CHAPTER III

RESEARCH METHODOLOGY

The objective of this work was to prepare water dispersible magnetite nanoparticles containing double-layer surface; hydrophobic inner layer and hydrophilic corona. To achieve the goal, poly(ethylene glycol)methyl ether (mPEG)-polyester block copolymer were first synthesized *via* a step-growth condensation polymerization. Hydrophobic polyester blocks were thought to physically adsorb onto the pre-synthesized oleic acid-coated magnetite nanoparticles, while hydrophilic mPEG blocks can protrude outward to provide steric repulsion and water dispersibility to the particles. In addition to solely physical interaction in the hydrophobic inner layer, a network structure in the hydrophobic inner shell was also prepared. Drug releasing profile was thus established to study the effect of crosslinking structure in the hydrophobic inner shell on its releasing behavior.

Therefore, this chapter thus covers the experimental details in the order of following; (Figure 17).

1. Synthesis of mPEG-polyester block copolymer
2. Preparation of oleic acid-stabilized magnetite nanoparticles as a primary surfactant-coated particle
3. Anchoring the as-synthesized mPEG-polyester copolymer onto the oleic acid-precoated nanoparticles
4. Partitioning indomethacin, the model drug, onto the copolymer-magnetite complex and investigating drug loading and entrapment efficiencies, as well as the drug-released behavior
5. Crosslinking of hydrophobic inner shell and study the effect of crosslinking structure on drug releasing behavior

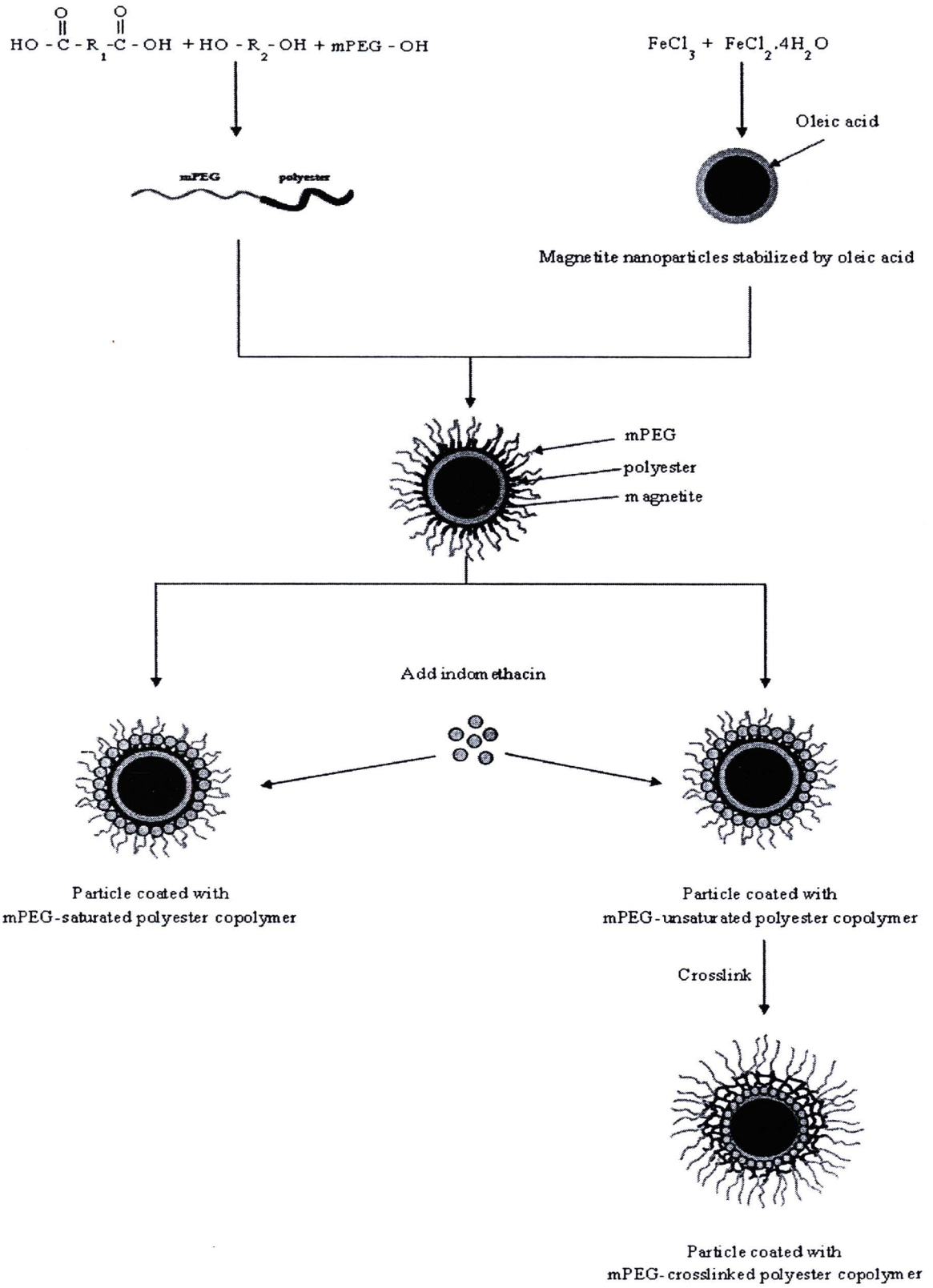

Figure 17 An experimental overview

Materials

Unless stated otherwise, all reagents and solvents were used without further purification. Poly(ethylene glycol) monomethyl ether (mPEG) with $\overline{M_n}$ 2,000 and 5,000 g/mol (Acros) was freeze-dried for 24 h. Iron (III) chloride anhydrous ($FeCl_3$) (Carlo Erba), iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$) (Carlo Erba), ammonium hydroxide (J.T. Baker, 28-30%), oleic acid (Fluka), ethylene glycol (99%, Acros), 1,6-hexane diol (99%, Acros), malonic acid (99%, Acros), maleic acid (99%, Acros), potassium peroxodisulfate ($K_2S_2O_8$, KPS) and indomethacin (90 %, Sigma), were used as received. Phosphate buffer solutions (pH 7.4) were prepared by mixing 0.1 M citric acid ($H_3C_6H_5O_7.H_2O$) with 0.2 M disodium phosphate ($Na_2HPO_4.12H_2O$). Cellulose dialysis tubing (Sigma-Aldrich) with molecular weight cutoff 12,400 was immersed in running water for 24 h before used.

Methodology

1. Synthesis of mPEG-polyester block copolymer

In the present work, hydrophobic polyester was prepared *via* a direct condensation reaction between diol and diacid compounds because of a wide variety of monomer structures commercially available, while hydrophilic mPEG was used due to its commercial availability of various molecular weights. From the summarized table in Table 1, malonic acid monomer was designated as "Ma", maleic acid as "Me", 1,6-hexanediol as "He", ethylene glycol as "Et", mPEG 2,000 g/mol as "M2" and mPEG 5,000 g/mol as "M5". An example procedure presented herein is for preparing a copolymer having 2,000 g/mol mPEG and polyester with targeted molecular weight of 5,000 g/mol (Appendix A-1). mPEG (2.00 g, 0.0010 mole), malonic acid (2.39 g, 0.023 mole), 1,6-hexanediol (2.60 g, 0.022 mole) and sulphuric acid (0.5 M, 0.25 ml) were charged into a reaction flask. The mixture was maintained at 180°C for 18 h under reduced pressure. The mixture was then slowly cool down to room temperature under N_2 atmosphere (Figure 18).

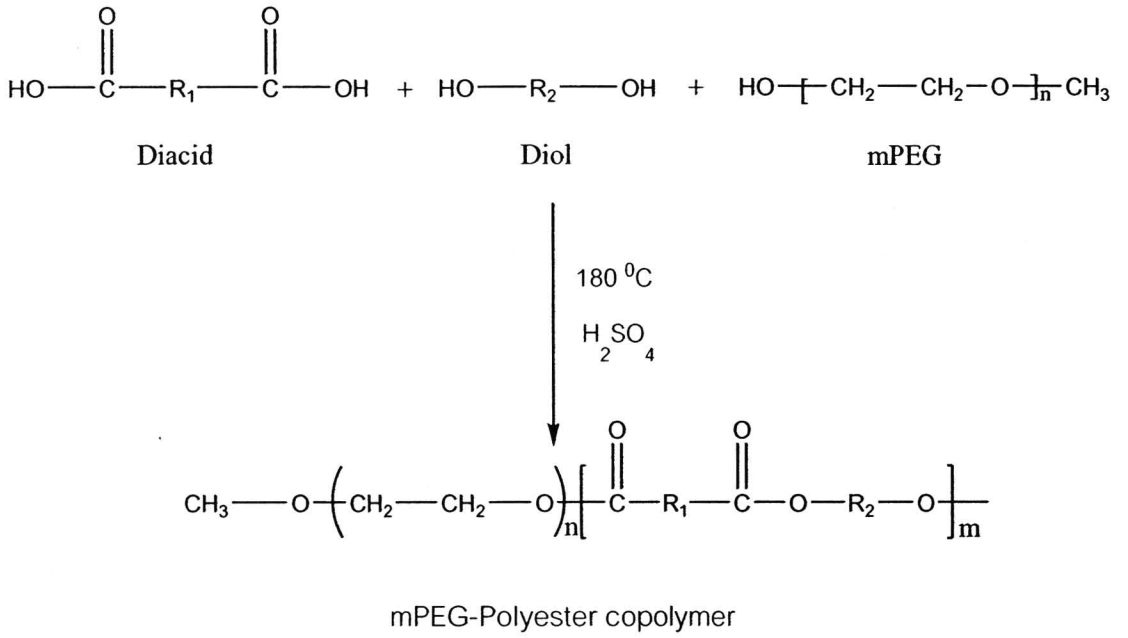

Figure 18 Synthesis of mPEG-polyester copolymer

Table 1 mPEG-polyester copolymer compositions

Copolymer name*	Type of polyester	$\overline{M_n}$ of mPEG (g/mol)
Ma/He/M2	malonic acid + 1,6-hexanediol	2,000
Ma/Et/M2	malonic acid + ethylene glycol	2,000
Me/He/M2	maleic acid + 1,6-hexanediol	2,000
Me/Et/M2	maleic acid+ ethylene glycol	2,000
Ma/He/M5	malonic acid + 1,6-hexanediol	5,000
Ma/Et/M5	malonic acid + ethylene glycol	5,000
Me/He/M5	maleic acid + 1,6-hexanediol	5,000
Me/Et/M5	maleic acid+ ethylene glycol	5,000

*Ma = malonic acid, Me = maleic acid, He = 1,6-hexanediol, Et = ethylene glycol, M2 = mPEG 2,000 g/mol and M5 = mPEG 5,000 g/mol

2. Preparation of the magnetite nanoparticles coated with oleic acid as primary surfactant

$FeCl_3$ (1.66 g) in deionized water (20 ml) and $FeCl_2.4H_2O$ (1.00 g) in deionized water (20 ml) were mixed with stirring. Black precipitant was observed once 25% NH_4OH (20 ml) were added into the solution, indicating the formation of magnetite nanoparticles. The dispersion was continuously stirred for another 30 min. to complete the reaction. The dispersion was centrifuged at 3000 rpm for 20 min. and the aqueous supernatant was discarded. An oleic acid solution in hexane (2.0 ml in 20 ml hexane) was then introduced into the magnetite dispersion with stirring. The dispersion was concentrated by evaporating hexane to obtain a black thick liquid of concentrated magnetite in hexane (Figure 19). Precise concentration of magnetite in the dispersion was determined by atomic absorption spectroscopy (AAS). An example of the calculation of the reagents used was shown in Appendix A-2.

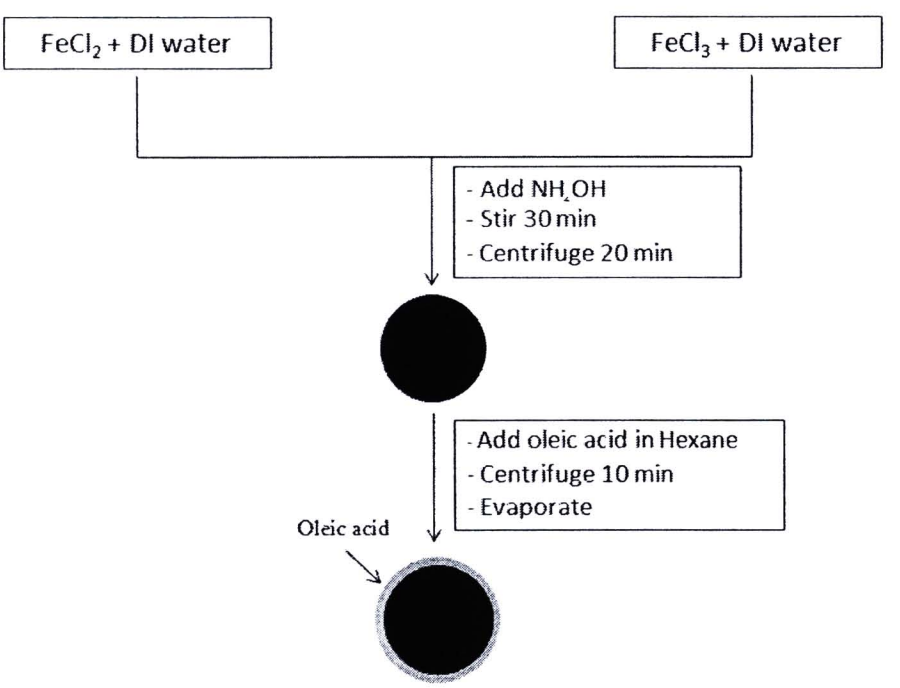

Figure 19 Preparation of the magnetite nanoparticles coated with oleic acid as primary surfactant

3. Synthesis of the copolymer-stabilized magnetite nanoparticles

To prepare the copolymer-stabilized nanoparticles, 0.5 ml of the oleic acid-coated particle dispersion was introduced into various concentrations of copolymer solutions in deionized water (5 ml) (1% w/v, 0.1% w/v, 0.01% w/v and 0.001% w/v of the copolymer solutions). The dispersions were then sonicated for 4 h, followed by centrifugation at 3000 rpm for 20 min. to remove agglomeration. The copolymer-stabilized magnetite nanoparticles in aqueous-based dispersion were dialyzed against deionized water for 24 h, and refreshed twice to remove an excess of the copolymers in the dispersion (Figure 20).

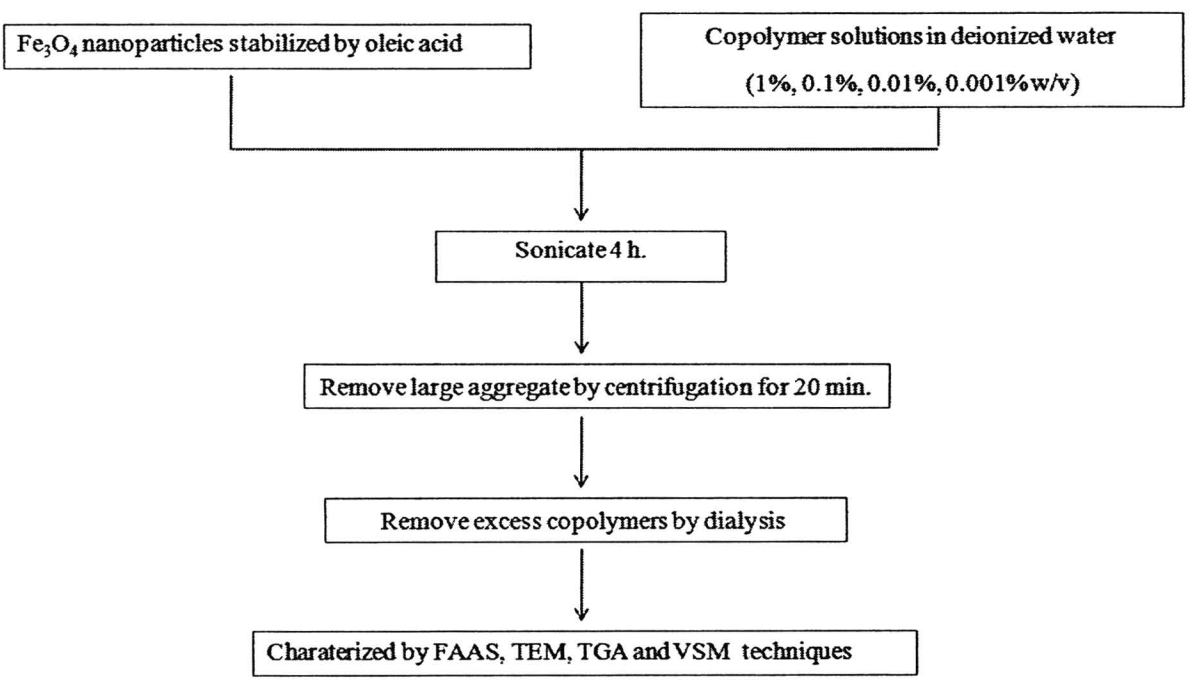

Figure 20 Synthesis of the copolymer-stabilized magnetite nanoparticle

4. Preparation of the particles coated with the copolymers containing unsaturated and crosslinked polyesters

The unsaturated copolymer was prepared using a similar procedure to those of the saturated copolymer with the use of a small amount of an unsaturated diacid compound (5% maleic acid, 95% malonic acid). It was then used as a polymeric surfactant of magnetite nanoparticle using a similar procedure described above.

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To chemically crosslinking the unsaturated portion presenting at the inner layer of the particle surface, a certain amount of potassium persulfate ($K_2S_2O_8$, KPS), a radical initiator, was added into the dispersion of the unsaturated copolymer-coated particle. The crosslinking reaction was carried out at 60 °C for 4 h under the nitrogen atmosphere (Figure 21).

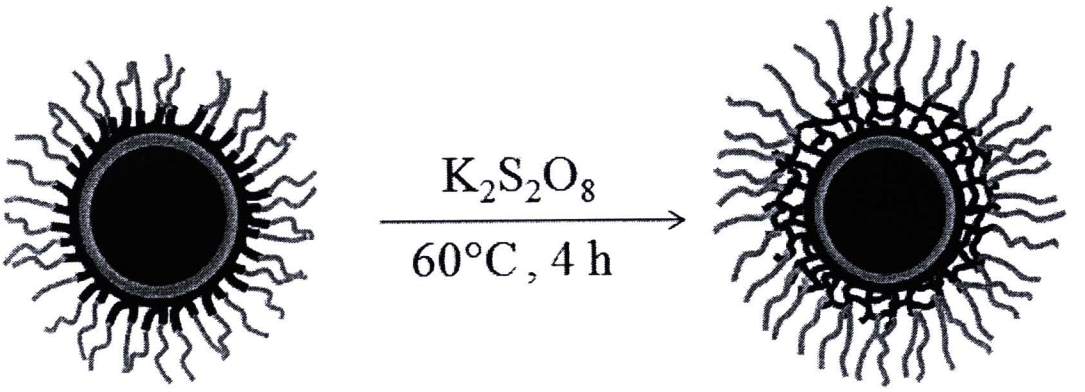

Figure 21 Schematic diagram for the crosslinking reaction

5. Investigation of indomethacin entrapment and loading efficiencies of magnetite nanoparticles

The chemical structure of indomethacin used as a model drug in the current studies is illustrated in Figure 15. To incorporate the drug to the particles, the drug solution (1 ml, 0.1 mg/ml in THF) was added dropwise with stirring to an aqueous dispersion of copolymer-magnetite complex (6.00 ml) (Figure 22). The mixture was stirred for 30 min. to allow fully partitioning the drug into the hydrophobic shell surrounding the particles. The excess drug was precipitated out from the mixture and was removed by centrifugation at 5000 rpm. The drug-entrapped magnetite was then separated using an external magnet. Due to a good solubility of indomethacin in a THF:ethanol solution (50:50 %v/v), the solvent mixture was used to repeatedly extract the entrapped drug from the particles. After centrifugation to remove aggregated particles, the drug concentration in the supernatant, reflecting the amount of the entrapped drug in the complex, was determined using UV-Visible spectrophotometer. Entrapment efficiency (%EE) and drug loading efficiency (%DLE) were determined from the following:

$$\%\text{Entrapment Efficiency}(\%EE) = \frac{\text{Weight of the entrapped drug in nanopartides}}{\text{Weight of the loaded drug}} \times 100$$

$$\%\text{Drug Loading Efficiency}\ (\%DLE) = \frac{\text{Weight of the entrapped drug in nanoparticles}}{\text{Weight of magnetite nanoparticles}} \times 100$$

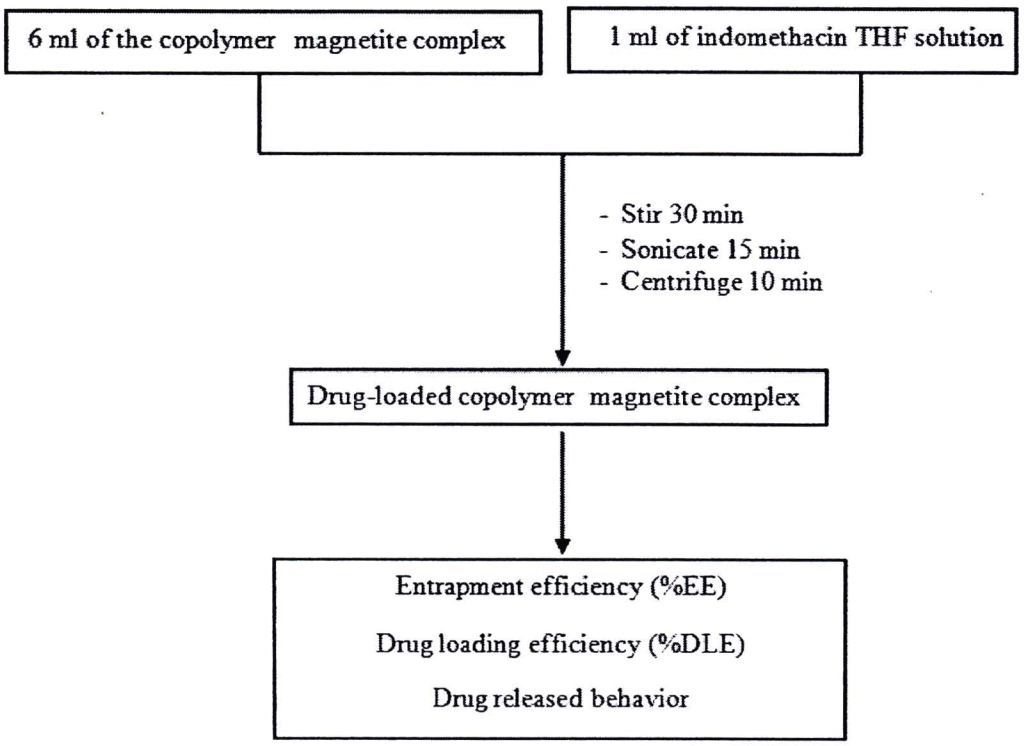

Figure 22 Loading procedure of indomethacin into the copolymer magnetite complex

In the *In vitro* releasing studies of the entrapped indomethacin in the copolymer magnetite complex, indomethacin loaded magnetite dispersions (5.00 ml) were introduced into a dialysis membrane bag immerged in a 300 ml phosphate buffer solution releasing media (pH 7.45) and it was consistently stirred at room temperature. At a predetermined time interval, 5.00 ml aliquots of the aqueous solution were withdrawn from the releasing media and 5 ml of phosphate buffer solution (pH 7.45) was replaced into the media. Concentrations of the released indomathacin were determined *via* UV–Visible spectrophotometer at 320 nm wavelength.

Characterization

1. Nuclear Manetic Resonance Spectroscopy (NMR)

Proton NMR spectra were performed on a 400 MHz Bruker NMR spectrometer using $CDCl_3$ as solvents.

2. Fourier Transform Infrared Spectroscopy (FT-IR)

FTIR was performed on a Perkin-Elmer Model 1600 Series FTIR Spectrophotometer in the wavenumber range of 4000-400 cm^{-1}. Liquid samples were directly cast onto potassium chloride plates. Solid samples were made by the pressed disc method after mixing dried solid samples with KBr.

3. Atomic Absorption Spectroscopy (AAS)

Magnetite concentrations in dispersions were investigated by treating the samples with hot concentrated nitric acid followed by concentrated perchloric acid to obtain complete dissolution. Iron concentrations were analyzed by flame atomic absorption spectroscopy (AAS) (Varian model SpectraAA200) and calculated from sample responses relative to those of standards and blanks.

4. Transmission Electron Microscopy (TEM)

Particle size and its size distribution were observed from Philips Tecnai 12 TEM operated at 120 kV equipped with Gatan model 782 CCD camera. The sample solution in water was cast onto carbon-coated copper grids and let to slowly evaporate at room temperature.

5. Thermogravimetric Analysis (TGA)

TGA was performed on SDTA 851 Mettler-Toledo at the temperature ranging between 25-600°C at a heating rate of 20°C/ minutes under oxygen.

6. Vibrating Sample Magnetometry (VSM)

Magnetic properties of the polymer-magnetite complexes were measured in the solid state at room temperature using a Standard 7403 Series, Lakeshore vibrating sample magnetometer (VSM). The magnetic moment of each sample was investigated over a range of applied magnetic fields from -10000 to +10000 G using 30 minutes sweep time. Mass specific magnetizations were calculated using the concentration of iron measured by atomic absorption spectrometer and assuming that all irons were in the form of magnetite.

7. UV-Visible Spectrophotometer

Indomethacin concentrations were determined using SPECORD S100 UV-Visible spectrophotometer (Analytikjena AG) coupled with a photo diode array detector. A standard curve at λ_{max} = 320 nm UV absorbance was established using identical conditions to calculate the amount of the drug entrapped on and released from the particles.

8. Gel Permeation Chromatography (GPC)

Molecular weights and polydispersity index (PDI) of mPEG-polyester copolymers were determined by GPC. GPC data was conducted on PL gel 10 μm mixed B2 columns and a refractive index detector. Tetrahydrofuran (THF) was used as a mobile phase with a flow rate of 1 ml/min at 30°C.

9. Photo Correlation Spectroscopy (PCS)

Hydrodynamic diameter of the particles was measured by PCS using NanoZS4700 nanoseries Malvern instrument. DI water used as a dispersing media was filtered through 0.22 μm nylon syringe filters before used. The aqueous dispersions of the particles were sonicated for 10 min before the measurement without filtration.