS. For the release pattern at pH 7.2, several oscillations in the concentration and poor release rate in the graphical representation as shown in Figure 8 are also found. Coming to the release profiles at pH 4.0 a lot more improved and sustained release profiles were achieved with its concentrations rising with respected to time throughout the test in a continuous manner. Thus, it is seen that the release kinetics of the curcumin incorporated PVP functionalized GO is best found in acidic pH rather than neutral and basic pH.

Keeping in mind the occurrence of acidosis[24,25] in dengue fever, the affinity of GO towards dengue virus[18] and the observed ideal drug release kinetics in acidic medium it would get easier for the drug delivery system, CUR-GO-PVP to release in the acidic bloodstream due to acidosis[24,25] and easily and target the liver, lungs and kidneys since main targets of the Dengue virus are the hepatocytes (Hep G_2 and Hep 3B) and the Kupffer cells of the liver,[18] the sodium bicarbonate ion channels of the glomeruli and the renal tubules of the kidneys[24,25] (main

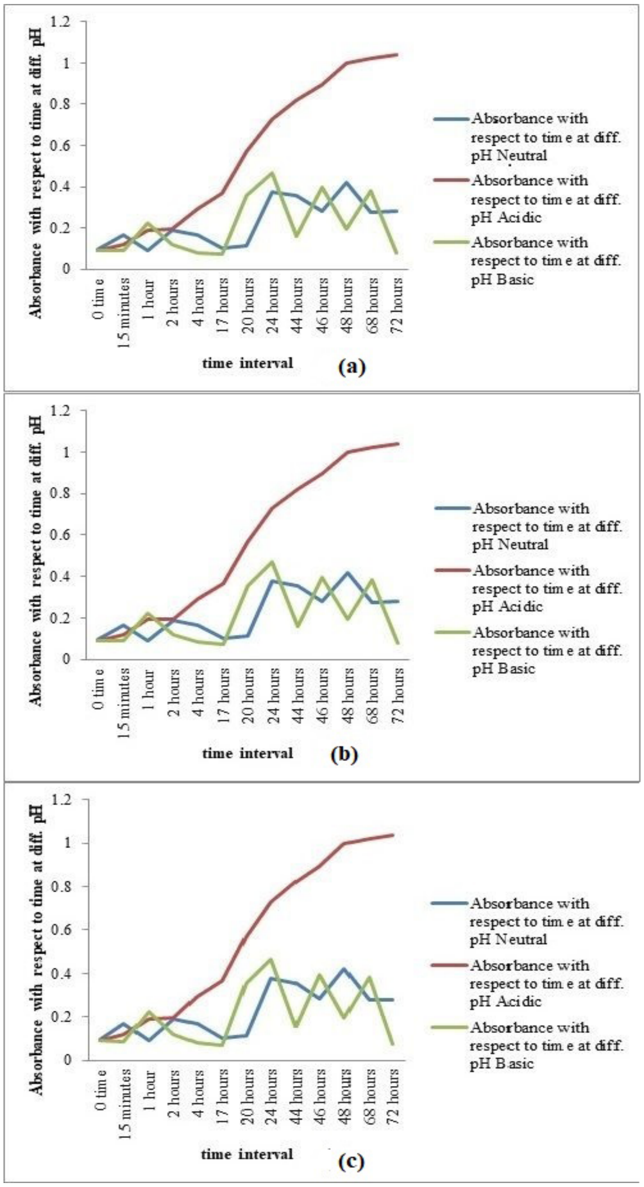

Figure 8: The release pattern of three formulations in different pH media: (a) formulation A, (b) formulation B and (c) formulation C

reason for acidosis) and the alveoli of the lungs.[26] Now as for being a nano sized particles with high surface area CUR-GO-PVP will invade the target site and taking into consideration the studies conducted by Padilla-S et al**.,** CUR having a tendency to inhibit the dengue virus by inhibiting it's ubiquitin proteasome system[21] and as mentioned by Afzali et al., GO has a tendency to increase the number of megakaryocytes in the liver,[18] who are the precursor of platelets thereby increasing the number of platelets as well with reduced toxicity due to the functionalization will biologically compatible polymer PVP.

CONCLUSION

A potential dual targeted nano drug delivery system has been formulated i.e., an antiviral combined with increase in the number of platelets with least toxicity which can be used as a perfect candidate for the treatment of dengue fever if proven ex vivo and in vivo.

Further Prospect of the Experiment

Here in this experiment, the results have been concluded on the basis of in vitro results. For further establishment of the formulation efficient Nano Drug Delivery System cell line studies in HepG2 and Hep3B strains infected with dengue virus were to be carried out and through subsequent preclinical and clinical trails the bedrock of this study is to be more solidified.

ACKNOWLEDGEMENTS

The authors acknowledge higher authorities of the institution for providing facilities and funding the experiment.

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