RESEARCH ARTICLE

Loading of Pemetrexed on Synthesized Fullerene C60 as a Promising Buckysomes

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ABSTRACT

Objective: This study utilizes C60 fullerene to prepare a new carrier (Buckysomes) for pemetrexed (for the first time) and investigate the contribution of such conjugates on the behavior, release, and anticancer activity against lung cancer cells through cell line study.

Methods: fullerene C60 was synthesized by laser irradiation of polycyclic hydrocarbons (PAHs) and loaded with pemetrexed (PMX). The pemetrexed loaded on fullerene C60 was evaluated by fourier transform infrared spectroscopy (FTIR), power x-Ray diffraction (PXRD), scanning electron microscopy (SEM), atomic force microscopy (AFM), differential scanning calorimetry (DSC), particle size analyzer, and zeta potential. The percentage of drug loading, in vitro release, and anticancer activity against lung cells A549 were also evaluated.

Results: Characterization technique showed the successful loading of pemetrexed on fullerene C60 nanocarrier, S9 was the optimum sample with percentage of yield reach to 90.17. Release profile shows that the percentage release of pemetrexed after 240 minutes equal 40% from pure PMX, while the release after 240 minutes was found to be 55% from pemetrexed loaded fullerene C60 nanocarrier, it is reached to 91.7% for pemetrexed loaded on fullerene nanocarrier versus 72% for pemetrexed at the end of 24 hours release. pemetrexed loaded on fullerene nanocarrier (S9) shows lower IC50 (1.03 μM) and higher cytotoxic effect than that of pure PMX (3.1 μM) and blank fullerene (75.5 μM) on A549 cancer cell at different times of exposure (24, 48, and 72 hours). The anticancer activity of pemetrexed against lung cancer cells A549 was improved upon loading on fullerene nanocarrier (80% cell death for S9) in comparison with the free drug (60% cell death) at 24 hours, although the fullerene showed limited anticancer activity (20% cell death after 24 hours) indicating that the loading process led to improve the cytotoxic activity of the drug for the tumor cells. Pemetrexed loaded on fullerene nanocarrier (S9) at 24 hours shows a higher percentage of cell death (52%) than the native pemetrexed (35%), this could be attributed to the synergistic effect of fullerene on the cytotoxic effect of pemetrexed in its complex (S9) which may lead to faster onset of action

Conclusion: This work succeeded to prepare Buckysomes for pemetrexed using fullerene C60 that may act as a promising nanocarrier for pemetrexed since it produced enhancement of anticancer activity, solubility and release profile.

Keywords: Anticancer activity, A549, Fullerene, Pemetrexed, Release.

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INTRODUCTION

Cancer is one of the leading causes of death worldwide. Despite efforts in recent decades to reduce risk factors, cancer rates have continued to rise.[1] Current cancer treatment protocols include precise staging, chemotherapy, radiation, and/or surgical resection. Chemotherapy and radiotherapy are known to have serious side effects.[2] Nanotechnology is being incorporated in the paradigm of 'nanomedicine' to achieve effective medication delivery, generate novel in vitro diagnostics, and produce nano-based implants.[3] Nanotechnology is opening up new possibilities for designing materials that have the potential to revolutionize drug delivery and change the landscape of cancer pharmacology.[4] Carbon's valency allows it to create a variety of allotropes (structurally distinct forms of the same element). Diamond and graphite are two well-known carbon compounds. So many allotropes have also been discovered and studied in recent decades, including ball forms like buckminsterfullerene and sheets like graphene. Carbon nanotubes, nanobuds, and nanoribbons are examples of larger scale carbon structures.[5] The C60 molecule, also known as fullerene, was the first of these carbon nanostructures to be discovered, and it was first identified in 1985. Several other fullerenes, including C20, C70, and even larger species, were found later, but C60 is by far the most studied to date[6] among all the carbon because they are

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predominantly made up of sp2 carbon atoms organized in a hexagonal network, nanoallotropes can be considered members of the same group. They all have certain common features as a result of their shared structure, but they also have substantial distinctions because to their diverse sizes and forms. Electrical conductivity, mechanical strength, chemical reactivity, and optical characteristics are all similar. The most significant distinction was their dispersibility in organic solvents; C60 is the only readily soluble nanostructure, while graphene can be dispersed in certain organic solvents. In organic solvents, many of the other components are just marginally dispersible, resulting in unstable suspensions.[7] Fullerenes, which are 0-dimensional carbon-based nanostructures with a closed-cage shape, have sparked widespread interest and investigation in a variety of sectors. Fullerenes, which are made up of covalently bound carbon atoms with a spherical molecule structure, have unique qualities including electron deficit and reactive exteriors that make them ideal for nanomedicine, drug delivery, cosmetics, organic, and perovskite solar cells.[8] Fullerenes are known as "radical sponges" because of their strong electron affinity, which allows them to effectively quench reactive oxygen species (ROS).[9] In 1985, Robert Curl, Harold Kroto, and Richard Smalley discovered that carbon can also exist in the form of very stable spheres, the fullerenes or Buckyball's. Each C60 molecule is made up of 60 sp2 carbon atoms organized in a circular (truncated icosahedral) shape made up of hexagons and pentagons.[10] Fullerenes are the tiniest stable carbon nanostructures known, and they exist at the intersection of molecules and nanomaterials.[11]

Fullerene could be used to deliver deoxyribonucleic acid (DNA), ribonucleic acid (RNA), small inhibitory ribonucleic acid (siRNA), locked nucleic acid (LNA), and plasmid DNA to specific cellular regions using targeted and regulated drug delivery methods such nucleic acid delivery and viral delivery. Nanoparticles, such as fullerenes, particularly cationic fullerenes, have been utilized to transport small compounds in several investigations due to their nonimmunological responses, low cost, and great efficacy.[12]

The anticancer drug PMX is a white slightly water-soluble crystalline powder with a molecular weight of 471.37 g/mol. It has two pKa the first pKa equal to 3.6 and the second pKa equal to 4.4 (carboxylic moieties).[13]

The PMX is a folate analog metabolic inhibitor that disrupts folate-dependent metabolic processes essential for cell replication. PMX inhibits several enzymes involved in the synthesis of pyrimidine and purine. Thymidylate synthetase is one of the principal targets of action (TS).[14] The folate-dependent enzyme thymidylate synthase catalyzes the conversion of deoxy uridine monophosphate to deoxythymidine monophosphate. When TS is inhibited, the amount of thymidine required for DNA synthesis is reduced. Among the most common side effects of pemetrexed were bleeding gums, chest pain, chills, coughing, fever, loss of coordination, lower back or side pain, painful or difficult urination, pains in the groin or legs, particularly in the calves of the legs, pale skin, pinpoint red spots on the skin, severe headaches of sudden onset, sore throat, sudden appearance of slurred speech, sudden vision changes, swollen glands and trouble swallowing.[15]

The aim of this study was utilizing C60 fullerene to prepare a new carrier (Buckysomes) for native pemetrexed (for the first time) and investigate the contribution of such conjugates on the behavior, release and anticancer activity against lung cancer cells through cell line study. This may lead to utilize such drug fullerene complex as a possible nano carrier that may contribute in targeting the drug to the required site and reducing its serious adverse effects.

MATERIAL AND METHODS

Materials

Pemetrexed® native and Polycyclic Aromatic Hydrocarbon were provided by Hyperchem China Ltd. Iso propyl alcohol was purchased from Sisco Research Laboratories India Pvt. Ltd. Benzene was purchased for Analytic reagents, UK.

Methods

Preparation of Fullerene C60

It has been discovered that by laser irradiation of polycyclic hydrocarbons (PAHs), it is possible to obtain new homologues of the fullerenes family that would otherwise be impossible to obtain in sufficient quantities through the uncontrolled process of graphite evaporation. The production of fullerenes is based on the use of polycyclic aromatic hydrocarbons (PAHs) that already contain the necessary carbon frameworks. Fullerenes are formed when PAH molecules are "rolled up" and heated to 1100°C in a flash vacuum pyrolysis (FVP) process. It has been shown that when a polycyclic aromatic hydrocarbon containing 60 carbon atoms is laser irradiated at 337 nm wavelength, it generates the fullerene C60.[16]

Loading of Pemetrexed on Fullerene C60

A total of 100 mL Erlenmeyer flask was filled with 80 mg of C60 fullerene and 25 mL of benzene. The mixture was stirred with a magnetic bar for 30 minutes and subjected to ultrasonication for 60 minutes. The solution was filtered through a filter paper and the filtrate and isopropyl alcohol were cooled separately in a refrigerator for 30 minutes. Subsequently, 25 mL of the saturated C60 fullerene solution and different volumes of iso propyl alcohol as illustrated in Table 1, were placed in a 100 mL vial. The mixed solution was ultrasonicated for 20 minutes then 0.05 mg of pemetrexed was added then ultrasonicated for further 20 minutes and then refrigerated for 20 hours. The cooled mixture was filtered through a filter paper and the precipitate was dried in an oven at 120°C for 3 hours.[17]

Calculation of %Yield and Drug Loading

The percentage yield (% yield) was calculated as a percentage ratio of the total weight of drug loaded nanocarriers to the weight of fullerene and drug fed initially in the reaction before incorporation,[18] as follows:

$$\%\text{Yield} = \frac{\text{Total weight of the obtained nanoparticles after drug incorporation}}{\text{weight of fullence and drug added}} \times 100\%$$

The percentage of drug loading (% drug loading) was calculated as a percentage ratio of the weight of drug in nanocarriers to

Table 1: Loading of pemetrexed on fullerene method

Sample Number	*Fullerene (g)*	*Isopropyl alcohol volume mL*	*Pemetrexed (g)*
S1	0.0843	5	0.05
S2	0.0843	10	0.05
S3	0.0843	15	0.05
S4	0.0843	20	0.05
S5	0.0843	25	0.05
S6	0.0843	30	0.05
S7	0.0843	35	0.05
S8	0.0843	40	0.05
S9	0.0843	45	0.05
S10	0.0843	50	0.05

the total weight of nanoparticles loaded with the drug,[19] as follows:

$$\%\text{Drug loading} = \frac{\text{Weight of drug loaded nanocarrier}}{\text{Total weight of nanocarrier loaded with the drug}} \times 100\%$$

The weight of PXM loaded on different nanocarriers was determined by dissolving 10 mg of drug loaded nanocarriers separately in 3 mL of benzene and then complete the volume to 50 mL with phosphate buffer pH 7.4 and scanned spectrophotometrically at λ_{max} 225 nm by UV spectrophotometer.

Choosing the Best Prepared Drug Loaded Nanocarrier

Sample S9 was selected as best prepared drug loaded nanocarrier since it showed the higher drug loading and % yield as 89.29, and 90.17, respectively.

Characterization of Pemetrexed and the Best Prepared Pemetrexed-fullerene C60 Nanocarrier

Fourier Transform Infra-Red (FTIR) Measurement

The FTIR spectroscopy (4000–500 cm^{-1}) with a potassium bromide disc was used to characterize pure pemetrexed and PMX loaded on fullerene (S9), which were each studied separately.[20]

X-Ray Diffraction (XRD) Measurement

Pure drug, fullerene, and drug loaded on fullerene (S9) were all subjected to X-ray diffraction (XRD) to assess the crystallinity of the compounds. It was equipped with Cu-Ka radiation (1.54060 nm), voltage (40 kv), and current (50 mA) to conduct XRD experiments (30 mA). With a scanning speed of (5°/min) and an axis-2 range of 0 to 60 degrees, the samples were subjected to an analysis.[21]

Differential Scanning Calorimetric (DSC) Measurement

Differential Scanning Calorimetric (DSC) analysis was carried out on pure drug, fullerene, and drug loaded on fullerene (S9) samples by dispersing 1–2 mg of each sample in 5 mL of phosphate buffer PH 7.4, then heating 1mL of the dispersed sample from 25 to 300°C at a rate of 10°C per minute under nitrogen gas carrier supplied at 10 mL/min for 30 minutes.[22]

Scanning Electron Microscopy (SEM) Measurement

The produced nanocarrier (S9), as well as the pure drug and blank fullerene, were all examined under a SEM to determine their properties. It was accomplished by taking 1 to 2 mg of powdered material and mounting it on a sample small aluminum holder, followed by coating the sample with gold (conductive metal), removing large molecules with nitrogen gas, and scanning the sample with a focused fine beam of electrons in a scanning electron microscope to capture images.

Particle Size Measurement

When it comes to routine particle size and distribution of nanoparticle width measurements, dynamic light scattering is the most powerful approach available. The native pemetrexed, fullerene, and best sample (S9) were placed in a disposable cuvette with a diameter of 1-cm to produce an appropriate scattering intensity. To further investigate this, the mean particle size (diameter nm standard deviation) and polydispersity index (PDI) of the formulations were computed using Brookhaven Instruments Corp90 PLUS software and the results were compared (ZetaPlus Particle Sizing, NY, Software, Version 5.34). A fixed scattering angle of 90° was used for the tests, which were carried out at room temperature (25°C).[23,24]

Zeta Potential (ξ) Measurement

Zeta potential (ξ) analysis for pure pemetrexed, blank fullerene, and fullerene loading with pemetrexed (S9) was accomplished by dissolving 2 mg from each sample individually in 10 mL of phosphate buffer pH 7.4 with sonication, filtering with a 0.2l filter syringe, and finally introducing each sample to the zeta potential analyzer to interpret the zeta potential data.[25]

Saturated Solubility

An excess amount of pure drug and pemetrexed loaded on nanocarriers best samples (S9) were added each separately to distilled water then leaving it stirred or shaken until equilibrium is established for 24 hours. Then the suspension was filtered, discard the first 2 mL of filtrate, then analyze the drug in the filtrate. The concentration was considered the saturation or equilibrium solubility of drug.

***In-vitro* Drug Release Study**

With the use of a USP type II rotating paddle apparatus at 370.5°C and a rotating speed of 100 rpm in 500 mL of phosphate buffer solution, an in-vitro release study for PMX-loaded fullerene (S9) was carried out in comparison to previously obtained PMX (pH 7.4,6.8 and 5.5). PMX equivalent to 100 mg PMX of the prepared drug loaded on fullerene (S9) and pure PMX were dispersed in the dissolution medium and samples of 5 mL were drawn out at predetermined time intervals and replaced with the same volume of fresh media after each withdrawal. The withdrawn samples were filtered, and the content of PMX was determined spectrophotometrically by using UV-visible technology.[26,27]

Cytotoxic Activity of Pemetrexed Loaded on Fullerene Nanocarriers Method

Cell Culture

Lung cancer cell line (A549) was obtained from the American Type Culture Collection (ATCC) in Middlesex, United Kingdom, and was stored in the Cell Bank of the

Biomedical Research Centre at Mustansiriyah University in Iraq. The A549 cell line was employed as a model cancer cell for this investigation.

Cell Maintenance

RPMI-1640 medium (GIBCO, Merelbeke, Belgium) was used to sustain A549 cells. It contained 0.5 percent fetal bovine serum FBS (Fisher Scientific, USA) and 1% L-Glutamine (Lonza, UK), as well as 1% Penicillin-streptomycin-amphotericin B 100X (Lonza, UK) for antiseptic purposes. During the incubation period, cells were grown in 75 cm^2 flasks at 37°C in a 5% CO2/95 percent humidified air environment. Flasks containing A549 cells were passaged under sterile circumstances once the cells had attained 90 percent confluence, according to the protocol. At 37°C, the cells were incubated for 2 minutes in trypsin solution to allow them to detach themselves from the bottom of the flask after being washed with 5 mL of phosphate buffered saline (PBS). An equivalent volume of complete growth media was added, and the cell suspension was placed into a conical tube measuring 50 mL in size. After that, the cells were centrifuged for 3 minutes at 1200 rpm. This was done by discarding the supernatant, then reconstituting the cell pellet in freshly supplemented growth media. The cells were then counted using a hemocytometer under a microscope and used as needed.[28]

Storage and Resuscitation of Cell Lines

Immediately after trypsinization of a confluent 75 cm^2 flask, the cell solution was centrifuged at 1200 rpm for 3 minutes to extract the trypsinized cells. Afterwards, the cell pellet was resuspended in 4 mL freezing media (Life Technologies), and 1-mL aliquots were applied to cryovials to create the cell suspension (Thermo Fisher Scientific, Loughborough, UK). The cells were kept frozen at -80°C for 24 hours before being placed in liquid nitrogen for long-term storage in a freezer. A rapid thawing at 37°C of cells preserved in liquid nitrogen was followed by the addition of 10 mL of fresh growth medium. Using centrifugation, the cells were extracted and resuspended in 25 mL of fresh media, which was then put to a 75 cm^2 flask and allowed to proliferate.[29]

Cell Viability and Inhibitory Concentration (IC_{50}) by MTT Assay

In order to determine the vitality of A549 cancer cells, the MTT assay was utilized in conjunction with fullerene, pemetrexed, and Pemetrexed+Fullerene. All cell suspensions (A549) were dispensed into 96-well flat-bottom tissue culture plates (Falcon, USA) at concentrations of 5 x 10^3 cells per well and incubated for 24 hours under standard conditions; 4 x 10^3 cells per well for 48-hours incubation; and 3 x 10^3 cells per well for 72-hours incubation under standard conditions. After 24 hours, the cells were treated with various concentrations of the C1, C2, and C3 chemokines (0.05, 0.15, 0.32, 0.75, 1.5, 3.12, 6.25, 12.5, 25, 50, and 100 μM). After a 24, 48, and 72-hours recovery period, the cell culture medium was removed and the cultures were incubated for 4 hours at 37°C with 30 mL of MTT solution (3 mg/mL MTT in PBS) (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide) (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenylt After 4 hours, the medium was removed by gently inverting the container and tapping it against the paper. Wells in the control group got only 100 mL of growth medium. Dimethyl sulfoxide (DMSO) was added to each well and the plates were allowed to sit at room temperature in the dark for approximately 15 to 20 minutes before being used. With a multiscan reader, the absorbance of each well was measured at a wavelength of 540 nm and corrected for background absorbance at a wavelength of 650 nm after which the results were analyzed. The optical density (OD) of the wells that did not contain extract was used to measure the viability of the cells. According to the investigators, the inhibitory concentration 50 percent (IC50) was defined as the smallest concentration of extract that reduced cell viability after 72 hours by fifty percent.[30]

RESULTS AND DISCUSSION

Synthesis of Fullerene C60 Nanocarrier

Higher fullerenes are produced via the traditional approach of graphite vaporization, however the process is plagued by challenges such as low yields, non-selective carbon cage creation, and purifying issues. It is necessary, as a result, to develop synthetic procedures that will yield a single isomer of the desired fullerene that will be devoid of impurities of other isomers or fullerenes of different sizes. Using planar polycyclic aromatic hydrocarbon precursor molecules that already contain the carbon framework required for the formation of the target fullerene cage, a promising route to achieve this goal for the selective synthesis of fullerenes has been identified for the selective synthesis of fullerenes. By irradiating the fullerene with a laser at 337 nm wavelength, the unfolded fullerene can be sewn back together through intramolecular cyclodehydrogenation to generate the appropriate fullerene isomer.[31] Black powder of fullerene C60 were obtained in this study (figure 1) and subjected for characterization.[32]

Loading of Pemetrexed on Fullerene C60

A dark brown powder was obtained for pemetrexed loaded fullerene C60 nanocarrier as a new compound that differ from the original colour of PMX (white) as well as from the original colour of fullerene C60 nanocarrier (black) resulting from

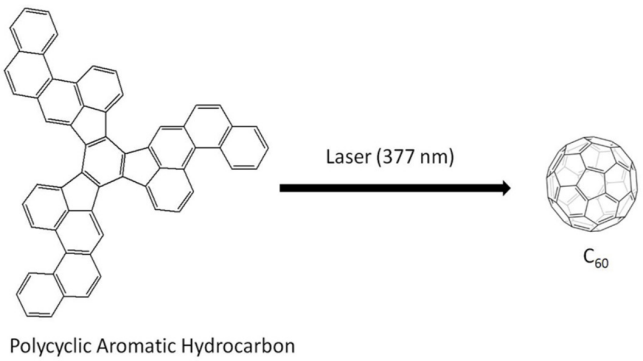

Figure 1: Direct synthesis of fullerene by laser irradiation of PAHs[16]

complex formation between the drug and the nanocarrier. The different volumes of isopropyl alcohol led to increase linear growth of fullerene C60 and increase the surface area available for drug loading therefore higher amount of drug loaded on the fullerene C60 Buckysomes leading to a higher percentage of yield but upon further increase of isopropyl alcohol (>45 mL) lead to non-significant decrease in % yield and drug loading and this could be due to saturation of the solution as shown in Table 2. The same result was observed when they check the influence of different alcohol such as isopropyl alcohol and propyl alcohol as dissolving agent on BSA nanocarrier which had a stronger coacervations ability to produce nanocarrier with higher yield.[33,34]

Characterization of Pemetrexed® loaded Fullerene Nanocarrier

FTIR Measurement

The FT-IR spectrum of fullerene C60 Buckysomes shows the characteristic band of C=C aromatic at 1531 cm^{-1} and C-C at 1427 cm^{-1} which is the same peaks observed with reported pristine fullerene C60.[35]

The FTIR spectrum of pemetrexed drug displace the characteristic band υ O-H of COOH at 3414 cm^{-1}, υ NH_2 at 3298 cm^{-1}, υ NH at 3170 cm^{-1}, υ C-H aromatic at 3050 cm^{-1}, υ C-H aliphatic at 2931 cm^{-1} , C=O adjacent to amide at 1743 cm^{-1}, C=O of COOH at 1698 cm^{-1}, C=N at 1624 cm^{-1}, C=C aromatic at 1500 cm^{-1} all peaks are the same observed with FT-IR of reported native pemetrexed.[36] The FT-IR spectrum of loaded fullerene-pemetrexed complex appeared with the same functional groups of free pemetrexed with small shifting which are υ O-H of COOH at 3417 cm^{-1}, υ NH_2 at 3298 cm^{-1}, υ NH at 3160 cm^{-1}, υ C-H aromatic at 3020 cm^{-1}, υ C-H aliphatic at 2738 cm^{-1}, C=O adjacent to amide at 1747 cm^{-1}, C=O of COOH at 1681 cm^{-1}, C=N at 1624 cm^{-1}, C=C aromatic at 1519 cm^{-1}.

Table 2: Percent yield and drug loading of pemetrexed on fullerene C60

Sample number	*Percent yield*	*%Drug loading*
S1	59.57	70.42
S2	63.29	72.46
S3	67.01	73.53
S4	67.76	78.13
S5	70.74	80.65
S6	73.72	82.10
S7	83.40	84.46
S8	84.88	85.76
S9	90.17	89.29
S10	85.63	86.96

Among the bonds found in a C60 fullerene are two that behave like single bonds: one that is shared between the hexagon and the pentagon and another that is shared between two hexagons that behaves like a double bond. These bonds coat the entire surface of any fullerene with a system of conjugated double bonds. Fully hexahedral fullerene structures with fewer hexagons have stronger SP3 bonding characteristics, such as higher strain and more reactive carbon sites, whereas fullerene structures with adjacent pentagons typically have lower stability and relative abundance than fullerene structures with isolated pentagon, where there is obvious delocalization of (pie) bonds throughout the structure.[37] Given that a fullerene's surface is curved, it is natural for its carbon atoms to be pyramidalized. Pyramidalization alters the hybridization of atomic orbitals at carbon atoms, such that (pie) orbitals contain varying amounts of S and P orbitals, allowing for a variety of chemical activities to take place.[38]

As opposed to the conjugated pie (π) - system seen in typical aromatic compounds, in fullerenes there are no replaceable hydrogen atoms that may facilitate replacement processes, which means that substitution reactions are not possible.[39] the contact between a carbon atom and an external chemical reagent may result in one or more of the following outcomes: orbital/hybrid changing towards SP3, pie (π) bond breaking, or a reaction between the free pie (π) orbital and the external chemical reagent.[40]

The FTIR spectra shows the same main functional groups of pemetrexed before and after loading with fullerene nano carrier with small shifting with less intense peaks for pemetrexed, indicating complex formation between pemetrexed and fullerene nano carrier.[41,42] The same result was less intense peaks for pemetrexed loaded on chitosan nanocarrier.[43]

X-Ray Diffraction (XRD) Measurement

The XRD technique was used to determine the purity of a blank fullerene C60 specimen (Figure 3-A). The FCC lattice of the fullerene C60 exhibited three different reflections, with diffraction peaks located approximately at 10, 17, and 20° angles, respectively, corresponding to 111, 220, and 311 crystal planes of FCC. XRD results of fullerene C60 were similar to those of the referred material in that all of the peak intensities were distinct,

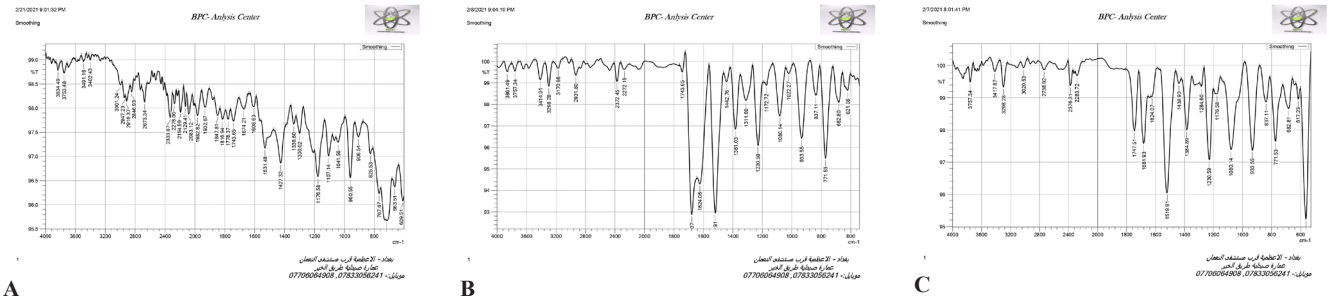

Figure 2: FT-IR of (A) blank fullerene, (B) pure pemetrexed and (C) Pemetrexed loaded fullerene nanocarrier (S9)

crisp, and intense without any peaks for impurities indicating crystalline property.[44] The XRD spectrum of free native pure pemetrexed (Figure 3-B) displays at 2Ɵ values at 5, 10, 20 to 30° and between 30 to 40° also a large number of peaks can be seen indicating its highly stable crystalline structure.[45]

While the XRD of pemetrexed loaded on fullerene nanocarrier (Figure 3-C) show less intense and less characterized sharp diffraction peaks with increase multiplicity in comparison with the XRD of free pemetrexed referring possibly to the more amorphous nature.[46,47] The same result was observed with lipid drug conjugate nanoparticle as a potential nanocarrier for pemetrexed.[48]

Differential Scanning Calorimetric (DSC) Measurement

The Differential Scanning Calorimetric (DSC) spectrum (Figure 4-A) of blank (pristine) fullerene nanocarrier display no sharp endothermic peak indicating outstanding thermal stability when heated to 550°C.[49] The DSC spectrum of free pemetrexed displays a sharp narrow, intense endothermic peak at 250°C that corresponds to the melting of the drug indicating its crystallinity and decomposition of the drug after this temperature.[50] The DSC spectrum of loaded pemetrexed fullerene nanocarrier shows a small non-intense peak at 96.5°C that indicates the initiation of melting of the complex and disappearance of pure melting point peak.[50,51] The same result was observed with docetaxel loaded poly D,L-lactic-co-glycolic acid (PLGA) nanoparticles and complex formation of pemetrexed with bile acid DCK.[52,53]

Scanning Electron Microscopy (SEM) Measurement

The images of the Scanning electron microscope of pristine fullerene (Figure 5-A) were captured to know the geometry and morphology of carbon nanostructures before and after loading and complexation.[54] The SEM of native pemetrexed indicates the crystalline form of pure pemetrexed as shown in figure (Figure 5-B).[55] The SEM images (Figure 5-C) of loaded pemetrexed with fullerene display a more compact surface with much less crystalline shape indicating the amorphous form of pemetrexed-fullerene complex in comparison with the free pemetrexed and blank fullerene nanocarrier.[56,57] The same result was observed with fullerene loaded with graphene oxide nanocomposite.[58]

Particle Size Measurement

The particle size measurements for fullerene and pemetrexed loaded on fullerene (S9) were done using Brookhaven Instruments Corp90 PLUS. The particle size of fullerene was

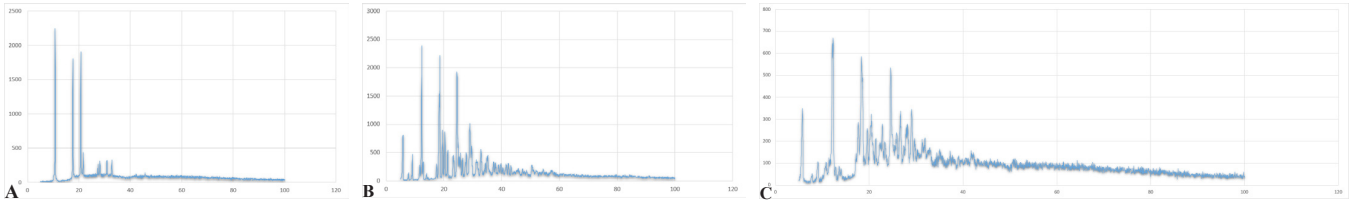

Figure 3: XRD of (A) Blank fullerene; (B) Pure pemetrexed and; (C) Pemetrexed loaded fullerene nanocarrier (S9)

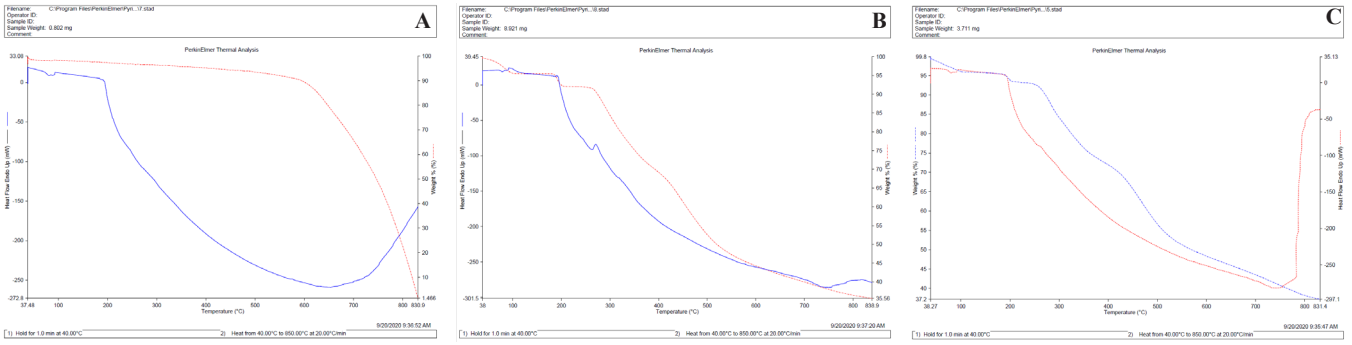

Figure 4: DSC of (A) blank fullerene, (B) pure pemetrexed, and (C) Pemetrexed loaded fullerene nanocarrier (S9)

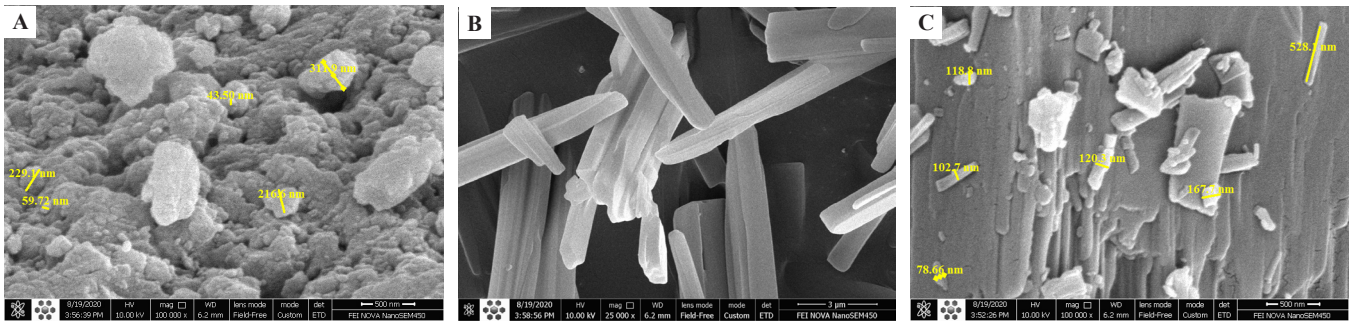

Figure 5: SEM of (A) blank fullerene, (B) pure pemetrexed and (C) Pemetrexed loaded fullerene nanocarrier (S9)

found 232.4 nm and for fullerene loaded with PMX (S9) is 301.5 nm. The results for non-covalent interactions occurred between pemetrexed and fullerene which might lead to hetero-aggregation between pemetrexed and fullerene or homo-aggregation of fullerene moieties that led to size enlargement distinctly, these aggregations due to the strong interactions were able to yield by $\pi-\pi$ stacking. This interaction could be studied by observing the change in particle size and PDI where PDI for PMX loaded on fullerene (S9) was 0.178 indicate homogenous distribution.[59] The same result was observed with loading of doxorubicin on single walled carbon nanotube (SWCNTs).[60]

Zeta Potential (ξ) Measurement

Zeta potential refers to the extent of repulsion or attraction between the particles, which was an important factor in predict dispersion electrostatic stability.[61] The zeta potential values were generated using water as the dispersion medium. All samples in the study produced negative zeta potential values (Table 3); the values was ≤ -30 mV predicting good physical stability of the prepared pemetrexed-fullerene complex (S9) as compared with native pemetrexed (ZP values higher than -30 mV), the detected stability of fullerene loaded with pemetrexed indicated that there is no additional aggregation occurs in dispersion medium which impart this good stability.[62,63]

Saturated Solubility

Fullerene loaded with pemetrexed led to slight improvement in the solubility of poorly soluble of drug (S9) (0.72 mg/mL) as compared with native pemetrexed (0.6 mg/mL). The same result was observed with fullerene loaded with docetaxel for breast cancer.[64]

In-vitro Drug Release Study

The in vitro release of PMX (Figure 6) from drug loaded nanocarrier (S9) in comparison to pure PMX shows that the percentage release of pemetrexed after 240 minutes equal 40% from pure PMX, while the release after 240 minutes was found to be 55% from pemetrexed loaded fullerene C60 nanocarrier (S9), it reached to 91.7% versus 72% for native pemetrexed at the end of 24 hours release. Also, fullerene improves the release of pemetrexed from drug-fullerene complex in the other pH (6.8 and 5.5).[65] The same result was observed with silibilin loaded on carbon nanotube.[66]

Cytotoxic Activity of Pemetrexed Loaded Nanocarrier

A549 cells were treated with increasing concentrations of pemetrexed before and after loading with fullerene nanocarrier as well as with blank fullerene nanocarrier, where each sample was dissolved in DMSO, knowing that DMSO showed no or negligible cytotoxic activity (inhibition rate percent IR%) on both tumor and normal cells.[67] PMX from pemetrexed fullerene nanocarrier (S9) shows lower IC50 (1.03 μM) and higher cytotoxic effect (high IR %) than that of pure PMX (3.1 μM) and blank fullerene (75.5 μM) on A549 cancer cell at different times of exposure (24, 48 and 72 hours) (Figure 7). The same result was observed with fullerene C60 loaded with cisplatin the led to improve anticancer activity of the drug for the tumor cells.[68]

The anticancer activity of pemetrexed against lung cancer cells A549 (Figure 8) was improved upon loading on fullerene nanocarrier (80% cell death for S9) in comparison with the free drug (60% cell death) at 24 hours, although the fullerene showed limited anticancer activity (20% cell death after 24 hours) indicating that the loading process led to improve cytotoxic activity of the drug for the tumor cells.[69] In general, the cytotoxic effect for pemetrexed loaded on fullerene was increased as the concentration increased at the different time intervals 24,48 and 72 hours.

Time response curve (Figure 9) showed that 12.5 μM of fullerene at 24 hours lead to (5% cell death), at 48 hours lead to (20% cell death) and 72 hours lead to (25% cell

Table 3: Zeta potential of pure pemetrexed, fullerene nanocarrier before and after loading with pemetrexed

Sample	*Zeta potential (mV)*
Pemetrexed	-25.1
Fullerene	-44.8
Fullerene loaded with pemetrexed (S9)	-48.8

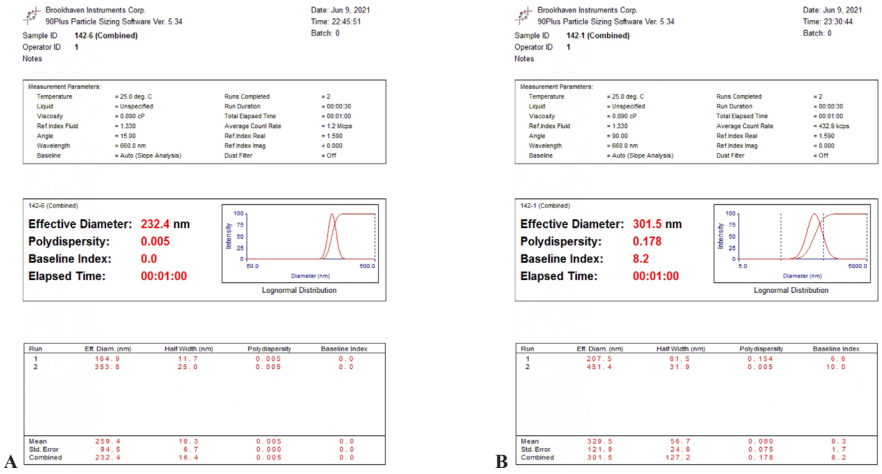

Figure 5: PSA of (A) blank fullerene and (B) Pemetrexed loaded fullerene nanocarrier (S9)

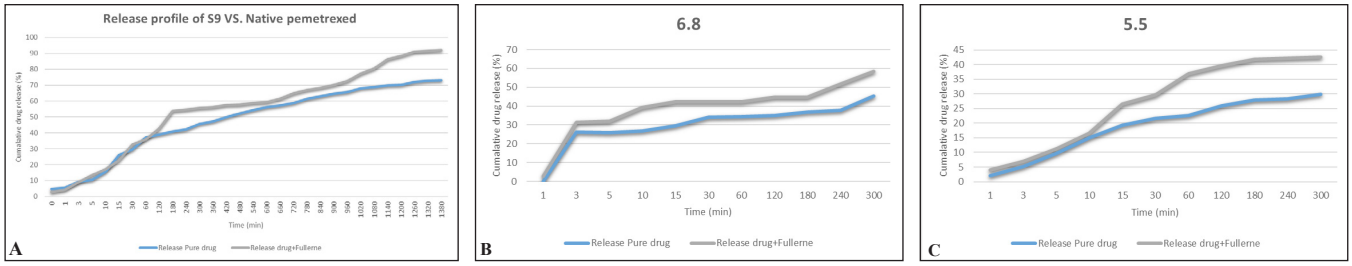

Figure 6: Comparative in vitro release profile of pure pemetrexed and pemetrexed loaded on fullerene C60 nanocarrier in phosphate buffer solution (A- pH 7.4, B- pH 6.8 and C- pH 5.5)

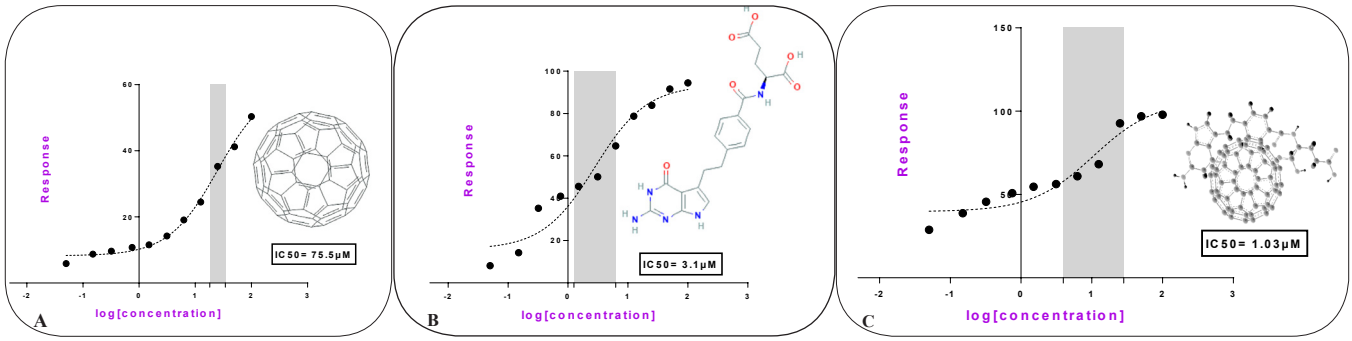

Figure 7: IC50 of (A) blank fullerene, (B) pure pemetrexed and (C) Pemetrexed loaded fullerene nanocarrier (S9)

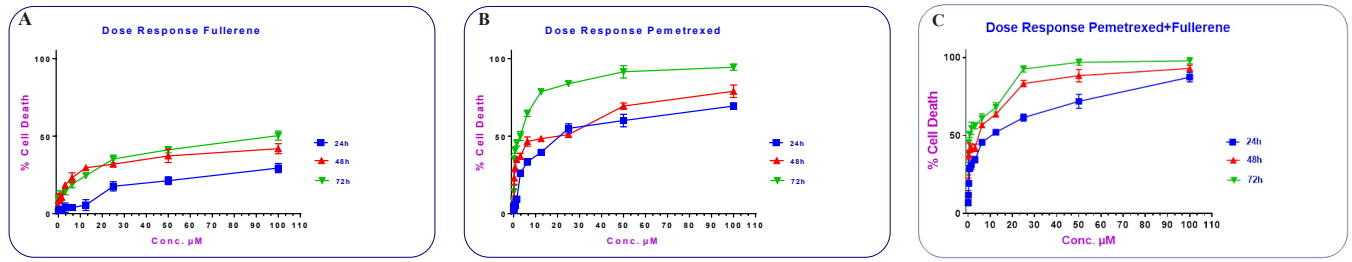

Figure 8: Dose response curve at different concentration in Lung cancer line A549 of (A) blank fullerene, (B) pure pemetrexed and (C) Pemetrexed loaded fullerene nanocarrier (S9) at 24,48 and 72 hr.

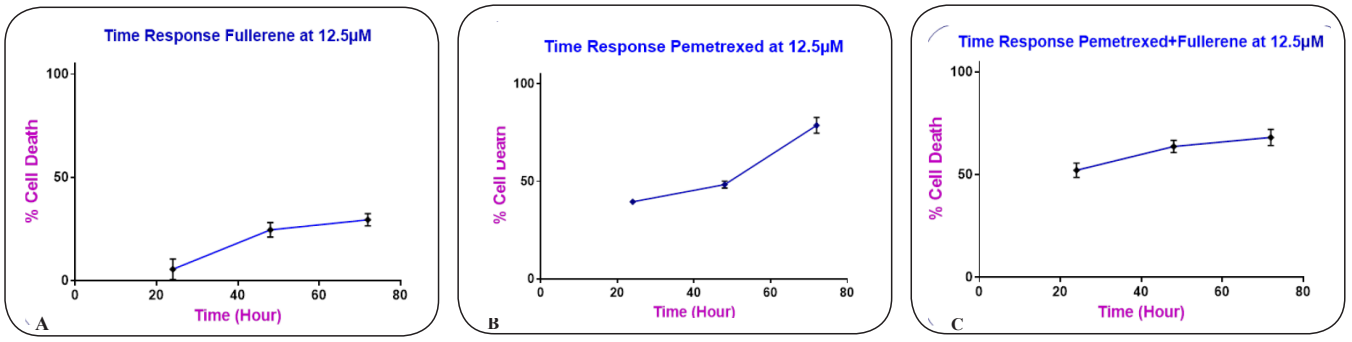

Figure 9: Time response curve at different time in Lung cancer line A549 of (A) blank fullerene, (B) pure pemetrexed and (C) Pemetrexed loaded fullerene nanocarrier (S9)

death). Native pemetrexed concentration 12.5 µM of at 24 hours lead to (40% cell death), at 48 hours lead to (45% cell death) and 72 hours lead to (78% cell death). Pemetrexed loaded on fullerene nanocarrier (S9) at 24 hours shows higher percentage of cell death (52%) than the native pemetrexed (35%), this could be attributed to synergistic effect of fullerene on the cytotoxic effect of pemetrexed in its complex (S9) which may lead to faster onset of action. The same result was observed with fullerene loaded with doxorubicin; this fullerene-drug complex will be led to enhance anticancer activity.[66]

CONCLUSION

This study results in interesting complex formation and loading of pemetrexed on fullerene nanocarrier with high %yield and high drug loading that improve the dissolution profile of

pemetrexed also amelioration in its anticancer activity which was acquired after loading with fullerene nanocarrier.

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