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FORMULATION AND EVALUATION OF METHOTREXATE IN NANO BASED DRUG DELIVERY SYSTEM

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ABSTRACT

In the present study, the main problem of cancer chemotherapy is the severe toxic side effects of anticancer drugs on healthy tissues that impose dose reduction, treatment delay along with health risks like hair loss, nausea and a major damage to the patient‘s immune system. The cancer drugs that are recommended in the second-line cancer chemotherapy, methotrexate (MTX) is considered as one of the most versatile drugs used in several types of cancer e.g. breast, head and neck and osteosarcoma along with rheumatoid arthritis, for delivery of MTX by parenteral or oral route is the poor bioavailability due to the limited absorption of the drug. In the current study ceramic-based nanoparticles layered double hydroxides (LDH) was synthesized by simple laboratory based wet chemical method (coprecipitation) and a well-known anticancer agent methotrexate (MTX) was intercalated by ex-situation exchange techniques to develop drug intercalated ceramic nanohybrid, abbreviated as LDH-MTX. The LDH-MTX nanohybrid was encapsulated with an approved biodegradable and biocompatible polymer poly (lactic-coglycolic acid) (PLGA) by double emulsion-solvent evaporation(W1/O/W2) techniques as well and was optimized by keeping some of the process parameters constant, while other process and formulation parameters e.g. the homogenization speed, concentration of polymer (PLGA), LDH-MTX and surfactants, aqueous and organic phase volume involved in the synthesis of the PLGA-LDH-MTX nanohybrids, were varied and evaluated to obtain the desired particle size range ~200nm and drug entrapment efficiency for specific use, this could be beneficial to realize the passive tumor-targeted drug delivery through enhanced permeability and retention (EPR) effects. The optimized drug-containing nanohybrids xxiv (PLGA-LDH-MTX) showed a sustained release profile till 240h (10days).

KEYWORDS

Anticancer drugs, LDH, PLGA, LDH-MTX and PLGA-LDH-MTX.

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INTRODUCTION

Cancer is a major health concern and the second common cause of death worldwide. The epidemiologic factors of cancer are multifold and can be extended to familial, genetic, racial, geographic, environmental, cultural, thereby spanning to age and gender specifically, that are predisposing in nature.

NANOPARTICLES

The prefix "nano" has found in last decade an ever-increasing application to different fields of knowledge. Nanoscience, nanotechnology, nanomaterials or nanochemistry are only a few of the new nano-containing terms that occur frequently in scientific reports, in popular books as well as in newspapers and that have become familiar to a wide public, even of non-experts. The prefix comes from the ancient Greek νᾶνος through the Latin nanus meaning literally dwarf and, by extension, very small. Within the convention of International System of Units (SI) it is used to indicate a reduction factor of 109 times.

Advantages of nanoparticle-based drug delivery system

Ability to improve bioavailability of cargo drug molecule by enhancing aqueous solubility of hydrophobic drugs, rendering them suitable for administration in intravenous route (Linhardt, 1989).

Act as safeguard to the cargo drug/other biomolecules, e.g., proteins, oligo/polynucleotide's (RNA/DNA) from the biological milieu (e.g., pH, temperature, enzymatic degradation etc) and thus, prevent hydrolytic degradation thereby enhancing their physical and chemical stability altogether (Haixiong and Ge, 2002).

Lead to controlled release of the drug during the transportation and at the site of action, thereby altering its biodistribution and decrease renal elimination/clearance of drug to achieve an enhanced biological half-life and therapeutic efficacy and reduction in side effects (Brigger *et al*, 2002; Couvreur *et al*, 1995).

Nanoparticles provide improved cell entry and extravasation of cargo drug molecules by endocytosis, phagocytosis mechanism (Haixiong and Ge, 2002).

A large surface area to a volume ratio provides a surface for chemical modification/functionalization by conjugation of selective targeting ligands/antibodies that allows for active targeting of the encapsulated drug directly in the diseased cell, without affecting the surrounding healthy cells, thereby largely enhancing its bioavailability (Lockman *et al*, 2002)[1].

DRUG INTERACTIONS

Methotrexate is an anticancer drug belonging to the category of dihydrofolic acid reductase inhibitor has been used clinically since 1953 for the treatment of many tumours including lymphoblastic leukaemia (ALL), choriocarcinoma, osteosarcoma, non-Hodgkin's lymphoma, Hodgkin's disease, head and neck, lung, metastatic choriocarcinoma and breast cancer (Genestier *et al*, 2000, Seo *et al*, 2009, Padmanabhan *et al*, 2009). Now a day it is a popular choice of drug for treatment of rheumatoid arthritis (RA) used as disease-modifying anti- rheumatic drug (DMARD) in low dose (Weinblatt *et al*, 1885)[2].

AIM OF THE WORK

The purpose of this study was to synthesize layered double hydroxide (LDH) (ceramic nanoparticles) by co precipitation method and to carry out structural, compositional characterization, to determine the phase, crystal size and other details of the crystallinity of the nanoparticles, electron microscopy (TEM, SEM/FESEM) to study the bulk crystallinity and phase in detail, TG-DTA/ DSC, and to determine the thermal stability of the phase, dynamic light scattering technique (DLS) to determine the particle size distribution, study of the surface charge distribution (zeta potential) to understand the electrokinetic stability of the particles in polar medium.

Objectives of the work

To synthesize the ceramic Nano particles

To encapsulate the drug loaded ceramic Nano particles.

To evaluate the methotrexate ceramic Nano particles.

Drug profile

Methotrexate

SYNONYM

Amethopterin, α-methopterin

IUPAC Name

N-[4-[[(2, 4-diamino-6-pteridinyl) methyl] methyl-amino] benzoyl]-L- glutamic acid.

Polymer profile

PLGA (Poly D, L-lactide-co-glycolide)

Characteristics

Biocompatible, biodegradable, non-toxic, anti-microbial and soluble in a wide range of solvents.

Solubility

Soluble in a variety of organic solvents including acetone, dichloromethane, chloroform

It carriers for both hydrophobic and hydrophilic drugs

PLGA is insoluble in neutral or alkaline media, hence it should be first dissolved in aqueous solution.

MATERIAL AND METHODS

Synthesis of pristine layered double hydroxide (LDH) was prepared by conventional coprecipitation method reported earlier by Choi *et al*. (Choi *et al*, 2004). A mixed metal solution was prepared by adding 500ml of decarbonated water to Mg(NO3)2.6H2O (32 milimol) and Al(NO3)3.9H2O (16 milimol) maintained at 25°C with constant stirring under a stream of nitrogen (XL grade, % purity, 99.999) gas. A basic solution of 0.5 M of NaOH (150ml) was added drop by drop to mixed metal solution and the pH of the resulting mixed metal solution was adjusted to 11.3 to get pristine LDH sus nitrogen gas blanket. The resulting LDH product was centrifuged (Heal Force, Neofuge 15R, China) at 10108g RCF for 5 min at 4°C to separate the precipitate. The precipitate was washed three times with decarbonated water, freeze dried (EYEL4, FDU2200, Japan, at -82°C and 20 Pa pressure) to get fine, free flowing nanocrystalline pristine LDH, denoted as sample A.

Optimized method for synthesis of PLGA-LDH-MTX nanohybrids by double emulsion (W1/O/W2)-solvent evaporation method

In this method, LDH-MTX (sample B) in lyophilized powder form (equivalent to 100mg of MTX present in LDH-MTX, loading calculated by HPLC analysis) was added to 5ml of decarbonated water and sonicated for 5 min to get a homogeneous suspension of LDH-MTX nanohybrids in aqueous medium. The suspension was then added drop wise through a 16 gauge needle syringe at a flow rate of 3ml /min (approximately) in 10ml of organic phase (DCM, Dichloromethane) containing a (200mg) weighed amount of PLGA (LDH-MTX: PLGA in 1:2 ratio by weight) and span 80 as surfactant (span 80, 0.5% w/v) under high speed homogenization at speed of 12,000rpm (IKA T25 ULTRA-TURRAX®, Germany) for 5 min, to get a primary emulsion (W1/O). The resulting water-in-oil (W1/O) emulsion was further added drop wise into 20ml of an aqueous solution of 2% (w/v) tween 80 (W2) used as an emulsion stabilizer to minimize coalescence of the emulsion and aggregation of the particles formed. The mixture was further homogenized for 5 min @ 12,500rpm to obtain a double W1/O/W2 emulsion.

Optimized method for synthesis of PLGA-MTX nanoparticles by single emulsion (O/W) -solvent evaporation method

Briefly, 500mg of PLGA and 250mg MTX (2:1 by weight) were dissolved in 10ml of acetone, and then added drop wise through a 16-gauge needle syringe to 15ml of a 2% (w/v) poly (vinyl alcohol) aqueous solution. The emulsion thus formed was homogenized (@ 12,000rpm) using a high-speed homogenizer for 5 min and was stirred for a period of 12 h for complete evaporation of the solvent, acetone, to precipitate the PLGA encapsulated MTX nanoparticles . The resulting sample was collected by centrifugation at 8519g RCF for 10 min and was washed several times with decarbonated water to remove the residual nonionic surfactant, PVA. Finally, the particles were resuspended in a cryoprotectant (1% w/v mannitol solution) and freeze dried at-82ºC with a vacuum pressure of 20 Pa. Each sample was prepared in triplicate to check reproducibility of the process (Musmade *et al*, 2014. Herrero-Vanell *et al*, 2000). The methods elaborated as above are optimized, w.r.t all the formulation and processing parameters.

Although PVA is a widely used polymeric surfactant in the external aqueous phase as an

emulsifier, the safety of PVA still appears to be a concern from the previous literature reports (Wangl, 2009, Liu *et al*, 2007[3]). Following repeated subcutaneous or intravenous administrations of PVA, various organ lesions and hypertension have been reported in rats, central nervous system depression and anemia followed by renal damage have also been reported in beagle dogs. Therefore, residual PVA present, if any, needs to be estimated and then removed by washing procedures such as repetitive centrifugation or filtration. In the present case, the residual amount of the surfactant PVA was determined by colorimetric method, resulting in formation of a coloured complex. In brief, 5mg of lyophilized powder of PLGA-MTX nanoparticles was taken and treated with 5ml of 0.5M sodium hydroxide solution for 15 min at 60ºC. Then the sample was neutralized with 2ml of 1N HCl and finally, the volume was made up to 10ml with distilled water.

RESULTS AND DISCUSSION

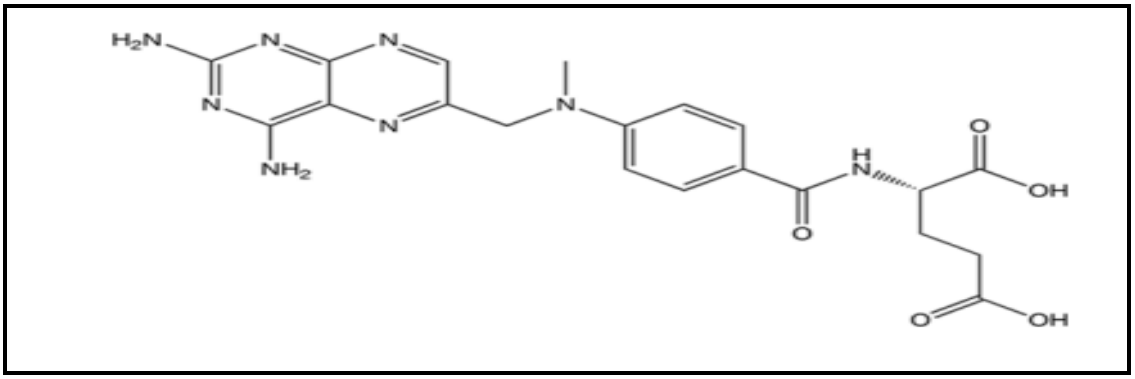
Powder X-Ray Diffraction of Sample A and B´

This is due to a stronger interaction between the anionic carboxylate groups of MTX and cationic brucite layer. Hence, the drug molecule is tilted at an angular configuration of 36.01° (Chakraborty *et al*, 2011[4]) within the layered framework of the brucite and is held in place by a charge-based interaction between the anionic counterpart of the MTX drug and the cationic brucite layers.

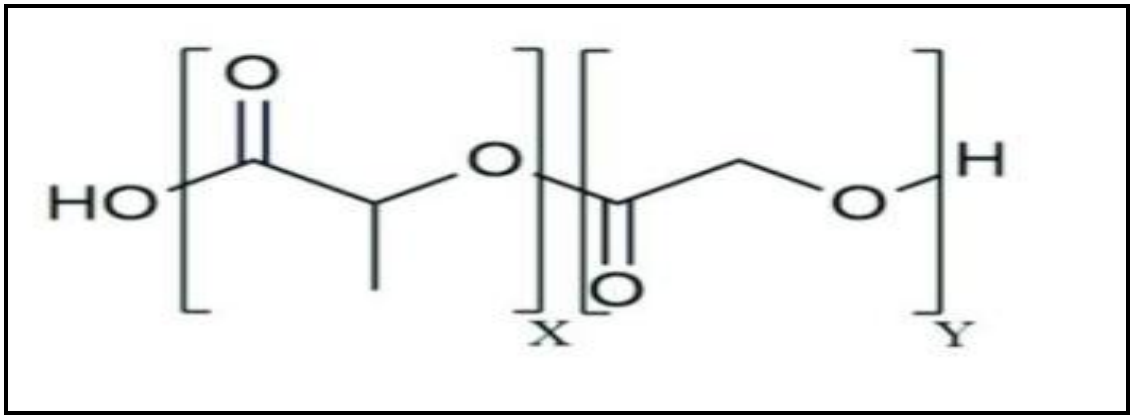
Field Emission Scanning Electron Microscopic (FESEM) Study Of Optimized PLGA-LDH-MTX Nanohybrids and PLGA-MTX Nanoparticles

FESEM micrographs of pristine LDH and LDH-MTX are shown in Figure No.8.3 which exhibits large clusters of dimension ~60nm of flaky LDH particles in the pristine sample. Incorporation of large drug molecule of MTX into interlayer space of LDH again resulted in exfoliation of the LDH layers, which in turn results in the increase in the average particle size to ~ 70nm to LDH-MTX (Sample B´). There was no significant change in particle morphology due to intercalation of MTX into interlayer space of LDH.

Molecular formula: C20H22N8O5



Structure



Synthesis of the pristine LDH

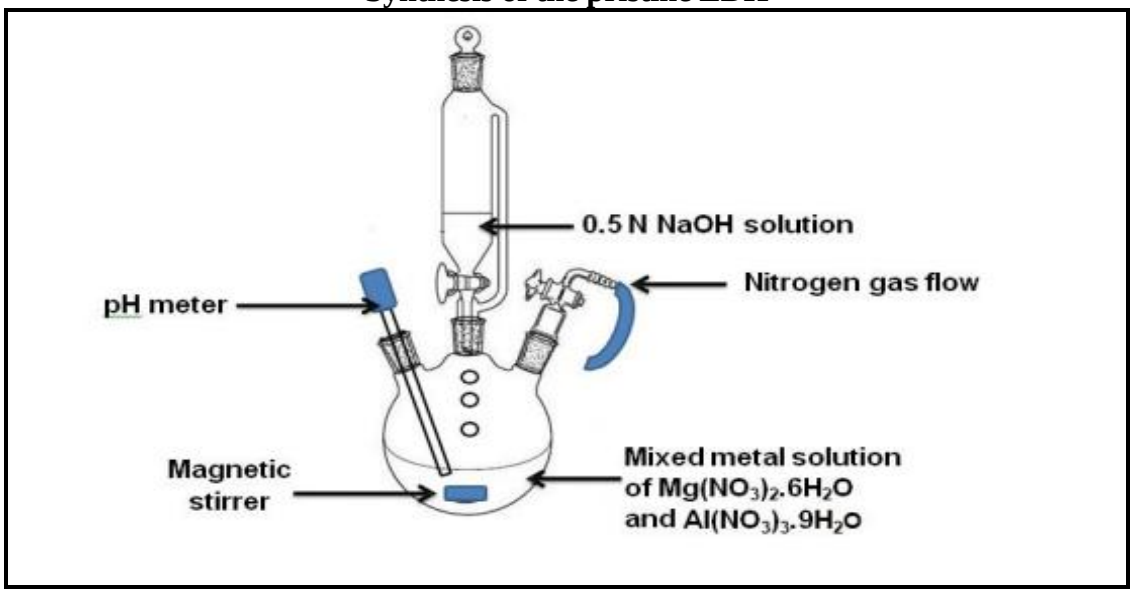


Figure No.1: Synthesis of layered double hydroxide

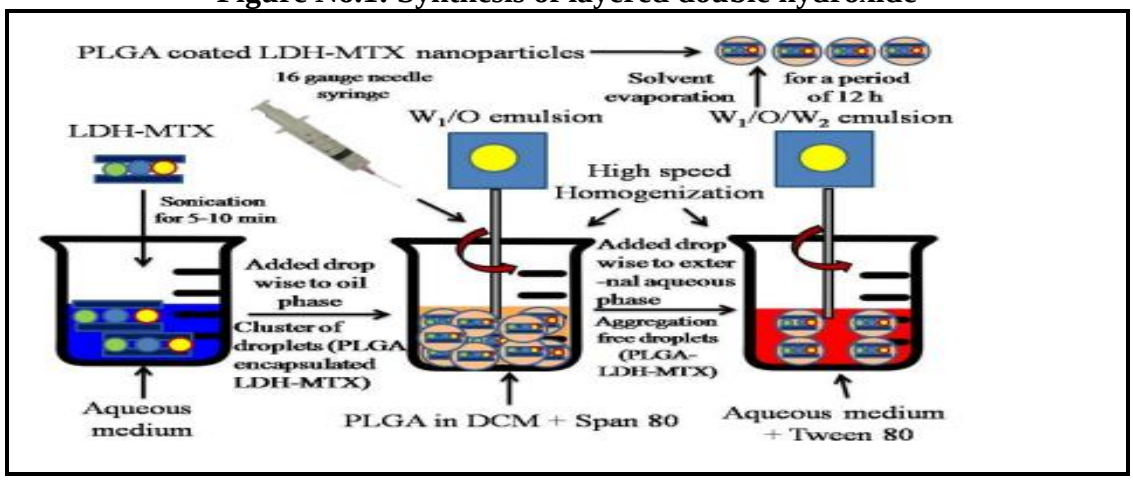


Figure No.2: Optimized technique for synthesis of PLGA-LDH-MTX nanohybrids by W1/O/W2 double emulsion- solvent evaporation method

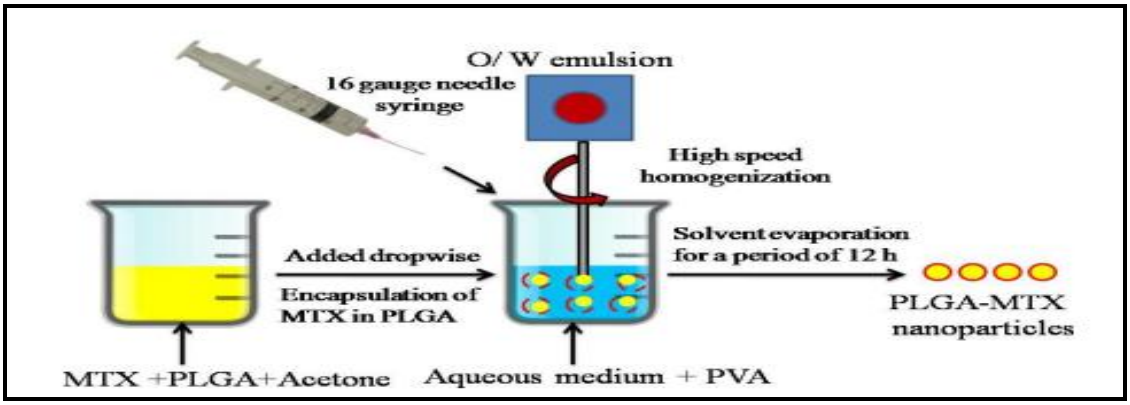


Figure No.3: Development of PLGA-MTX nanoparticles by O/W single emulsification- solvent evaporation method

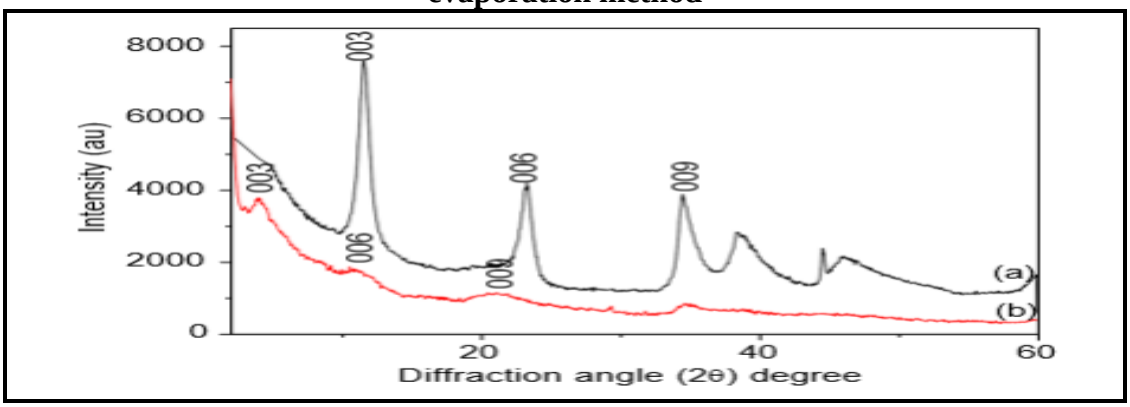


Figure No.4: Powder X-ray diffraction patterns of (a) pristine LDH (sample A) and (b) LDH– MTX (sample B´) nanohybrids

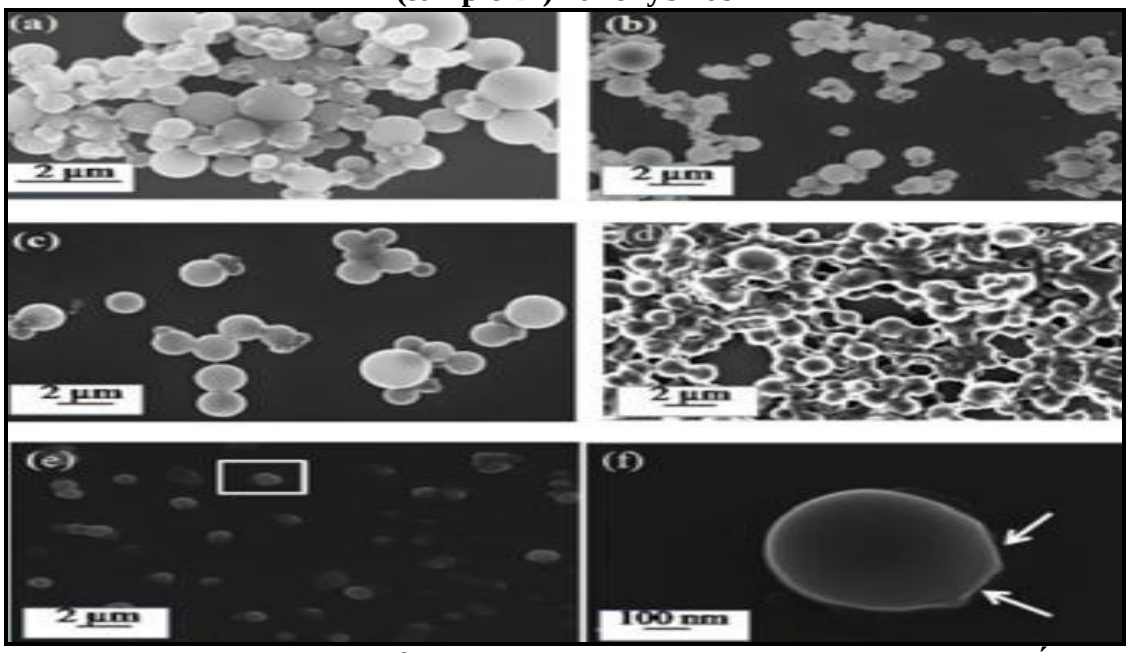


Figure No.5: FESEM micrographs of (a) pristine LDH (sample A) and (b) sample B´ (LDH-MTX) nanohybrids

DIFFERENTIAL SCANNING CALORIMETRY (DSC) OF SAMPLE A, B AND C

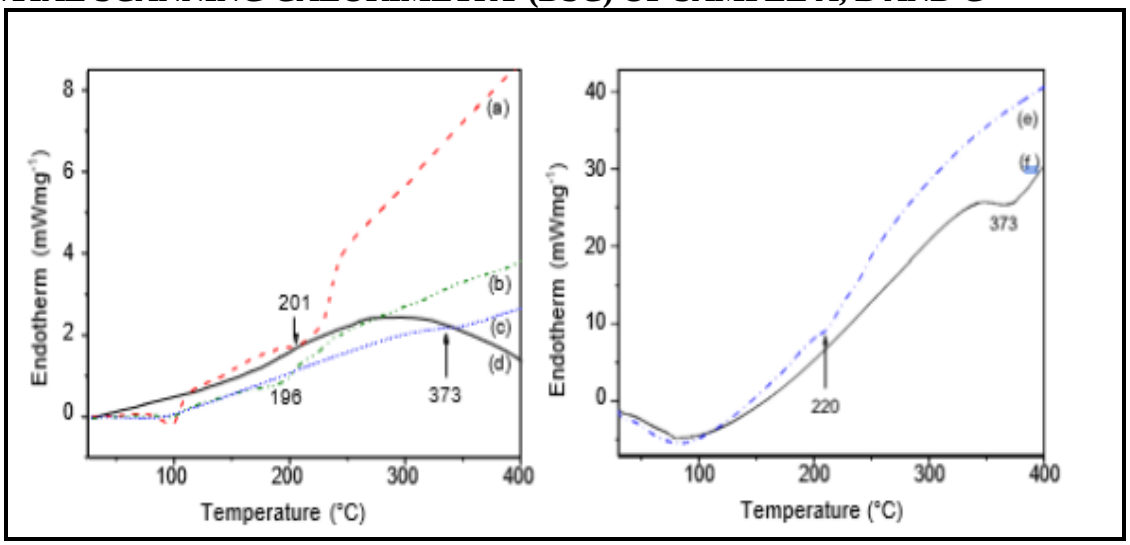


Figure No.6: Differential scanning calorimetry (DSC) of (a) MTX (API drug) (b) LDH: MTX physical mixture 1:1 ratio (c) LDH-MTX nanohybrids (sample B) (d) pristine LDH (sample A) (e) PLGA and (f) PLGA coated LDH-MTX nanohybrids (sample C). A medium intensity endotherm at 373°C corresponds decarboxylation during the spinellization process leading to nucleation of magnesium aluminate spinels and thereby transformation of hexagonal LDH moiety to oxide spinels (Chakraborty *et al*, 2013, Chakraborty *et al*, 2013). Further similar results also obtained from the TG-DSC data shown in panels (c) and (d) (arrow marked)

***In-vitro* Drug Release Study of Bare Drug MTX, LDH-MTX (Sample B), PLGA-LDH- MTX (Sample C) and PLGA-MTX (Sample D)**

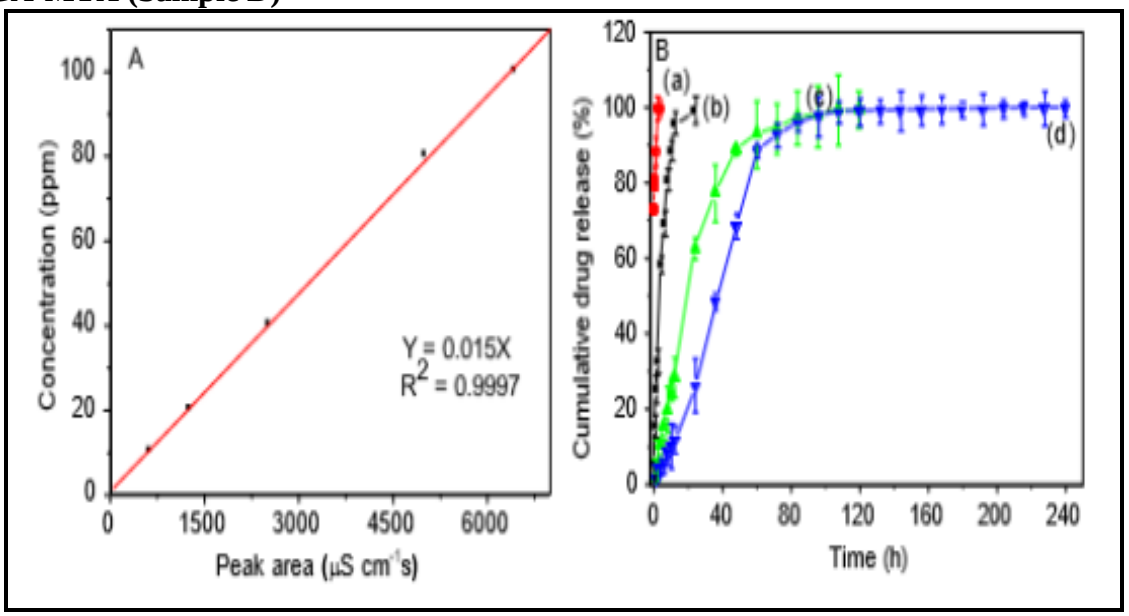


Figure No.7: (A) Calibration plot of methotrexate in phosphate buffer saline (PBS) at pH 7.4.(B) Cumulative release profiles of MTX (a) physical mixture (LDH: MTX in 1:1 ratio by weight) and (b) LDH-MTX nanohybrids (sample B) (c) PLGA-MTX nanoparticles (sample D) and (d) PLGA-LDH-MTX nanohybrids (sample C)

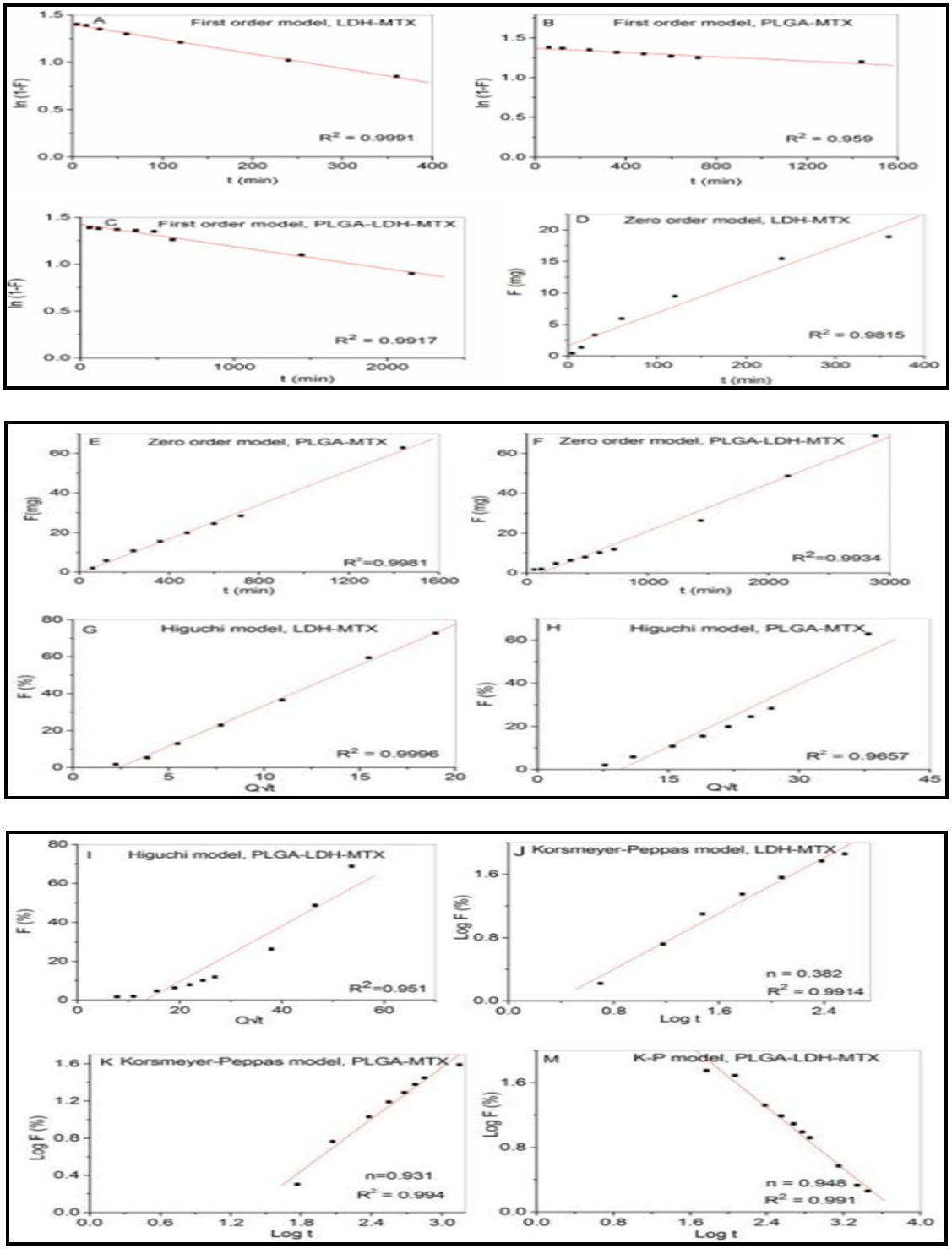


Figure No.8: (B) kinetic models and the fitting curves of release of MTX from the samples B (LDH-MTX), C (PLGA-LDH-MTX) and D (PLGA-MTX). The obtained *in vitro* release data as above were fitted in the different release kinetic models: Zero order, first order, Higuchi's square root plot and Korsmeyer-Peppas (Narashimhan *et al*, 1999, Peppas, 1985, Dash *et al*, 2010, Bhaskar *et al*, 1986) and the corresponding correlation coefficients and the kinetic exponents

Differential Thermal Analysis of Optimized PLGA-LDH-MTX and PLGA-MTX Nanoparticles

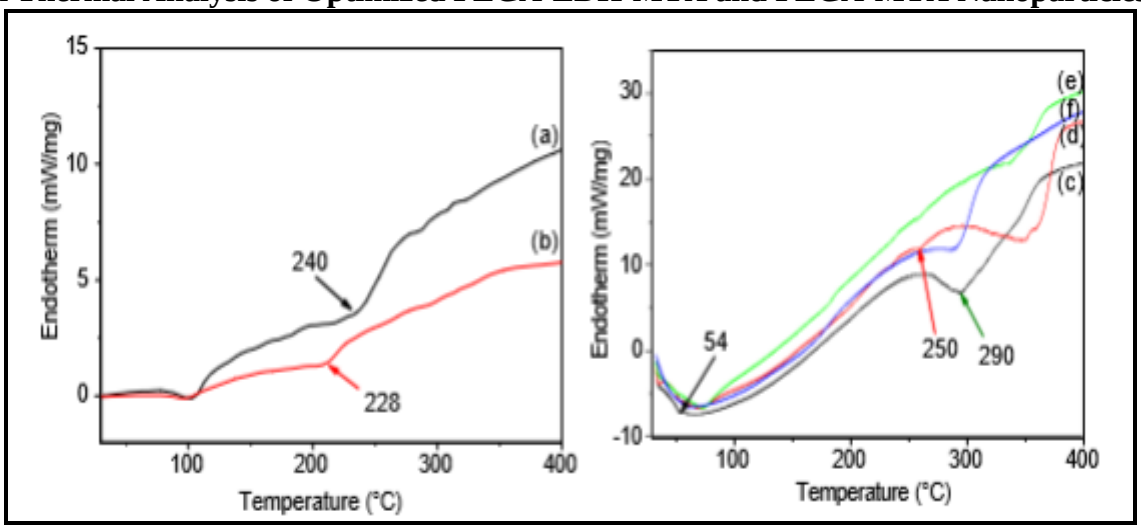


Figure No.9: DSC of (a) MTX (b) LDH-MTX (c) PLGA (d) PLGA-MTX physical mixture (e) PLGA-MTX nanoparticles and (f) PLGA-LDH-MTX nanohybrids

Table No.1: Correlation coefficients (R^2) of different models of release kinetics of sample B, C and D (LDH-MTX, PLGA-LDH-MTX nanohybrids and PLGA-MTX nanoparticles)

S.No	Formulation	Zero Order	First Order	Higuchi	Korsmeyer Peppas	N Values
1	LDH-MTX	0.9815	0.9991	0.9996	0.9914	0.382
2	PLGA-MTX	0.9981	0.9590	0.9657	0.9940	0.931
3	PLGA- LDH-MTX	0.9934	0.9917	0.9510	0.9910	0.948

Table No.2: Physiochemical properties of the unoptimized PLGA-MTX nanoparticles (L and H), PLGA-LDH-MTX nanohybrids (L and H) where the drug: Polymer ratio and other process parameters are constant and the homogenization speed only was varied (for unoptimized nanoparticles)

S.No	Un-Optimized Formulation	Drug: Polymer Ratio (w/w)	Homogenization speed (rpm)	Particle Size (nm)	Zeta Potential	% Yield	% DEE
1	PLGA-MTX	1:2	15000	112±1.79	-32.04	45.40±1.74	15.84±2.16
2	PLGA-MTX-H	1:2	5000	1865±1.90	-34.14	59.33±3.39	62.28±1.33
3	PLGA-LDH-MTX-L	1:2	15000	110±2.22	-33.76	51.33±6.14	4.39±4.36
4	PLGA-LDH-MTX-H	1:2	5000	1980±3.43	-34.33	63.88±1.09	89.96±4.36

SUMMARY AND CONCLUSION

Nanotechnology is an emerging field with the potential to revolutionize drug delivery. Advances in this area have allowed some nanomedicines in the market to achieve the desirable pharmacokinetic properties to reduce toxicity and improve patient compliance, as well as clinical outcomes.

The X-ray diffraction (XRD) spectra confirm the characteristic of the hydrotalcite-like phase comprising hexagonal lattice with rhombohedral space group with D3D symmetry, as evident from the symmetric reflections (00l) of (003) plane at 2θ= 10.21° for Mg-Al LDH. The d spacing corresponding to the (003) plane of the pristine Mg-Al LDH at 8.25 Å shifted considerably on intercalation of the methotrexate drug to 21.35Å confirming the successful encapsulation of the same within the LDH layers. Considering the thickness of the brucite layers to be 4.8 Å, the gallery height of the LDH-MTX nanohybrid is found to be 16.55 Å (21.35-4.8) Å. As it is known that the longitudinal molecular length of MTX is 21.2 Å; Hence, the drug molecule is tilted at an angular configuration.

The particle size distribution obtained by dynamic light scattering (DLS) technique exhibited a narrow range unimodal distribution of the size in the range 50-120nm for both LDH and LDHMTX nanohybrids. The D50 value in the range in between 60 - 80nm for pristine LDH and LDHMTX respectively with the polydispersity index (PDI) of 0.2 are indicative of a narrow distribution in the colloidal size range and is attributed to an effective charge neutralization of the precipitated nanohybrids at neutral pH condition of the medium.

Hence, it can be concluded that the biodegradable biocompatible polymer (PLGA) encapsulated anticancer drug loaded ceramic nanoparticles have the capability to release the drug MTX in a controlled manner till 240 h and has higher cell inhibition capability compared to the bare MTX drug. Apart from that, PLGA-LDH-MTX nanohybrid also shows a remarkable efficacy in terms of in vivo antitumor activity than bare MTX and PLGA-MTX on administration of the same dose based on MTX drug content, thereby reducing the possibility of side effects. Hence all the results in consolidation suggest that the newly developed PLGA-LDH-MTX nanohybrid drug delivery system, reported in the present work has great potential in anticancer application, with an enhanced *in vivo* therapeutic efficacy, when compared to bare MTX and has possibility to replace the existing chemotherapeutic agents and thereby revolutionize the entire drug delivery concept.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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