International Journal of Drug Delivery 6 (2014) 24-35
http://www.arjournals.org/index.php/ijdd/index

Original Research Article

ISSN: 0975-0215

Fabrication and characterization of etoposide loaded magnetic polymeric microparticles

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Abstract

The purpose of this study is to develop a targeted drug carrier system. Magnetic poly (-caprolactone) (PCL) microspheres were prepared using classical oil-in-water solvent evaporation method by loading magnetite nanoparticles and anticancer drug etoposide. The prepared magnetic microspheres were smooth, free flowing, individual and homogenous in nature. Fourier transformed infrared spectroscopy studies revealed the absence of any potential incompatibility of drug with other excipients. DSC studies were conducted to study the state of etoposide in the formulation. Further the magnetic microspheres were characterized for entrapment efficiency, drug loading, invitro release studies and subjected to particle size analysis and scanning electron microscopy. The magnetite nanoparticles were well dispersed in polymer matrix, which are responsible for magnetic response. The magnetic property of the prepared microspheres was measured by using vibrating sample magnetometer. The amount of magnetite in the formulation was estimated quantitatively by atomic absorption spectroscopy which was about 31.5%. The experimental results proved that the magnetic microspheres exhibited superparamagnetic behavior and the saturation magnetization was 7.26 emu/g. The optimized formulations exhibited a narrow size distribution which were below 10 μm and are evident from SEM analysis. Formulation batches prepared with drug/polymer ratio 1:10 showed a maximum encapsulation efficiency and the invitro release profile in phosphate buffer (pH 7.4) solution showed an extended release of etoposide up to 76.25% at the end of 21 day. Histopathological studies proved that the etoposide loaded magnetic microspheres were nontoxic and safe.

Keywords: poly (-caprolactone), solvent evaporation, superparamagnetic, saturation magnetization

Introduction

The rationale behind the development of polymeric controlled delivery systems is to make a therapeutic agent do its best when administered into the body. These systems are one of the most attractive areas in drug delivery and drug targeting [1,2]. Despite several advantages offered by controlled drug release, a major problem associated with all these systems so far developed is uniform biodistribution throughout the body, lack of drug specificity, requirement of excess dose to achieve high local concentration, non-specific toxicity and adverse side effects. Hence the development of drug system that deliver the drug molecules to the tumor site, without a concurrent increase in its concentration near healthy cells of the tissues, is one of the most active area of investigation in cancer research [3].

Among all the existing targeting drug delivery systems, a promising one will be the system associated with external or feedback control such as magnetic control [4,5]. The technique is based on the principle that the drug is encapsulated with magnetite nanoparticles, covered by a polymer shell. The existence of magnetic particles in magnetic microspheres will make them specifically transported to the target site under the influence of external magnetic field. On reaching the target organ or tissue, the magnetic vehicles release the drug in a temporal manner. The magnetically targeted therapy can be effective to lower the toxic effects and to enhance the therapeutic effect of drug because of the characteristics of driven magnetic accuracy, targeting and high drug capacity[6-8].

Magnetic particles are usually made of magnetite (Fe_3O_4), maghemite ($\gamma - Fe_2O_3$), cobalt ferrite (Fe_2CoO_4), chromium dioxide (CrO_2) etc. Among them magnetite is extensively used, properly characterized in various aspects, whose toxicity has been demonstrated to be low and well tolerated in human body[9,10].

Biocompatible and biodegradable polymers have been widely used for the controlled drug release [11,12]. The polymeric shell can consist of different kinds of polymers such as poly (-caprolactone) (PCL), polylactides (PLA) and polyglycolide (PGA) are of great interest in design of carrier based drug delivery. The biocompatibility, biodegradability and greater permeability of PCL make it suitable for controlled drug delivery and wide use in medical applications. Its compatibility with wide range of drugs enables uniform drug distribution in the formulation matrix and its long term degradation facilitates drug release up to several months[13,14]. The investigation on PCL formulations revealed

that the mechanism of drug release is often dominated by diffusion from microsphere matrix, by which the lipophilic drug release can be controlled in vivo from PCL formulations for much longer duration [15,16].

Etoposide is a semisynthetic derivative of podophyllotoxin that exhibits superior antitumor activity. Etoposide inhibits DNA topoisomerase II, thereby inhibiting DNA synthesis at the premiotic stage of cell division. Etoposide is cell cycle dependent and phase specific, affecting mainly the S and G2 phases of cell division and cause cell death. Etoposide is considered as major standard cytotoxic drug for small lung cancer [17]. The chemotherapy regimens that utilize etoposide are more effective when the drug is given over an extended period of time [18, 19].

The present work is aimed to develop etoposide loaded PCL magnetic microspheres that ensure the delivery of concentrated dose of etoposide directly into the tumor, which will eliminate the need for patient to consume large quantities of the drug. The developed magnetic microspheres were characterized for morphology, size distribution, encapsulation efficiency, magnetic property and in vitro release.

Materials and Methods

Poly (-caprolactone) M.Wt.45000, polyvinyl alcohol (M.wt 15000-20,000), Magnetite (iron II, III oxide) nanopowders < 50nm was purchased from sigma – Aldrich chemical co. (USA). Etoposide was a gift sample obtained from Cedilla India ltd., Mumbai. Potassium dihydrogen orthophosphate and sodium hydroxide were purchased from SD fine chemicals ltd., Mumbai. All solvents (dichloromethane, methanol) used in the preparation were of analytical grade and purchased from Qualigens fine chemicals, Mumbai.

Preparation of drug loaded PCL magnetite microspheres by O/W emulsion method.

The magnetic loaded PCL microspheres containing etoposide were prepared by O/W emulsion- solvent evaporation technique using polyvinyl alcohol as the external aqueous phase. The method was similar to the procedure previously reported for the development of etoposide loaded controlled release PCL microspheres [20]. Briefly, the required amount of PCL, magnetite and drug were dissolved in 10 ml of dichloromethane. The organic phase was added slowly to the 40 ml of aqueous phase containing 1% PVA as stabilizer. The mixture was emulsified with the help of a high speed homogenizer [Turrax T25, IKA] at 5000 rpm. The formed O/W emulsion was stirred under a magnetic stirrer for 3 h at 1000 rpm under room temperature to make free of organic phase. The magnetic microspheres so formed were collected with the help of placing a magnet of 8000G strength at the bottom of the beaker, washed thrice with distilled water, filtered and dried at 45^{o}C. All formulations were transferred to glass vial and stored in a desicator.

Morphology and particle size

The studies on morphology of samples were carried out using scanning electron microscopy (SEM, JOEL-JFC 5300). Magnetic microspheres were dispersed in distilled water, dripped in aluminium foil and evaporated. The dried magnetic microspheres were mounted on a copper stub and coated with gold palladium under vacuum using an ion sputter coater (JEOL JFC 1100E) for observation under scanning electron microscope. The formulated magnetic microspheres were characterized by optical microscopy for particle size and size distribution. The eye piece micrometer was calibrated with the help of a stage micrometer. The average particle size was determined using Edmundson's equation $D_{mean} = \Sigma nd/\Sigma n$, where n is the number of microspheres counted and d is the mean size range.

Determination of drug loading and encapsulation efficiency

The formulated magnetic microspheres were estimated for their loading and encapsulation efficiency by using the equations 1 and 2 respectively.

$$\text{Drug loading (\%)} = m_c/m_t \times 100 \quad \text{---------- 1}$$

$$\text{Encapsulation efficiency (\%)} = m_c/ m_\theta \times 100 \quad \text{---------- 2}$$

where m_c is mass of etoposide in magnetic microspheres, m_θ is the total mass of the drug and m_t is the total mass of magnetic microspheres.

An accurately weighed amount of drug loaded magnetic microspheres (100 mg) were dissolved in 10 ml of dichloromethane: methanol mixture in screw cap (Teflon tube). The tubes were shaken vigorously for 1 min. The contents were filtered by using a 0.1μ millipore filter assembly and suitably diluted with respective solvent system. The UV absorbance of the solution was measured using the UV/ Vis spectrophotometer (Perkin Elmer-LAMBDA 25) at 283 nm and the concentration was calculated according to the standard regression.

Fourier Transformed Infrared Spectroscopy (FTIR) analysis

The FTIR spectroscopy was used to characterize the drug, polymer, magnetite and the formulated magnetic microspheres. The samples were recorded on a Perkin Elmer-(Spectrum RX) using the conventional KBr pellet method. All samples were scanned in the IR range from 400- 5000cm^{-1}at 25^{o}C.

State of etoposide in magnetic microspheres

Differential scanning calorimetry was used to determine the thermal behavior of etoposide, polycaprolactone, magnetite and magnetic microsphere formulations. Samples were scanned for the melting temperatures in nitrogen atmosphere by Perkin Elmer DSC-7. Samples were placed in hermetically sealed aluminium pans and heated at a scan speed of 10 ^{o}C per min over a temperature range of 30- 500^{o} C at a chart speed of 10 ml/min. The heat of fusion was calibrated with indium.

Determination of magnetic property

The magnetic properties of Fe_3O_4/PCL microparticles were measured by using a Vibrating Sample Magnetometer (VSM) [DMS 1600]. The samples in the form of powder were placed in Teflon sample holder. The magnetic properties were then determined by an increasing magnetic field over the sample. The measurements were carried out in the field range of ± 1 T at room temperature.

Determination of magnetic content

The content of magnetite in formulations was estimated quantitatively by atomic absorption spectroscopy (AAS) [SL 173, Elico]. A preweighed (100 mg) amount of magnetic microspheres was digested with 5ml of HCl and 5ml of HNO_3 in CEM microwave digester using MARSX press at 800psi and 200°C. The digested solution was made up to 500ml using de-ionized water and was thoroughly filtered using whatman 40 filter paper. The clear solution was assayed for iron by AAS at 248 nm. The weight percentage of iron content in the formulation can be calculated from the equation

$$\text{Wt \%} = \frac{\text{ppm (mg/L) X Volume in mL X dilution factor X } 10^{-4}}{\text{Weight of sample in grams}}$$

where, ppm (mg/L) is the result obtained from the instrument, Volume in ml- is the volume required for digestion, weight of sample in gm- 0.100gm for all the samples.

In vitro drug release studies

The in vitro drug release studies for formulated magnetic microspheres were carried out in phosphate buffer (pH 7.4) at 37.5 ± 0-5° C. Dried samples of drug loaded polycaprolactone magnetic microspheres (35 mg) were put into a dialysis bag immersed in 50 ml of phosphate buffer (pH 7.4) in a conical flask. [21, 22] The flask was placed in an orbital shaker incubator [C 24, Remi] and rotated at 50 rpm. At predetermined time intervals the flasks were taken out of the shaker, 5 ml aliquots of the medium were withdrawn and the same volume of fresh medium was added to the bulk to maintain the sink condition. The amount of drug released in media was analyzed by using UV- Visible spectrophotometer [Lambda 25, Perkin Elmer].

Histological studies

Presence of excipients in the formulation may sometimes produce toxicity which may be immediate or delayed toxicity. In order to ascertain the safety, the formulations were administered to group of Wistar Albino rats and the vital organs like liver, kidney, heart, brain and lungs were removed. The histopathological evaluation of the tissues with etoposide loaded PCL magnetic microspheres administered rats were fixed in 10% formalin, routinely processed and embedded in paraffin. Sections were cut on glass slides and stained with hematoxylin and eosin.[23] The sections were examined under a light microscope to detect any damage occurred to the tissue and were compared with similar sections of tissues isolated from untreated rats. Studies were performed under OECD guidelines no 423.

Results and Discusssion

Characterization of magnetic microspheres

The prepared polycaprolactone magnetic microspheres loaded with etoposide were characterized for their morphology and size distribution. Figure 1 shows the morphology of magnetic microspheres obtained by scanning electron microscopy. The microspheres obtained are free flowing. As evidenced from the figure the microspheres are smooth and spherical in shape.

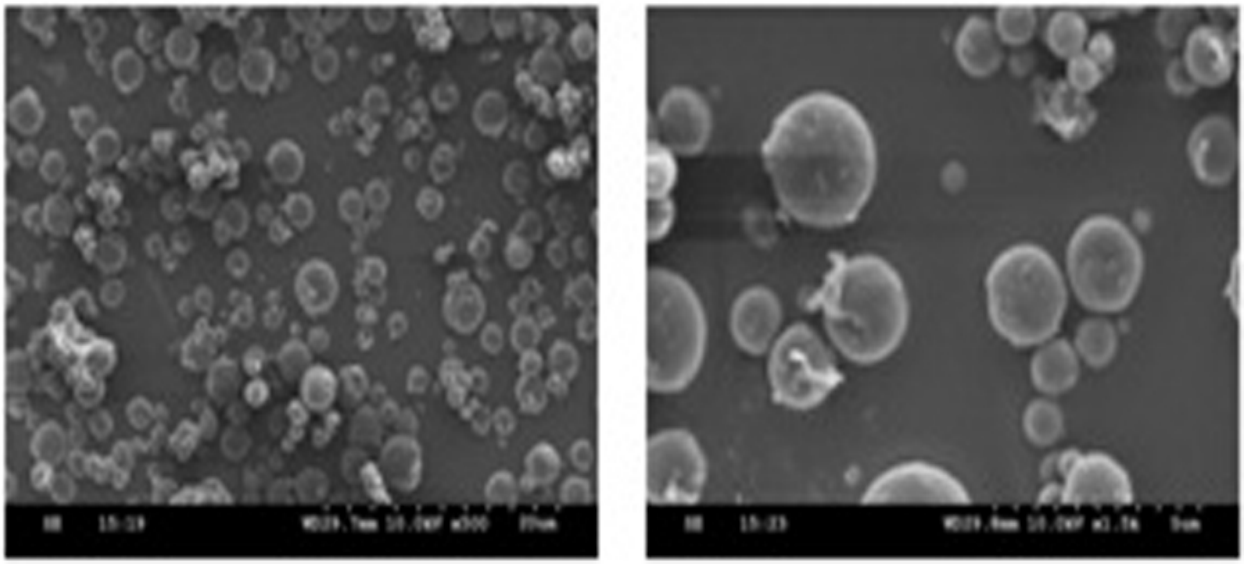

Figure 1: SEM images of polycaprolactone magnetic microspheres

The magnetic microspheres were formulated with different concentrations of PCL as shown in table 1. The amount of polymer is varied in formulations to investigate the influence of particle size and size distribution. The mean particle size of polycaprolactone magnetic microspheres of etoposide with different dug/polymer ratio was shown in table 1 and figure 2, 3 and 4. The particle size distribution of prepared microspheres ranged from 4.8μm to 9.08μm. Increase in the concentration of polymer resulted in increase in mean particle size. It could be suggested that the higher concentration of polymer may lead to increased frequency of collisions, resulting in fusion of semi particles and finally produce bigger particles thereby increasing the size of microspheres [24]. Polyvinyl alcohol at a concentration of 1% was used as a surfactant in the formulation. The coalescence and agglomeration, which were common occurrence in preparation of microspheres by solvent evaporation process were probably due to increase in viscosity and gradual decrease in the volume as a result of solvent evaporation [25]. The presence of polyvinyl alcohol reduces the coalescence and agglomeration by forming a thin film around the emulsion droplets, thus preventing their approach towards each other and its consequences.

Table 1: Composition, mean particle size, drug loading, encapsulation efficiency and magnetite concentration of prepared microspheres

Formulation code	Drug/polymer/magnetite ratio	Mean Particle Size (μm)	Drug Loading (%)	Encapsulation efficiency (%)	Magnetite Concentration (%)
F 1	1:1:1	4.80	4.00	48.2	13.6
F 2	1:1:2	5.10	4.67	47.4	14.7
F 3	1:1:3	5.27	3.92	44.3	13.5
F 4	1:1:4	4.68	3.60	40.2	15.2
F 5	1:5:1	6.05	5.84	61.4	14.3
F 6	1:5:2	6.19	5.10	59.5	17.7
F 7	1:5:3	6.40	4.80	53.8	19.3
F 8	1:5:4	6.80	4.10	53.0	22.7
F 9	1:10:1	9.08	6.12	69.4	17.7
F 10	1:10:2	8.22	5.70	69.0	21.9
F 11	1:10:3	7.88	5.21	64.0	31.5
F 12	1:10:4	8.42	4.84	62.3	30.8

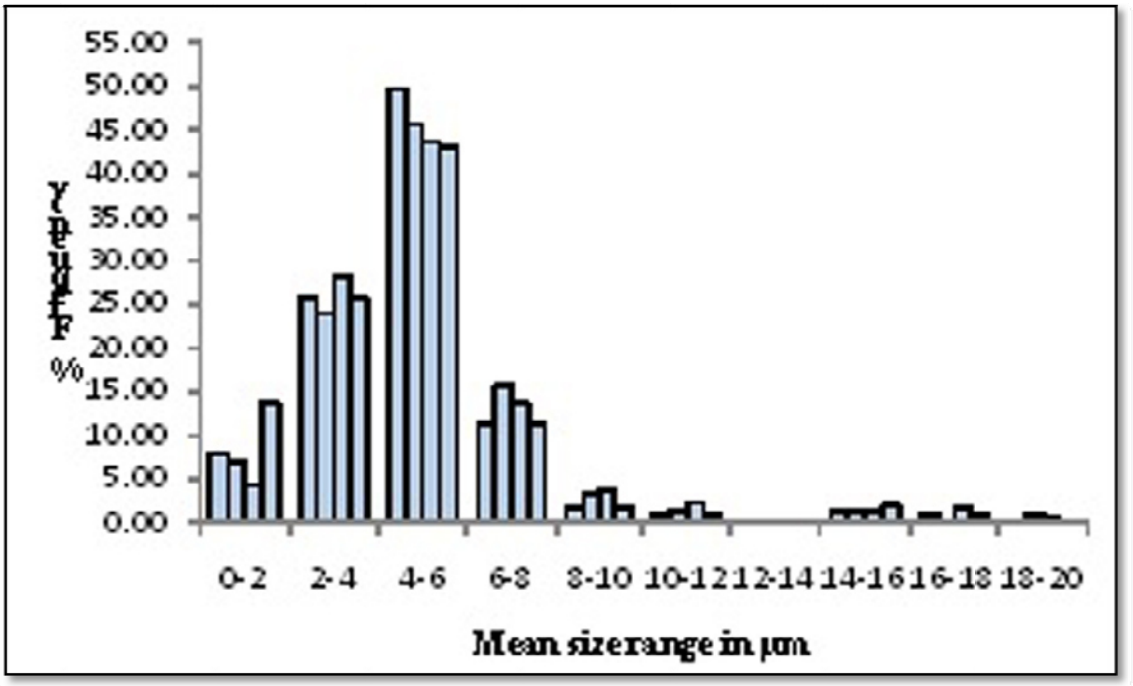

Figure 2: Particle size distribution of etoposide loaded polycaprolactone magnetic microspheres with drug/polymer ratio 1:1

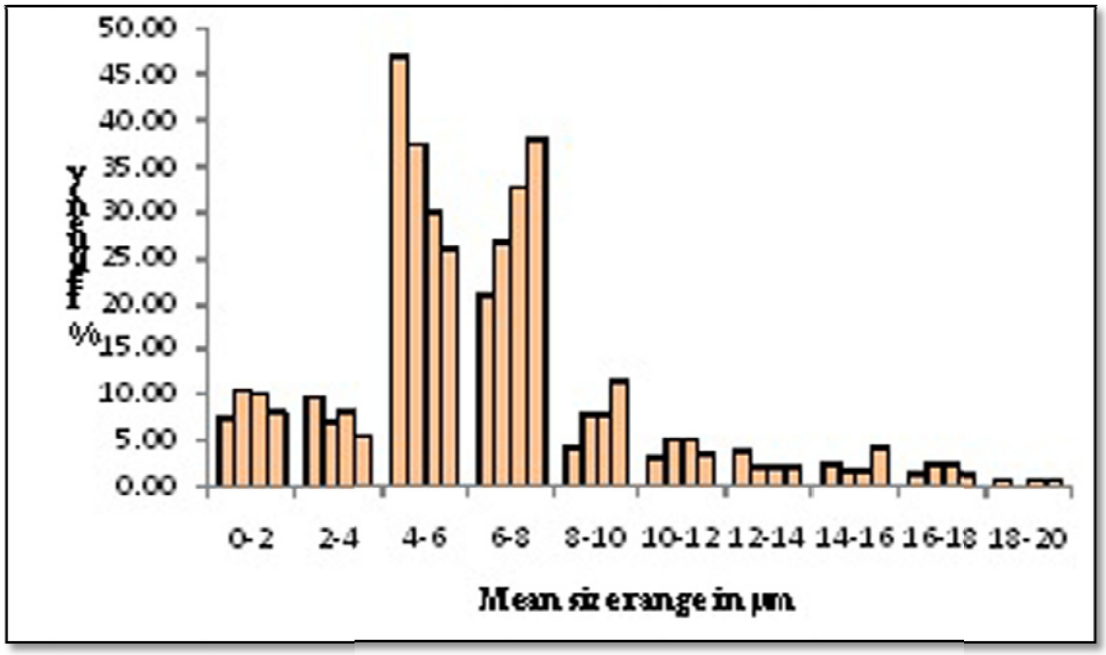

Figure 3: Particle size distribution of etoposide loaded polycaprolactone magnetic microspheres with drug/polymer ratio 1:5

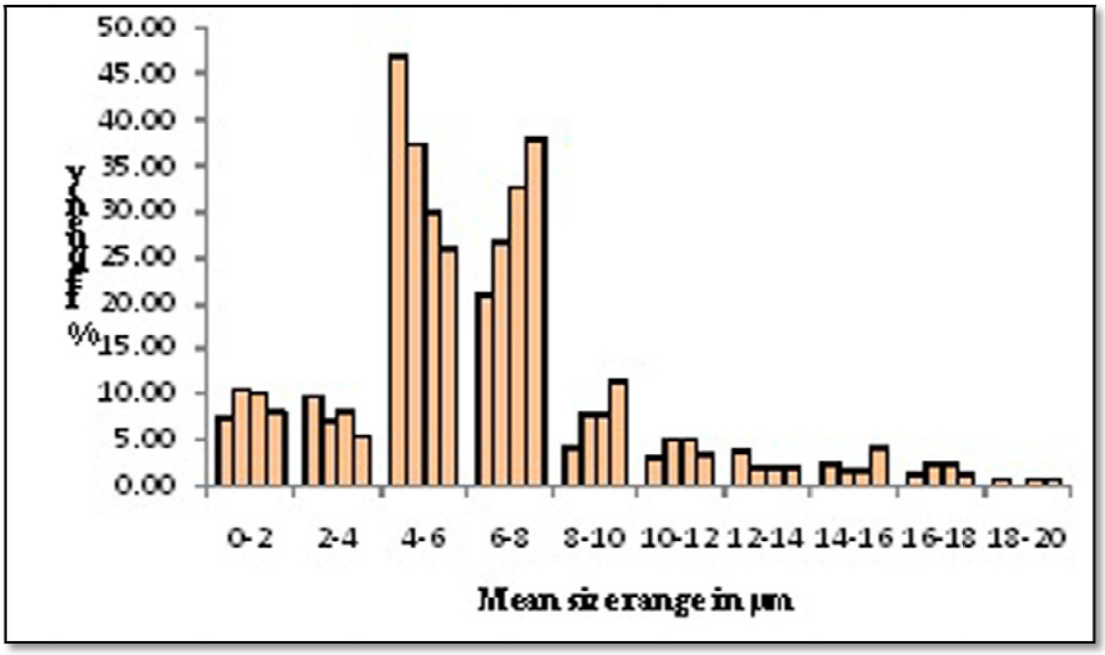

Figure 4: Particle size distribution of etoposide loaded polycaprolactone magnetic microspheres with drug/polymer ratio 1:10

Efficiency of encapsulation

The amount of drug encapsulated in each of polymeric magnetic microspheres was determined by calculating the amount of drug that could be recovered after dissolving the microspheres in dichloromethane: methanol mixture. The efficiency of encapsulation of all formulations were investigated and reported in table 1. An increase in concentration of polymer resulted in the increased entrapment efficiency. This effect was also observed by Benoit [24]. The enhancement in polymer concentration leads to increase in viscosity of organic phase which in turn restrict the migration of drug to the external water phase.

Fourier Transformed Infrared Spectroscopy

Figure 5 shows the FTIR spectra of magnetic polycaprolactone microspheres prepared by o/w emulsion method. The FT-IR spectra of etoposide, PCL, Fe_3O_4 nanoparticles and the magnetic microspheres were analyzed. The functional groups of PCL were very important for diverse applications. The FTIR spectra of etoposide (Figure 5 A) exhibits a broad band between 3400-3700 cm^{-1} which is characteristic of phenolic - OH group and a characteristic intense doublet of ether showed C=C stretched band at 1614 cm^{-1}. The C=C stretching of vinyl ethers occurs in the 1660 – 1610 cm^{-1} region. This band is characterized by its higher intensity compared with the C=C stretching band in alkene. A characteristic broad absorption band at 3412 cm^{-1} is due to the presence of hydrogen bonded OH group. Figure 5 B shows a strong carbonyl stretching band at 1724 cm^{-1} revealed the ester carbonyl bond that come from PCL. The spectra of Fe_3O_4 nanoparticles (Figure 5 C) exhibit at low frequency region (600 – 400 cm^{-1}) due to the iron oxide structure. Depending on Fe (II) (III) content the pattern of magnetite (Fe_3O_4) spectrum shows at 570 cm^{-1}[26]. In the IR spectrum of magnetic microspheres (Figure 5 D) the characteristic absorption bands of etoposide and PCL existed in the same wave number. This indicates the absence of chemical interaction between polymer and drug in magnetic microspheres and the presence of drug as a molecular dispersion in the polymer matrix.

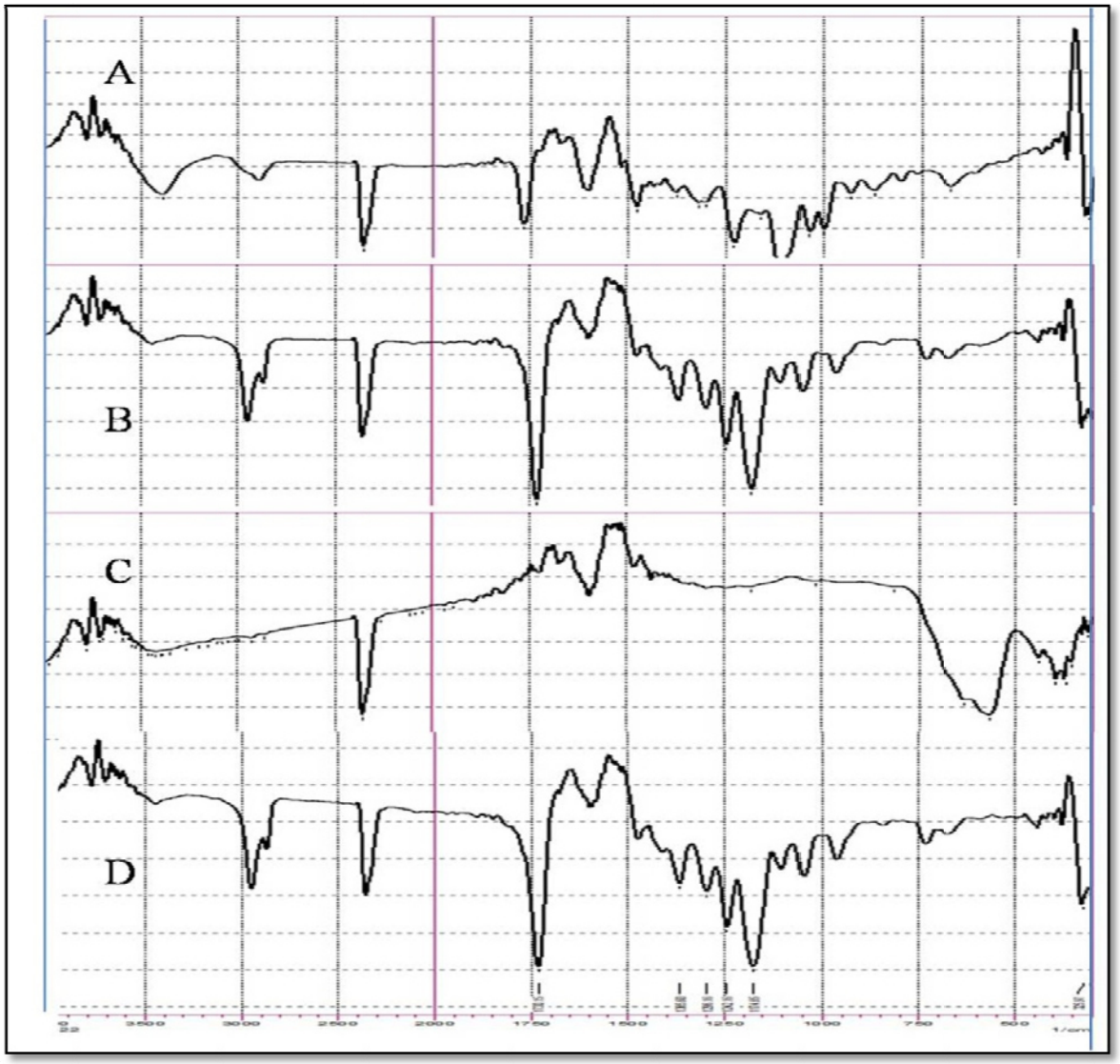

Figure 5: Infra red spectrum of A) pure drug etoposide B) Polycaprolactone C) Fe_3O_4magnetic nanoparticles and D) Etoposide loaded magnetic microsphere formulation.

Differential Scanning Calorimetry

The DSC method of analysis is quite informative about the quality, quantity and physico chemical status of the drug in the formulation. DSC thermograms for free drug, PCL, polymer, magnetite and drug loaded PCL magnetic microsphere formulations were given (Figure 6 A -D). The glass transition temperature of pure drug and polymer were present at 283.7ºC and 77.7ºC. The thermogram of drug loaded magnetic microsphere exhibits similar shape and position to that of PCL polymer and did not show any detectable endotherm corresponding to melting temperatures of free drug between 200 - 300ºC. However, the absence of detectable melting peak of drug in the formulation indicates the presence of drug in amorphous state or molecular dispersed state which proves to enhance the solubility so as reach improved biological activity. The amorphous state of etoposide in magnetic microsphere contributes to the strong intermolecular forces between etoposide and PCL during the formulation of microspheres.

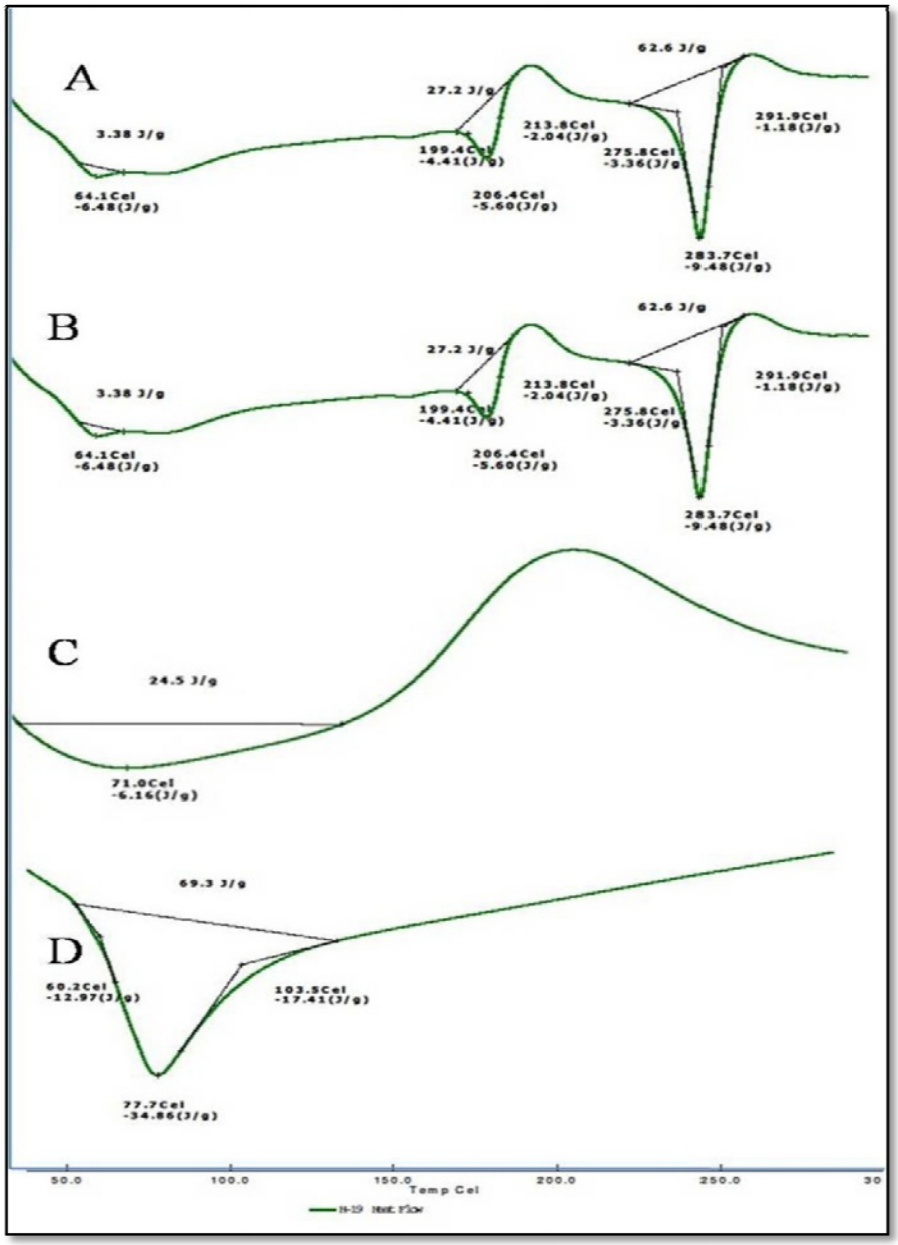

Figure 6: DSC Thermograms of formulated magnetic microspheres: A) Pure drug; B) PCL polymer; C) Magnetic nanoparticles and D) Magnetic microsphere formulation

Magnetic property of magnetic microspheres

The superparamagnetic property of polymer magnetic microsphere is critical for their application in biomedical and bioengineering fields which prevents polymer magnetic microspheres from aggregation and enables them to redisperse rapidly when the magnetic field is removed [6]. The magnetization curves of the naked Fe_3O_4 particles (figure 7(a)) and Fe_3O_4 drug loaded polycaprolactone magnetic microspheres (figure 7(b)) recorded with VSM are illustrated in figure 7. As shown in the figure the magnetization of the samples would approach the saturation values when the applied magnetic field increases to 10,000 Oe. The saturation magnetization (s) of magnetite was found to be 24.82 emu/g and that of microsphere formulation was found to be 7.26 emu/g. The saturation magnetization of magnetic microspheres was much less than that of bulk magnetite as reported in the literature for this material [27]. Figure 7(a) and Fig 7(b) represents a typical characteristic of superparamagnetic material with no detected remainance or coacervity at room temperature indicated that the single domain magnetic Fe_3O_4 nanoparticles remained in the prepared magnetic microspheres.

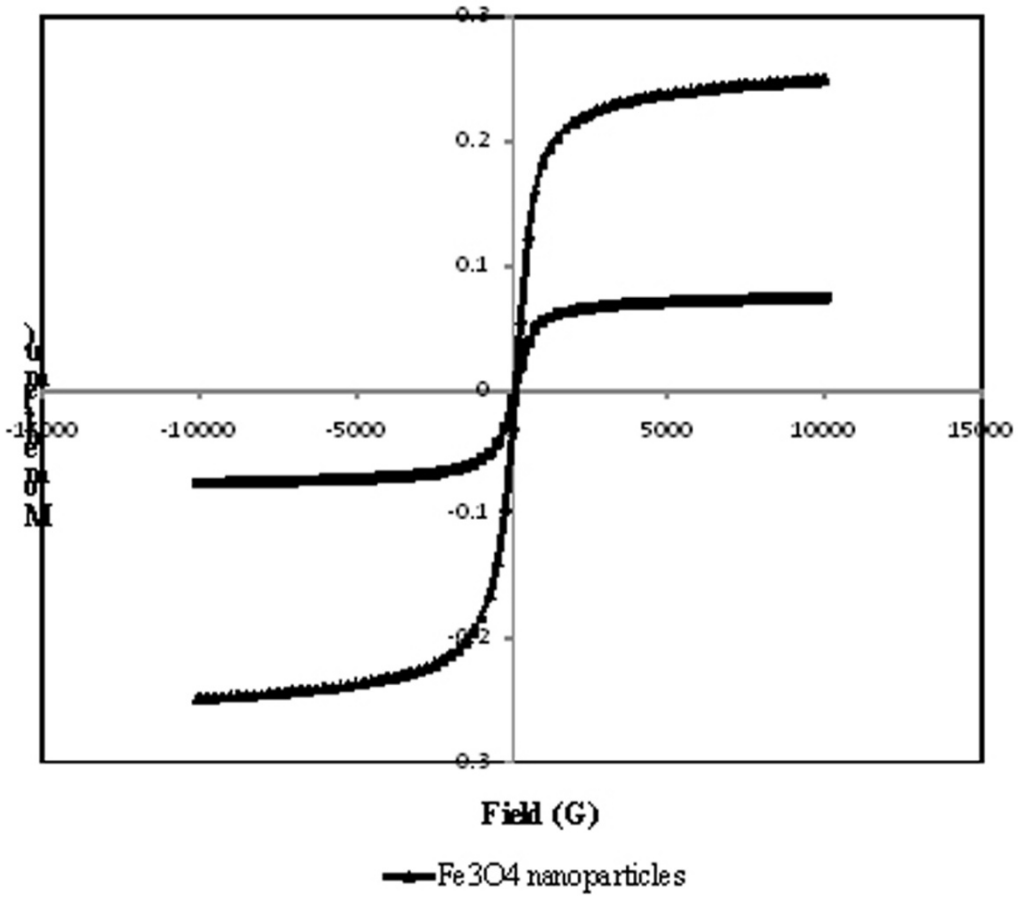

Figure 7: Magnetization curves obtained by vibrating sample magnetometer (VSM) at room temperature. (a) Fe_3O_4 nanoparticles (b) Fe_3O_4 / PCL magnetic microspheres.

Content of magnetic particles

The study includes the incorporation of Fe_3O_4 nanoparticles along with drug and encapsulated with polycaprolactone. The presence of magnetic particles can carry the drug to a specific target site quickly under the external magnetic field. The concentration of magnetite included within the polymeric carriers was estimated in terms of weight percentage by atomic absorption spectroscopy. The amount of magnetite in the polymeric microspheres increases with the increase in the polymer concentration which was illustrated in table 1. Microspheres fabricated with 1:10 drug/polymer ratio could accommodate highest amount of magnetite. On the other hand, the magnetite and drug compete with each other to dwell into the matrix space of the polymeric droplets during its formation. Accordingly the ability of the microspheres to entrap the drug decreases with increase in magnetite concentration.

The average amount of magnetite within the spheres was found to be 19.4%w/w. This concentration of nanoparticulate magnetite within the polymeric vehicles could be sufficient to direct the microspheres to reach their destination target site. Such a

observation was previously reported by Gupta and Hung [28] demonstrated that a 15 – 20% w/w magnetite is sufficient to achieve 100% retention of the magnetic carrier using 8000G magnet for an arterio-capillary flow of 0.005 – 0.1 cm/s. A similar finding was made with gelatin magnetic microspheres, where a higher amount of magnetite (upto 30%) was considered sufficient to withstand arterial pressure under a magnetic field [1]. Thus in the view of the above mentioned research findings the 31.5 w/w of magnetite included in microspheres in the present study could achieve the expected degree of localization of the microspheres.

In-vitro drug release studies

Figure 8 - 10 shows the percentage of accumulative drug released from the polymeric magnetic microspheres as a function of release time. The release of etoposide from magnetic microspheres with drug/polymer ratio 1:1, 1:5 and 1:10 containing 1% polyvinyl alcohol indicated a quick release within 48 hrs. The release rate becomes slower and constant after 48 hrs. The average burst release for the formulations were found to be 38%. Zhou et al reported about the drug release in microspheres and revealed that the release involved two different mechanisms of drug molecules diffusion and polymer matrix degradation [29]. The cause for initial burst release was probably due to small amount of poorly encapsulated drug bound to the microparticles surface which easily diffused from the dialysis bag [30, 31]. Further the burst release was followed by constant slow release. The reason for slow release may be due to diffusion of drugs from polymer as well as due to erosion of polymer [32]. At the end of 3rd week period the total amount of etoposide released from the magnetic microspheres with drug/polymer ratio 1:10 was found to be 76.25%.

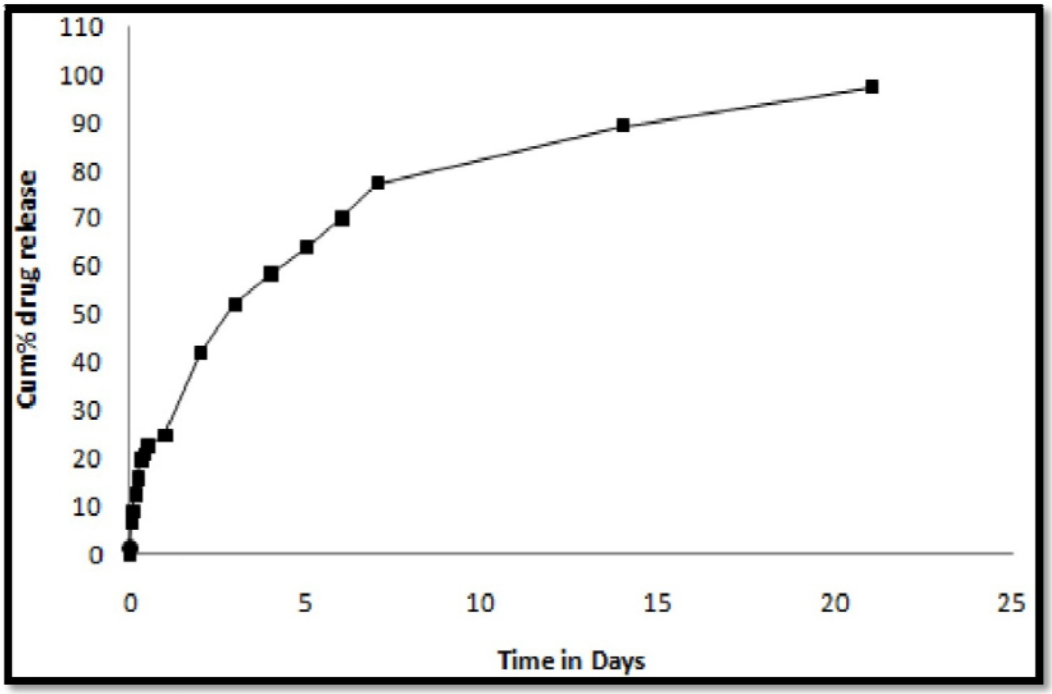

Figure 8: Drug release profile of etoposide from PCL magnetic microspheres of drug/polymer ratio 1:1

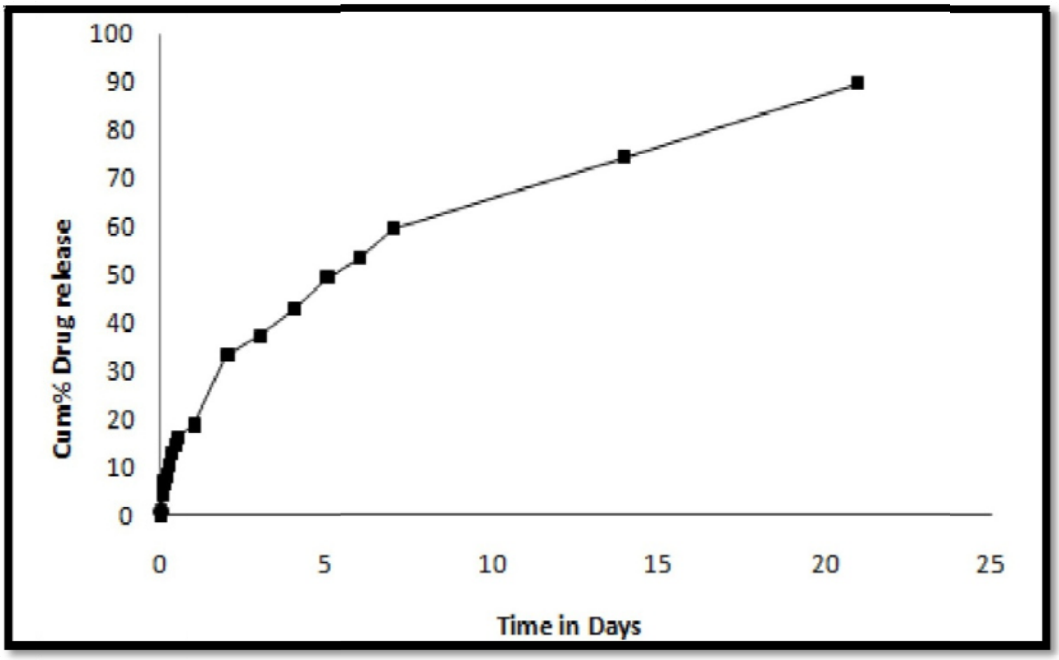

Figure 9: Drug release profile of etoposide from PCL magnetic microspheres of drug/polymer ratio 1:5.

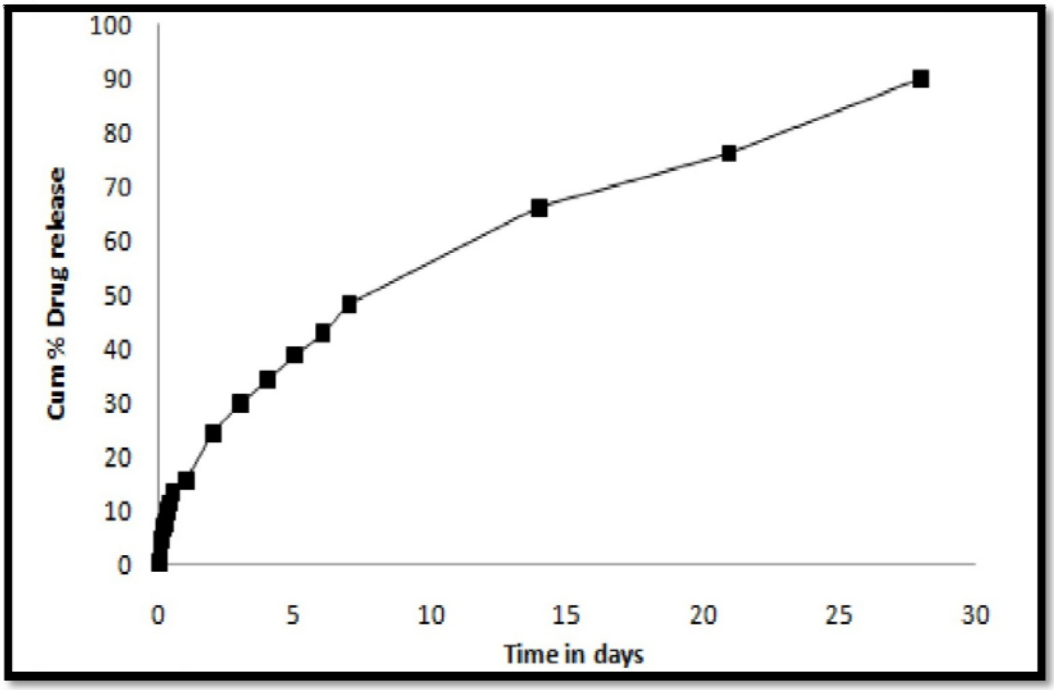

Figure 10: Drug release profile of etoposide from PCL magnetic microspheres of drug/polymer ratio 1:10.

The concentration of polymer plays a major role in release pattern. The release of drug from microspheres with lower concentration of polymer was much more rapid than those with higher polymer concentration. An increase in concentration of polymer will develop very dense and least polymer matrix resulting in slower release rate.

Histopathological analysis

Histopathological analysis of various organs like brain, heart, liver, lungs and kidney of animals treated with encapsulated etoposide was illustrated in figure 11a-11e.The vital organs were assessed for toxic effects of etoposide loaded polycaprolactone magnetic microspheres. The cross section of brain, heart and liver of treated animals were examined for cell necrosis, cellular hoemostasis and inflammation on hepatic cells. None of these signs was detected, revealing there was no significant toxicity produced by the formulations (Figure 11a, 11b and 11c). Enlarged airway spaces and necrosis of alveoli on cross examination of the cells of the lungs were found to be absent, hence signifying no toxic effects (Figure 11d). A toxicity produced kidney is identified by reversible lesions such as interstitial fibrosis. The cross sections of the kidneys showed no such effects, thus signifying no toxic effects of the drug (Figure 11e). Hence it was concluded from the microphotographs that no histological alterations were observed in various organs of animals treated with encapsulated etoposide.

Conclusion

The poly (- caprolactone) microspheres containing magnetite and anticancer drug were successfully formulated by O/W emulsion solvent evaporation method with maximum drug encapsulation and desired release profile. The method adopted favored the formation of smooth, spherical shaped microspheres with optimal particle size. The formulated magnetic microspheres were magnetically responsive and this synthetic approach is applicable for producing magnetic polymer microspheres with high magnetite contents. The possession of sufficient paramagnetic property was confirmed by VSM. The histopathological studies proved that the etoposide loaded magnetic microspheres was nontoxic and safe. The results suggested that the magnetic PCL magnetic microspheres may have potential as a highly versatile carrier for targeted delivery approach.

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