RESEARCH ARTICLE

Glutaraldehyde-Mediated Synthesis of Asparaginase-Bound Maghemite Nanocomposites: Cytotoxicity against Human Colon Adenocarcinoma Cells

G Baskar*, Garrick Bikku George

Abstract

Drugs processed using nanobiotechnology may be more biocompatible, with sustainable and stabilised release or action. L-asparaginase produced from fungi has many advantages for treatment of lymphocytic leukemia with lesser side effect. In the present work, maghemite nanobiocomposites of fungal asparaginase were produced using glutaraldehyde-pretreated colloidal magnetic nanoparticles. Formation of nanobiocomposites was observed using laser light scattering and confirmed by UV-visible spectrophotometry with the absorption peak at 497 nm. The specific asparaginase activity was increased from 320 U/mg with crude asparaginase to 481.5 U/mg. FTIR analysis confirmed that primary amines are the functional groups involved in binding of asparaginase on magnetic nanoparticles. The average size of the produced nanobiocomposite was found in the range of 30 nm to 40 nm using histogram analysis. The magnetic nanobiocomposite of asparaginase synthesised using glutaraldehyde showed 90.75% cytotoxicity against human colon adenocarcinoma cell lines. Hence it can be used as an active anticancer drug with an augmented level of bioavailability.

Keywords: Nanobiocomposite - asparaginase - magnetic nanoparticle - adenocarcinoma cells

Asian Pac J Cancer Prev, **17 (9)**, 4237-4240

Introduction

Nanotechnology has been taking predominant steps in all the scientific background that focuses mainly in bringing out the solution for environmental and medicinal problems (Williams et al., 2008). The metallic nanoparticles copper, iron, gold and silver have been used as efficient carriers for imaging and medicinal diagnostics (Kaushik et al., 2010). Nanotechnology plays a major role in the health care system. Nano drugs have superior qualities such as increased aqueous solubility of the drug, protects the drugs from the degradation, prolonged release of the drug thereby increasing bioavailability of the drug and appropriate form in all routes was well administered (Suphiya et al., 2012; Shiho et al., 2013).

Passive targeted drug delivery provides enhanced permeability as the nanoparticles move into the porous cancerous cells (Matsumura et al., 1986; Maeda et al., 2000). The N-2-hydroxypropyl methyacrylamide given as an interveinal drug to EL-4 lymphoma cells of a mouse has produced cent percent success on treatment (Chytil et al., 2008). An oligonucleotide sequence called aptamers bound with paclitaxel-polylactide composite enhanced the process of cancer treatment (Tong et al., 2010). Polysorbate 80 coated poly-n-butyl cyanopolyacyrlate nanoparticles bound with doxorubicin selectively adsorbs to the plasma proteins, apoproteins E and B or A-I increases the porosity and releases the drug by receptor mediated endocytosis. They produced effective results when tested on glioblastoma bearing rats. This efficiency was further enhanced when it was replaced by poly-lactide-co-glycolide coated with polysorbate or poloaxmer 188 with doxorubicin (Steiniger et al., 2004; Michaelis et al., 2006; Wohlfart et al., 2011).

Gold Nanoparticles was synthesized by the presence of generation 4-polyamidoamine dendrimer was bound to jacalin. This has showed significant anticancer activity in breast, bladder and prostate cancer (Valeria et al., 2013). Gold coated silica nanoparticle providing rapid damage to the cancer cells with minimal damage to normal surrounding cells (Suphiya et al., 2012). Graphite encapsulated with alloys of iron with cobalt or gold with iron have higher magnetic properties when compared with iron oxide nanoparticles. These nanoparticles can be used for better imaging of cancer and treatment of cancer cells (Robert et al., 2013).

Asparaginase converts the free circulating asparagine in blood into aspartic acid and ammonia. Asparaginase can be effectively used for the treatment of the cancer. The fungus Aspergillus terreus was reported as a potential for asparaginase production (Baskar and Renganathan, 2011). Thus the present work was focused on developing

*Department of Biotechnology, St. Joseph's College of Engineering, Chennai, India *For correspondence: basg2004@gmail.com*

magnetic nanocomposite of fungal asparaginase from *Aspergillus terreus* as its anticancer activity against and testing their therapeutic efficacy against human colon adenocarcinoma (HT-29) cell lines.

Materials and Methods

Microorganisms used

The fungus Aspergillus terreus MTCC 1782 was obtained from IMTECH Chandigargh, India. Aspergillus terreus was cultivated in modified Czapek agar slants at 35-37°C for 4 days and used as inoculum for the synthesis of asparaginase.

Reagents and cancer cell lines used

The ferric chloride and ferrous Sulphate were purchased from CHEMSPURE, India. The glutaraldehyde was purchased from SD FINE CHEM Ltd., Mumbai, India. The agar used in Czapek Dox was purchased from HIMEDIA Pvt. Ltd., Mumbai, India. The amino acid asparagine was obtained from LOBA CHEMIE Pvt. Ltd., Mumbai and proline was purchased from QUALIGENS, Mumbai, India. The other laboratory reagents used are sourced from SISCO RESEARCH LABORATORIES Pvt. Ltd., Mumbai, India. The HT-29 cell lines were obtained from Veterinary College, Chennai. The cells were maintained in minimal essential medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO2 at 37°C.

Fungal synthesis of asparaginase

Aspergillus terreus is inoculated in a 500 ml Erlenmeyer flask containing 200 ml of modified Czapek-Dox liquid medium containing (g/100ml of distilled): 2.0 L-Proline, 1.0 L-Asparagine, 0.2 glucose, 1.0 sodium nitrate, 0.052 potassium chloride, 0.152 Di-potassium hydrogen phosphate, 0.001 zinc sulphate, 0.001 copper sulphate, 0.001 ferrous sulphate and 0.052 magnesium sulphate, maintained at pH 6.2. The fungus was grown aerobically by agitating in an orbital shaker at 160 RPM at 32°C for 4 days. After the incubation period the culture was filtered under vacuum through Whatman #2 filter paper which is rich with L-asparaginase (Baskar and Renganathan, 2012).

Chemical synthesis of maghemite nanoparticles and maghemite nanobiocomposite of asparaginase

Maghemite nanoparticles were prepared by co-precipitation method. The solution of 0.2 M ferric chloride and 0.1 M ferrous sulphate are mixed together and stirred continuously. Then 10% (w/v) NaOH was added drop wise to the mixture continuously. The brown precipitate obtained was washed with de-ionized water, separated by magnetic decantation and re-suspended. Maghemite nanofluid of 100 ml of was mixed with 1% glutaraldehyde for 2 hr at 30°C and then mixed with 50 ml of asparaginase filtrate for 30 min at 30°C. Then it was centrifuged at 4°C and 10,000 rpm to separate the maghemite nanobiocomposites, freeze dried and stored at 4°C for further characterization.

Estimation of L-asparaginase activity

The tube containing of 0.1ml crude enzyme/ nanobiocomposite was added with 0.9ml of 0.1M phosphate buffer along with 1ml of 0.04M of L-asparagine. This mixture is incubated at 37°C for 10 min. Later the reaction was stopped by adding 0.5 ml of 15% Trichloroacetic acid, after this thorough mixing, it was centrifuged at 6000 rpm for 10 min at 4°C.Then 0.1ml of supernatant was taken in a separate tube made up to 8ml, along with this 1ml of 2 M NaOH and 1ml of Nessler's reagent was added. This mixture was incubated for 10 min at room temperature and absorbance was measured at 480 nm (Wriston and Yellin, 1973).

Estimation of protein by Bradford's method

Bradford method is based on interaction of dye, Coomassie Brilliant Blue. Each samples consisting 20µl was mixed with 180µl of distilled water along with 2 ml of Bradford's reagent separately. The mixture was incubated for 10 min at 37°C in an incubator and absorbance was measured at 595 nm (Bradford, 1974).

Characterization of synthesized maghemite nanobiocomposite of asparaginase

The optical property of the nanobiocomposite was investigated by SYSTRONICS Double Beam UV-Visible spectrophotometer 2201 by obtaining spectrum values from 300 to 800 nm. The Fourier Transform Infra-red (FT-IR) spectroscopy is used to analyze the functional groups present in the nanobiocomposites using BRUKER α-T FT-IR Spectrometer. Sample pellets pellets were prepared by gently mixing 1mg of the lyophilized sample with 100mg of KBr. These discs were scanned from 400 to 4000 cm^{-1} to obtain the FT-IR spectra. The particle size distribution of nanocomposite was analyzed. The X-ray diffraction (XRD) analysis was used to determine the crystallinity, and metallic nature and cubic structure of magnetic iron nanoparticles. The sample was prepared by centrifugation of the nanobiocomposite solution at 10000 rpm for 10 min. The supernatant was discarded, and the pellet was lyophilized for XRD analysis.

Cytotoxicity of maghemite nanobiocomposite against human colon adenocarcinoma cells

Cells (1×10^5/well) were plated in 24-well plates and incubated in 37°C with 5% CO_2 condition. After the cell reaches the confluence, the various concentrations of the maghemite nanobiocomposite of asparaginase was added and incubated for 24 hr. Then the samples were removed from the well and washed with phosphate-buffered saline (pH 7.4). 100 µl/well (5 mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hr. After incubation, 1 ml of DMSO was added into all the wells .The absorbance at 570 nm was measured with UV- Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC50) was determined graphically. The cell viability was calculated using the following formula (% cell viability = A570 of treated cells / A570 of control cells × 100). Graphs are plotted using the % of Cell Viability at Y-axis

and concentration of maghemite nanobiocomposite of asparaginase in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments (Mosmann, 1983).

Results and Discussion

Activity of maghemite nanobiocomposite of asparaginase

The maghemite nanoparticles obtained were very fine and brown colour in nature. It was observed that the magnetic nanofluid is consisting of fine maghemite nanoparticles. The intensity of the colour was increased to dark brown on the addition of glutaraldehyde. The formation of magnetic nanobiocomposite of asparaginase was observed using laser light scattering method and confirmed using UV-Visible Spectrophotometer with the absorption peak at 497.6 nm. The specific asparaginase activity was increased from 320 U/mg of crude asparaginase to 481.53U/mg of maghemite nanobiocomposite of asparaginase prepared by glutaraldehyde functionalizing of maghemite nanoparticles. The presence of asparaginase on the synthesized maghemite nanobiocomposite was confirmed. Thus the synthesized maghemite nanobiocomposite found to have good asparaginase activity. Hence it can be used as potential anticancer drug.

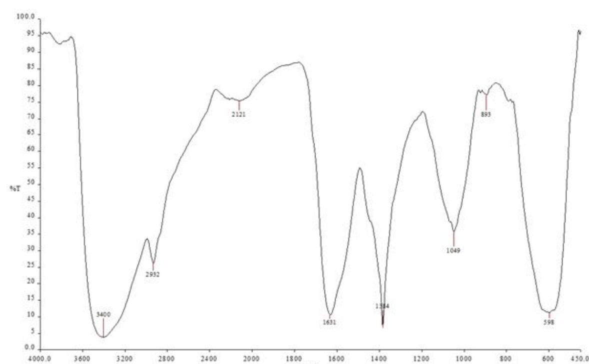

Figure 1. FTIR Analysis of Maghemite Nanobiocomposite of Asparaginase

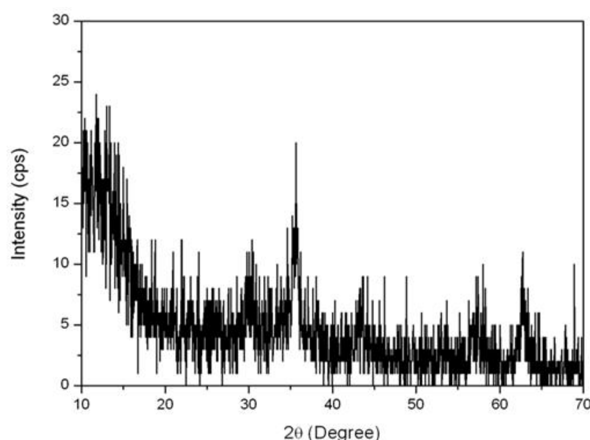

Figure 2. XRD of Maghemite Nanobiocomposite

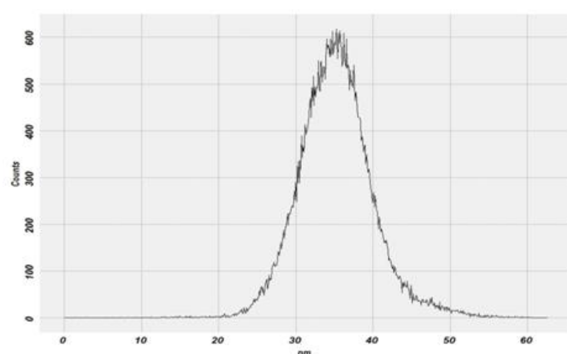

Figure 3. Histogram Analysis of Maghemite Nanobiocomposite

FT-IR Analysis of maghemite nanobiocomposite of asparaginase

The interaction between asparaginase and maghemite nanoparticles were analysed by Fourier Transform Infrared (FTIR) spectroscopy. The spectra represented an average of 50 scans, recorded from 400 to 4000 cm^{-1} with a resolution of $4cm^{-1}$ (Figure 1). There were eight peaks observed. The amino groups observed peak 3400 cm^{-1} and 1631 cm^{-1} showed the strong NH stretching in $–NH_2$ in aromatic amines, primary amines and amides. The presence of strong carbon and hydrocarbons ($–CH_3$ and $–CH_2$ in aliphatic) were observed at $2932cm^{-1}$. The functional groups at this peak indicate that they have CH anti-symmetrical and symmetrical stretching mode of vibration. The N=C isonitriles are found in the peak 2121 cm^{-1}(N=C stretching). The peak at1631 cm^{-1} showed C=O and NH_2 in primary amides. This indicated two bands from C=O stretching and NH_2 deformation. The 1384 cm^{-1} showed SO_2 in sulfonyl chloride where the functional group SO_2 possed and the peak at 1049 cm^{-1} showed SO_3H in sulfonic acid having SO_3 symmetrical stretching mode of vibration. The peak 893 cm^{-1} showed N-C=O in amides were observed. The peak at 598 cm^{-1} showed other smaller miscellaneous groups like silicone, fluorine and chlorine/bromine. The strong NH group indicates the presence of asparaginase maghemite nanobiocomposite.

XRD analysis of maghemite nanobiocomposite

The XRD pattern (Figure 2) of the maghemite nanoparticle bound asparaginase by glutaraldehyde pretreatment showed peaks at 18.4, 30.40, 35.64, 43.68, 53.48, 56.8 and 62.76 2θ having (220), (311), (400), (422), (511) and (400), JCPDS file No. 19-062990. Thus the crystalline structure of the maghemite nanocomposite was confirmed. The nanocomposite showed cubic structure.

Particle size analysis of maghemite nanobiocomposite

The histogram analysis is used to determine the size of the nanobiocomposite. It was observed histogram analysis that the magnetic nanobiocomposite was distributed in the range of 30 to 40 nm (see Figure 3).

Cell viability of human colon adenocarcinoma cells using MTT assay

The maghemite nanobiocomposite of asparaginase was tested for its anticancer activity on HT-29 (Human colon adenocarcinoma) cells. The concentration of nanobiocomposite was taken in eight different levels at 1000, 500, 250, 125, 62.5, 31.2, 15.6, and 7.8 μg/ml. The cancer cell viability at different concentration of nanobiocomposite using MTT assay is shown in Figure 4. The cell viability was 9.25%, 20.37%, 29.62%, 35.18%,

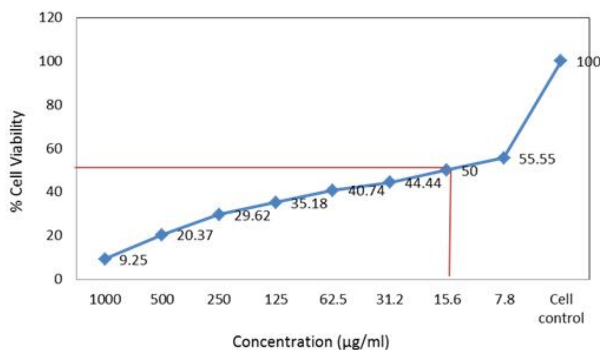

Figure 4. MTT Assay of Maghemite Nanobiocomposite of Asparaginase on HT-29 Cell Line

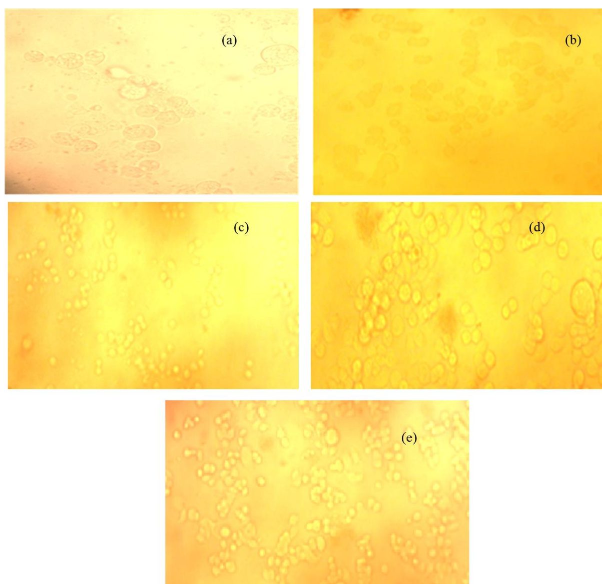

Figure 5. Cytotoxic Effects of Various Concentrations of MGA on HT-29 Cancer Cell Line. (a)Toxicity-1000 μg/ml (b) Toxicity-125μg/ml (c) Toxicity-62.5μg/ml (d) Toxicity-31.2μg/ml (e) Normal HT-29 Cell line

40.74%, 44.44%, 50% and 55.55% for 1000, 500, 250, 125, 62.5, 31.2,15.6 and 7.8 μg/ml of nanobiocomposite respectively. The anticancer effect of nanobiocomposite on HT-29 cell line is shown in Figure 5. The decrease in number of viable cancer cells was observed for increase nanobiocomposite concentration. Only very few viable cancer cells were observed at the concentration of 1000μg/ml of nanobiocomposite, while the 15.6 μg/ml concentration comparatively showed increased number of viable cancer cells. There was a gradual decrease in the cell viability on increase in the concentration of the drug showed stable cytotoxic effect of the maghemite nanobiocomposite of asparaginase.

Conclusions

Fungal asparaginase was bound to maghemite nanoparticles by glutaraldehyde pretreatment method. The peaks in the UV spectroscopy indicated the presence of gamma iron oxide in maghemite nanobiocomposite of asparaginase. The FT-IR peaks elucidated the involved of NH group in binding of asparaginase on maghemite nanopartilcle. The XRD showed cubical crystalline structure of the nanocompositeand it is partial crystalline due to the presence of asparaginase. The in-vitro assay displays the strength of the synthesized nanocomposite of asparaginase drug against HT-29 cancer cell lines.

References

Baskar G, Renganathan S (2011). Optimization of media components and operating conditions for exogenous production of fungal L-asparaginase. *Chiang Mai J Sci*, **38**, 270-79.

Baskar G, Renganathan S (2012). Optimization of L-asparaginase production by Aspergillus terreus MTCC 1782 using response surface methodology and artificial neural network linked genetic algorithm. *Asia-Pac J Chem Eng*, **7**, 212-20.

Bradford M (1974). A rapid and sensitive method method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**, 248-54.

Chytil P, Etrych T, Konak C, et al (2008). New HPMA copolymer-based drug carriers with covalently bound hydrophobic substituents for solid tumour targeting. *J Control Release*, **127**, 121-30.

Kaushik NT, Snehit SM, Rasesh YP (2010). Biological synthesis of metallic nanoparticles. *Nanomedicine*, **6**, 257-262.

Maeda H, Wu J, Sawa T, et al (2000). Tumor vascular permeability and the EPR effect in macromolecular therapeutics: A review. *J Control Release*, **65**, 271-84.

Marangoni VS, Paino IM, Zucolotto V (2013). Synthesis and characterization of jacalin-goldnanoparticles conjugates as specific markers for cancer cells. *Colloids Surf B: Biointerfaces*, **112**, 380-86.

Matsumura Y, Maeda H (1986). A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res*, **46**, 6387-92.

Michaelis K, Hoffmann MM, Dreis S, et al (2006). Covalent linkage of apolipoprotein e to albumin Nanoparticles strongly enhances drug transport into the brain. *J Pharmacol Exp Ther*, **317**, 1246-53.

Mosmann, T (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods*, **65**, 55-63.

Parveen S, Misra R, Sahoo SK (2012). Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. *Nanomedicine*, **8**, 147-66.

Sinclair R, Li H, Madsen S, Dai H (2013). HREM analysis of graphite-encapsulated metallic nanoparticles for possible medical applications. *Ultramicroscopy*, **134**, 167-74.

Steiniger SC, Kreuter J, Khalansky AS, et al (2004). Chemotherapy of glioblastomain rats using doxorubicin-loaded nanoparticles. *Int J Cancer*, **109**, 759-67.

Tokonami S, Yamamoto Y, Shiigi H, et al (2012). Synthesis and bioanalytical applications of specific-shaped metallic nanostructures: A review. *Anal Chim Acta*, **716**, 76-91.

Tong R, Yala L, Fan TM, et al (2010). The formulation of aptamer-coated paclitaxel-polylactide nanoconjugates and their targeting to cancer cells. *Biomaterials*, **31**, 3043-53.

Wohlfart S, Khalansky AS, Gelperina S, et al (2011). Efficient chemotherapy of rat glioblastoma using doxorubicin-loaded PLGA nanoparticles with different stabilizers. *PLOS One*, **6**, 19121.

Wriston JC Jr, Yellin TO. (1973). L-asparaginase: A review. *Adv Enzymol Relat Areas Mol Biol*, **39**, 185- 248.

Yu BY, Kwak SY (2010). Assembly of magnetite nanoparticles into spherical mesoporous aggregates with a 3-D wormhole-like porous structure. *J Mater Chem*, **20**, 8320-28.