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ORIGINAL ARTICLE

Encapsulation of Doxorubicin and Chrysin on magnetic PCL-PEG-PCL nanoparticles: Optimization of parameters and drug delivery evaluation

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Abstract

Iron oxide nanoparticles are smart materials that have been commonly used in medicine for diagnostic imaging, drug delivery, and therapeutic applications. In this study, Iron oxide nanoparticles and Doxorubicin (DOX)- Chrysin (Chr), were absorbed into triblock copolymer (PCL-PEG-PCL) for narrow behavior. PCL-PEG triblock copolymers were synthesized by ring-opening polymerization of ε-caprolactone (ε-CL) with polyethylene glycol (EG) as an initiator. The bulk properties and chemical structure of these copolymers were characterized using Fourier transform infrared spectroscopy and ^{1}H-NMR. In adding together, the consequential particles were characterized by scanning electron microscopy, X-ray powder diffraction, vibrating sample magnetometry, and zeta potential measurement. Response surface methodology (RSM) was employed to study the effects of the three most important parameters on encapsulation efficiency, namely DOX and Chr weight (2-18 mg), ε -CL weight (0.6-3.8 g), and sonication time (15-75s). The optimum encapsulation conditions were: 11.2 mg for DOX and Chr weight, 3.75 g for ε -CL, and 48.15 s for sonication time. The highest encapsulation efficiency in these conditions predicted by the equation is 95.68% and the release profile was controlled. There is potential for the application of Fe3O4- PCL-PEG_{4000} magnetic nanoparticles for biomedical purposes.

Keywords: *Chrysin; Doxorubicin; Iron Oxide Nanoparticles; PCL-PEG; Triblock Copolymer.*

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INTRODUCTION

Nanotechnology and one of its most important branches, medical nanotechnology, has been able to advance rapidly in the medical field and replace traditional treatment methods with new, more modern, cheaper, and more effective systems in the fight against diseases. In recent years, the development of nanoparticles in the field of controlled drug release has attracted much attention and has become one of the best choices in biomedical and bioengineering applications. Compared to cancer cells, nanoparticles are much smaller in size, which gives them the ability to reach the site of the disease and cross cellular barriers through enhanced permeability and retention, which is very useful in passive drug delivery [1-4].

Magnetic nanoparticles (MNPs), especially iron oxide nanoparticles (Fe_3O_4), have become one of the most important and exciting nanostructures in the field of nanomaterials that have many applications in targeted drug delivery, increasing the contrast of MRI images and killing cancer cells through hyperthermia. MNPs have a high potential in the field of targeted drug delivery, in that they can be accumulated in the target tissue under the influence of an external magnetic field and

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eventually release the drug carrier into the target area. Therefore, healthy tissues are not exposed to highly toxic drugs such as chemotherapy drugs, and as a result, the side effects of treatment are significantly reduced [5-8].

In addition, the results of preclinical and clinical trials demonstrate the high biocompatibility of MNPs, and several formulations of them were found to be used in MRI and drug delivery such as Combidex® Resovist®, Endorem®, and Sinerem® [9-11]. Polymeric nanoparticles, based on the type of application and type of drug encapsulated, are prepared with various methods. Biodegradable polymer nanoparticles are highly preferred over other types of carriers because of their promising feature in drug delivery. These nanoparticles have the characteristics of controlled and continuous release, have a size in cellular dimensions, and are biocompatible with tissues and cells [12-15].

Flavonoids are natural polyphenolic materials generally considered due to several properties such as anti-inflammatory, anti-oxidant, and anti-proliferative proficiencies [16]. Chrysin (Chr), (5, 7-dihydroxyflavone or 5, 7-dihydroxy-2-phenyl-4-Hchromen-4-one), as a natural flavonoid is known as a biologically active compound that is found mainly in honey, propolis, and passion fruit [17]. Glucuronide and chrysin sulfate as the main metabolites of chrysin are substrates for ABCC2 (MRP2) [18, 19]. However, their poor water solubility limits their pharmaceutical applications [18, 20]. Therefore, to overcome these biological limitations, several procedures based on polymeric nanoparticles have been developed. In this study, magnetite-coated PCL–PEG–PCL nanoparticles as a successful drug delivery system are reported. Therefore, the produced triblock copolymer through the ring-opening polymerization was modified by the organized magnetic nanoparticles. Then, the process of Dual drug encapsulation of DOX and Chr in nanoparticles was followed by the double emulsion method (w/o/w). The nanoparticle properties were specified in the expression of size, and in vitro release of DOX and Chr. Response surface methodology (RSM) was employed to study the effects of the three most important parameters on encapsulation efficiency (EE), namely DOX and Chr weight, ε -CL weight, and sonication time [21].

EXPERIMENTAL

Materials and Methods

Ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), and ammonium hydroxide were bought from Fluka. ε-Caprolactone $(PCL)_{1000}$, polyethylene glycol $(PEG)_{4000}$, stannous octoate $(Sn(Oct)_2$, and polyvinyl alcohol (PVA) were obtained from Sigma-Aldrich (USA). Chrysin and Doxorubicin hydrochloride were purchased from Sigma-Aldrich (USA). Analysis of scanning electron microscopy (SEM) was achieved by the use of the KYKY-EM3200. The magnetic properties were measured by using a magnetometer (Meghnatis Daghigh Kavir). Zeta potential measurements were made with a zeta potential analyzer (Shimadzu, Japan). Infrared spectra were recorded at room temperature with BRUKER series FTIR. ^{1}H-NMR Bruker Avance 400 instrument was used to identify the chemical structure of copolymers. The drug-loading capacity was investigated using an ultraviolet-visible spectrometer (Shimadzu).

preparation of Fe_3O_4 nanoparticles

Fe_3O_4 nanoparticles were obtained using an improved chemical coprecipitation method [22]. At the first step, $FeCl_2 \cdot 4H_2O$ (0.008 mol, 1.5868 g) and of $FeCl_3 \cdot 6H_2O$ (0.014 mol, 3.7842 g) were dissolved in DI water (320 mL). Then the reaction was followed under N_2 at 80 °C for 1 h. After that, ammonium hydroxide (32 wt.%) (40 mL) was quickly added to the solution and stirred under N_2 for another 1 h. Finally, the obtained nanoparticles were washed three times with hot water and dried under vacuum at 70 °C.

Preparation of PCL (1000)-PEG (4000)-PCL (1000) triblock copolymers

Triblock copolymer (PCL-PEG-PCL) was obtained through ring-opening polymerization (Fig. 1). First, the reaction was followed with ethylene glycol (1.5 g) as initiator and ε-caprolactone (ε -CL) (according to the experimental design and Table 1) in a dry three-necked flask at 130 ºC under nitrogen atmosphere to complete melting. Then, the polymerization was completed in the presence of $Sn(Oct)_2$ (0.05% (w/w) at 180 ºC for 6 h [23]. Subsequently, the product was dissolved in dichloromethane and precipitated in cold diethyl ether, and the copolymer was dried under vacuum for 48 h at 50 ºC.

Preparation of drug-loaded PCL-PEG-PCL magnetic nanoparticles

Drug-loaded magnetic nanoparticles were

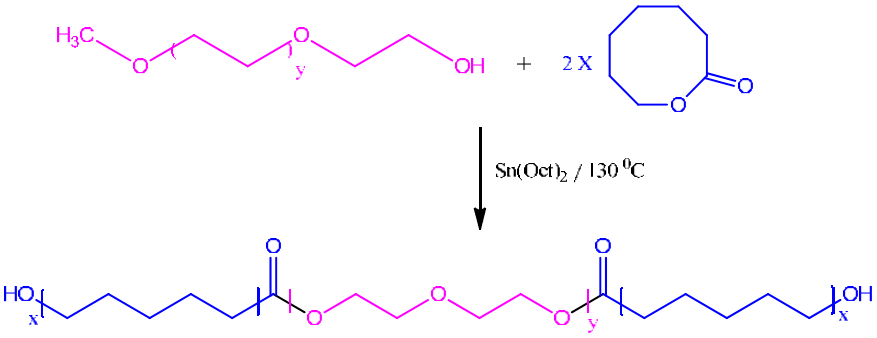


Fig. 1. Preparation of PCL -PEG -PCL triblock copolymers.

Table 1. The main independent variables and their levels used in central composite design.

Variable	-2	-1	0	+1	+2
A: DOX and Chr weight (mg)	2	6	10	14	18
B: ε -CL weight (g)	0.6	1.4	2.2	3	3.8
C: sonication time (s)	15	30	45	60	75

prepared by the dual emulsion (w/o/w) method. At first, Doxorubicin and Chrysin with equal weight ratio, and Fe_3O_4 nanoparticles (10 mg) were added to 200 mg copolymer in 15 ml dichloromethane. The weight of each drug (DOX and Chr in mg) was according to Table 1. The obtained mixture was sonicated (20,000 rpm, 15-75 s) for the formation of the emulsion (w/o). Then, the mixture containing the emulsion (w/o) and PVA (polyvinyl alcohol) (50 ml, 0.5%) was sonicated (70,000 rpm, 2 min) to obtain the emulsion (w/o/w).

After the evaporation of the organic solvent of emulsion (w/o/w), the nanoparticles were centrifuged at least three times at 12000 rpm for 15 minutes in each one of the cycles. Next, the participated nanoparticles were freeze-dried. Finally, the filtration of nanoparticles was achieved by using a 1.2 mm filter (Millipore, Bedford, MA). To determine the encapsulation efficiency of drugs in modified Fe_3O_4 magnetic nanoparticles, nanoparticles were disintegrated in dichloromethane [7]. The concentration of drugs was determined by spectrophotometer at 484, 348 nm for DOX and Chr respectively [24, 25]. The encapsulation efficiency and drug loading were calculated according to Equations (1) and (2) [26]:

$$\text{Encapsulation efficiency}(\%) = \frac{\text{actual drug loading}}{\text{theoretical drug loading}} \times 100 \quad (1)$$

$$\text{Drug loading}(\%) = \frac{\text{weight of remained drug in the nanoparticles}}{\text{weight of nanoparticles}} \times 100 \quad (2)$$

Experimental design

In this study, the weight of DOX and Chr (A), ε- CL weight (B), and sonication time (C) were introduced as RSM input variables. The experimental ranges of factors are shown in Table 1. For Drug-loaded magnetic nanoparticles, a 2^3 factorial central composite design with six axial (α=2) points, eight cube points, and six central points resulting in a total of 20 experimental points was used. Design Expert 11 software was used for experiment design and data analysis. The independent variables and their levels are shown in Table 1. A second-order model (Eq. 3) was used to express encapsulation efficiency (EE) as a function of studied variables.

$$Y = b_0 + \sum_{i=1}^{n}(b_i X_i) + \sum_{i=1}^{n}(b_{ii} X_{ii}^2) + \sum_{ij=1}^{n}(b_{ij} X_i X_j) \quad (3)$$

Where b_o is the value for the fixed response at the central point of the experiment; b_i, $b_{ii,}$ and b_{ij} are the linear, quadratic, and interaction coefficients, respectively; and n is the number of variables. Y and X represent the response and independent variables, respectively. ANOVA was used to fit the second-order polynomial equation. A small

P-value ($p<0.05$) represents the significance of each term in the model on the response variable. Encapsulation efficiency (EE) was taken as a response. The experimental runs were performed in a randomized manner to eliminate the effects of the error. Experiments were performed again under optimal conditions to confirm the validity of the model.

In Vitro Drug Release

To study drug release, the first 3mg of lyophilized nanoparticles containing doxorubicin and chrysin were suspended in phosphate buffered solution (5.0 mM, 30 ml, pH = 7.4) at 37 ºC. The release medium (~3 ml) was removed at specified intervals and analyzed by a UV–Vis spectrometer to control the total of doxorubicin and chrysin released (λex 484 and 348 nm for DOX and Chr respectively). And also, to investigate the effects of pH and temperature on drug release, the release process was followed at pH = 5.8 and 40 ºC [27].

RESULTS AND DISCUSSION

Characterization

According to Fig. 2 (a, b, c), the FTIR spectrum of magnetic nanoparticles showed absorption bonds at 576, and 3433 cm^{-1} attributed to the Fe–O bonds

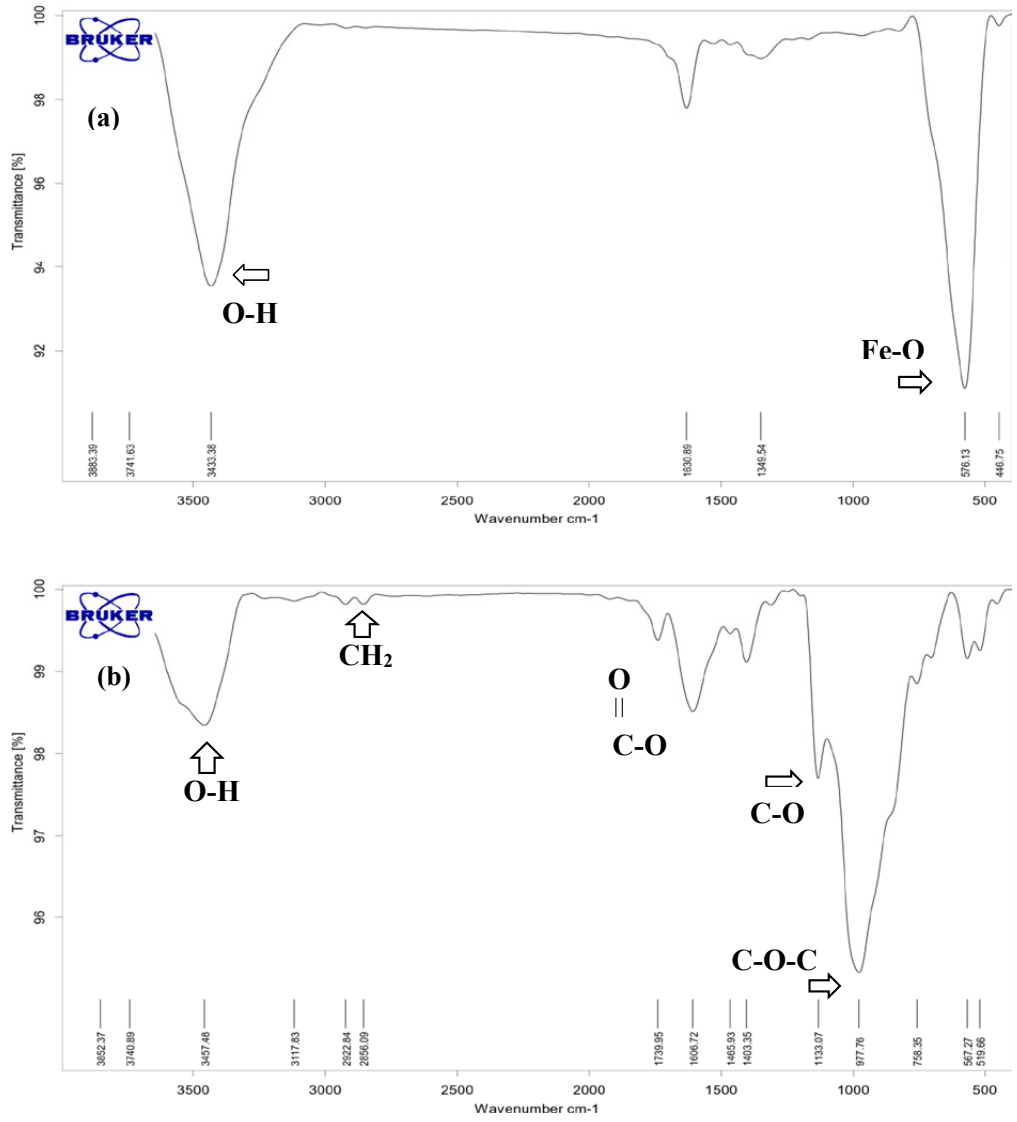
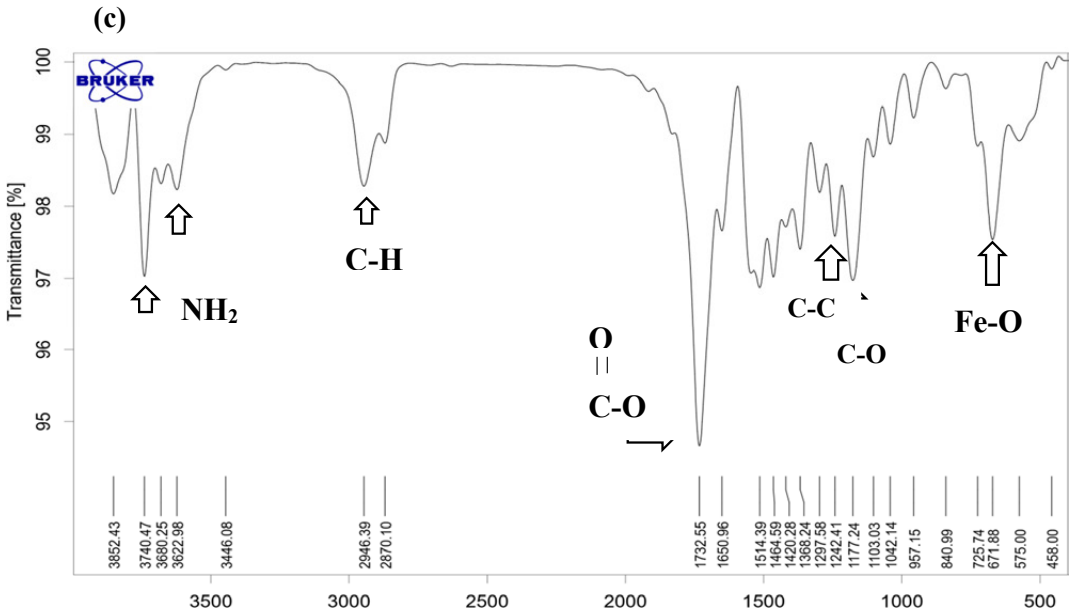


Fig. 2. a) FTIR spectrum of magnetite nanoparticles (Fe_3O_4), b) PCL_{1000}-PEG_{4000}-PCL_{1000} copolymer, c) and drugs loaded with magnetic nanoparticles – copolymer.



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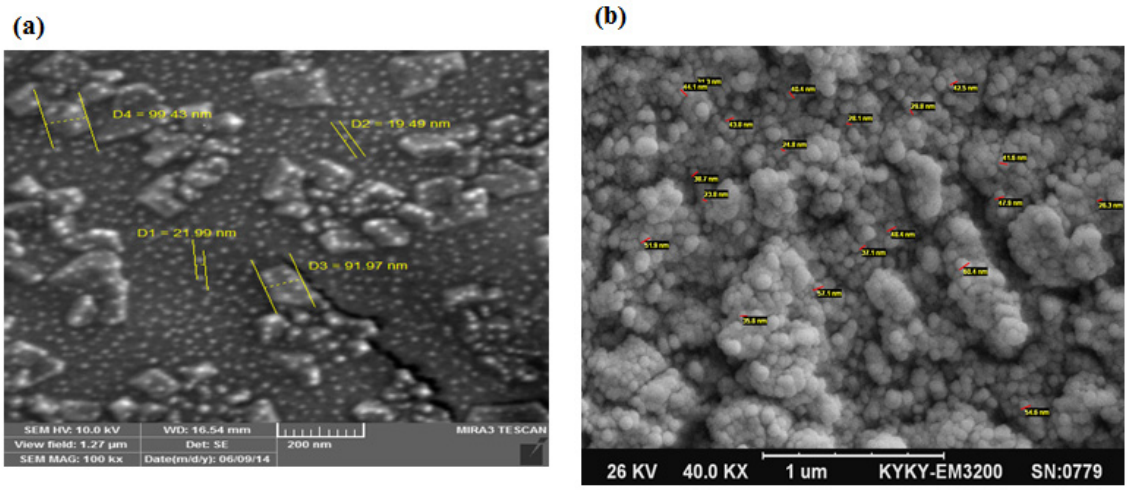

Fig. 3. a) SEM image of pure magnetic nanoparticles (Fe_3O_4), b) drugs loaded with magnetic-copolymer nanoparticles (Fe_3O_4- PCL_{1000}-PEG_{4000}- PCL_{1000}- Dox-Chr).

and the hydroxyl groups of Fe_3O_4, respectively. The CH stretching vibrations were observed at 2869 and 2950 cm^{-1}. Additionally, the absorption bands at 977, 1113, and 1739 cm^{-1} were related to the C–O and C=O bonds in polymeric magnetic nanoparticles. Also, the stretching vibrations at 3622 and 3680 cm^{-1} were related to the NH_2 group in drug-polymeric magnetic nanoparticles [21,23].

The morphology of nanoparticles was investigated by scanning electron microscope (SEM). As shown in (Fig. 3a), the photograph of the pure magnetic nanoparticles is well aggregated, with a particle size of about 40 nm. The SEM results revealed that the particle size of the drug-encapsulated magnetic modified nanoparticles was less than 100 nm (Fig. 3b).

The ^{1}H-NMR spectrum of the triblock copolymers is shown in Fig. 4. The chemical

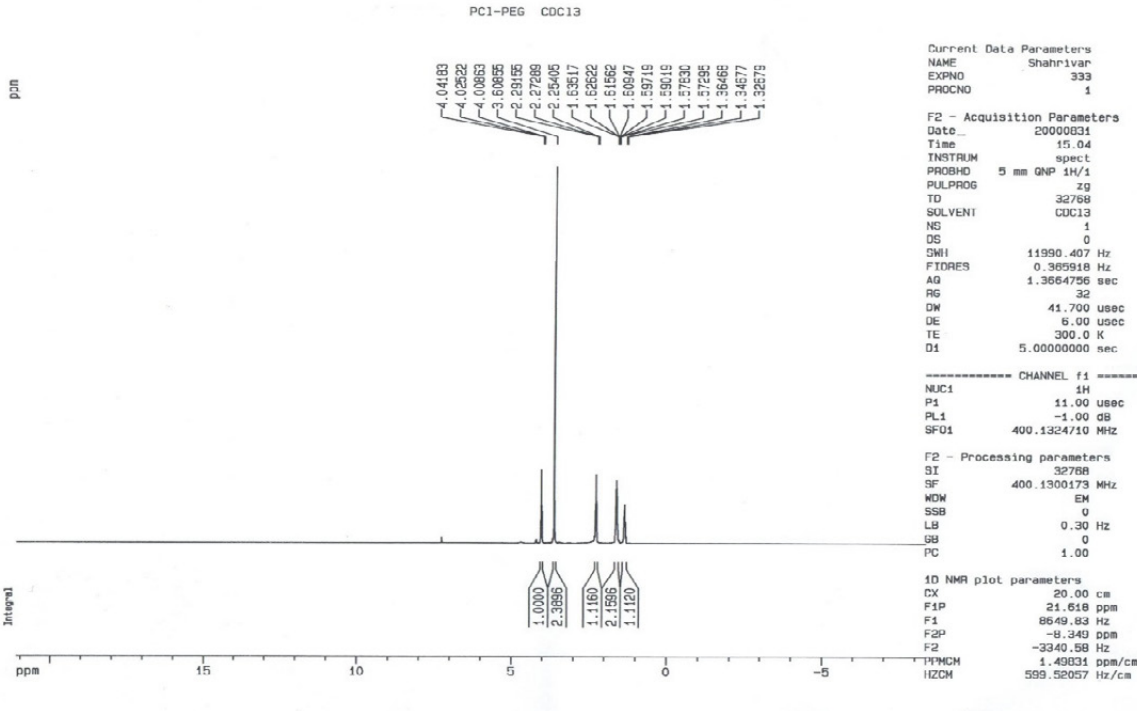

Fig. 4. ^{1}H-NMR of PCL-PEG-PCL triblock copolymers.

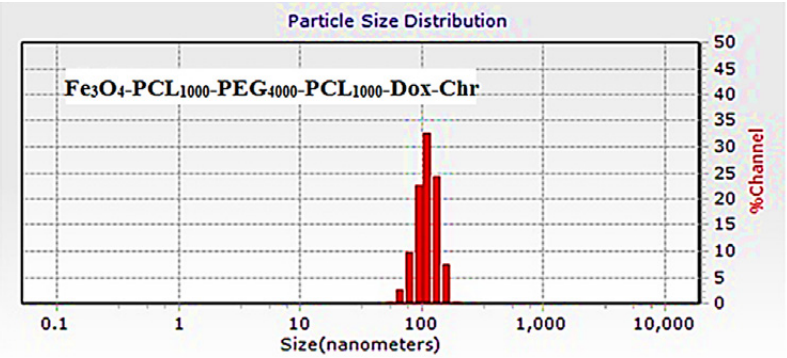

Fig. 5. The size distribution of drugs loaded with magnetic-copolymer nanoparticles.

displacement of protons 1.24, 1.2, 1.3, and 4.06 corresponds to -(CH_2)$_3$-, -- CH_2OOC-- and -OCCH_2- in polycaprolactone. The sharp peak at 3.66 ppm is related to the chemical displacement of protons in polyethylene glycol. The chemical displacements at 4.1 ppm are protons of O-CH_2-CH_2 polyethylene glycol [28].

The distribution size of nanoparticles was measured through the Dynamic light scattering (DLS) (Fig. 5). The hydrodynamic size of particles obtained 100 nm with a polydispersity index (PDI) 0.5, signifying a reasonably fine particle size distribution.

The surface charge of nanoparticles was measured by the zeta potential. The zeta potential of nanoparticles as shown in Fig. 6 was negative and equal to -0.8 mV before drug loading and -16.2 mV after drug loading [29]. Zeta potential and DLS data results are shown in Table 2. The results were in good agreement with SEM images.

The magnetic nature of the nanoparticles was characterized through the vibrating-sample magnetometer (VSM). The hysteresis loops of the samples are displayed in Fig. 7. The

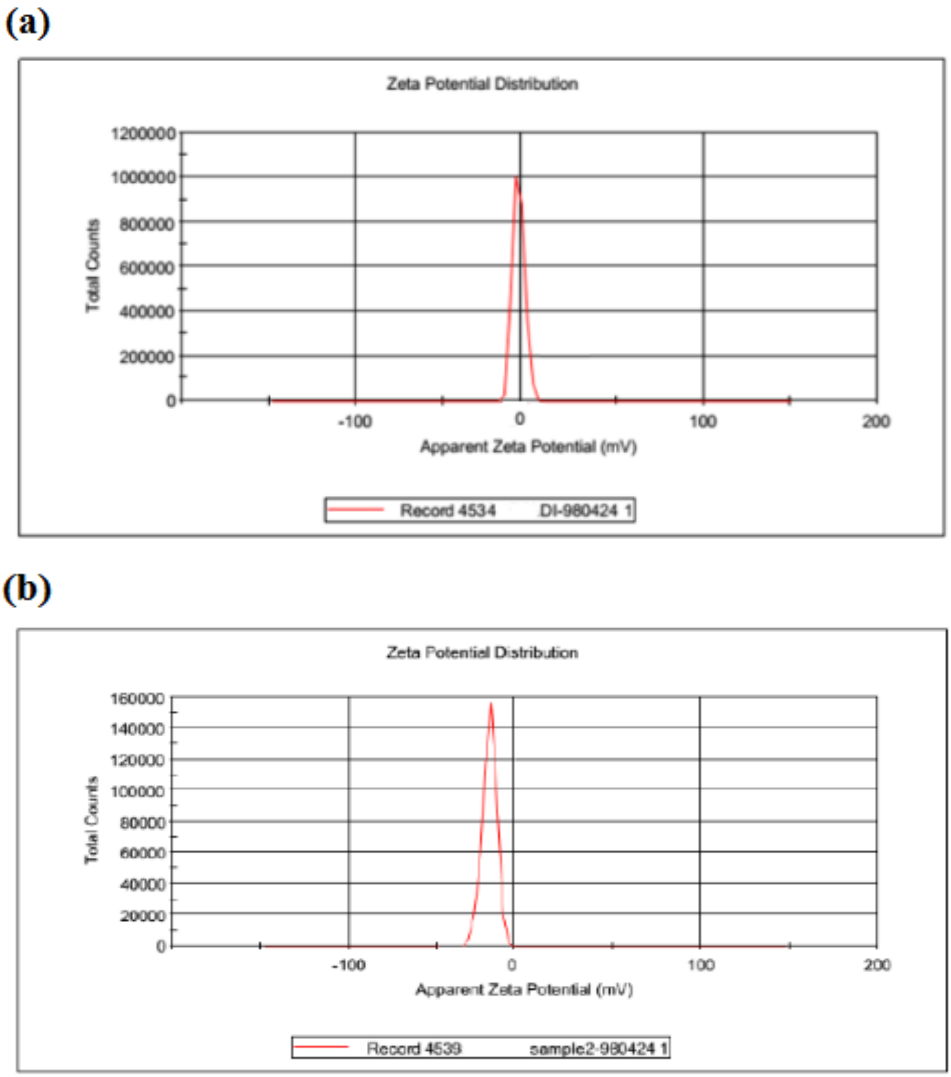

Fig. 6. The zeta potential changes of nanoparticles a) before, b) after drug loading.

saturation magnetization of copolymer coatings containing doxorubicin-chrysin was found to be 5 emu/g, which was much lower than pure iron magnetization (60 emu/g). Thus, it is promising to distinct them from the drug-encapsulated magnetic nanoparticles in a magnetic field. In addition, the nanoparticles exhibited the superparamagnetic (SPM) properties due to the lack of hysteresis loop, coercivity, and remanent magnetization at room temperature [21, 23].

CCD model and residuals analysis

The encapsulation efficiency obtained from experimental runs is shown in Table 3. As shown in Table 3, encapsulation efficiency varied from 73.5 to 96.5 %. ANOVA table for the Quadratic model for encapsulation efficiency is given in Table 4.

As clearly observed in Table 4, except sonication time, the linear and quadratic terms of all independent variables had significant ($p<0.05$) effects on encapsulation efficiency. The results indicated that DOX and Chr weight, ε -CL weight, and sonication time had more significant effects on encapsulation efficiency, respectively, due to their high F ratio values (Table 4). The correlation between the experimental data and the predicted responses is evaluated quantitatively by the correlation coefficient (R^2). The results showed that the predicted values matched the experimental values reasonably well with

Table 2. Zeta potential and DLS data results for nanoparticles.

Name	Polarity	Zeta potential(mv)	PDI	Size (nm)
NP before drug loading	Negative	-0.8	-	40 nm
NP after drug loading	Negative	-16.2	0.5	100 nm

Table 3. The 3-factor central composite design matrix and the value of response function (Encapsulation Efficiency %).

Std	A: DOX and Chr weight (mg)	B: ε-CL weight (g)	C: sonication time (s)	Encapsulation Efficiency (EE) %	
				Experimental	predicted
1	6	1.4	30	81.2	82.4
2	14	1.4	30	90.2	91.4
3	6	3	30	85.2	86.2
4	14	3	30	89.2	90.6
5	6	1.4	60	81.9	82.5
6	14	1.4	60	88.2	89.2
7	6	3	60	89.57	90.3
8	14	3	60	91.6	92.4
9	2	2.2	45	73.5	72.7
10	18	2.2	45	84.9	83.7
11	10	0.6	45	84.2	83.2
12	10	3.8	45	91.2	90.2
13	10	2.2	15	92.8	91.4
14	10	2.2	75	93.9	93.3
15	10	2.2	45	94.6	95.2
16	10	2.2	45	94.8	95.2
17	10	2.2	45	96.5	95.2
18	10	2.2	45	95.5	95.2
19	10	2.2	45	95.3	95.2
20	10	2.2	45	96.5	95.2

Table 4. ANOVA table for the Quadratic model for Encapsulation Efficiency.

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	693.39	9	77.04	41.57	< 0.0001	significant
A-DOX and Chr weight	121.72	1	121.72	65.68	< 0.0001	
B- ε -CL weight	49.25	1	49.25	26.57	0.0004	
C-sonication time	3.68	1	3.68	1.98	0.1893	
AB	10.74	1	10.74	5.80	0.0368	
AC	2.73	1	2.73	1.47	0.2531	
BC	8.14	1	8.14	4.39	0.0625	
A^2	453.73	1	453.73	244.83	< 0.0001	
B^2	113.33	1	113.33	61.15	< 0.0001	
C^2	12.69	1	12.69	6.85	0.0257	
Lack of Fit	15.20	5	3.04	4.56	0.0607	not significant
R^2	0.9740					
Adjusted R^2	0.9505					

R^2=0.974. It meant that 97.4 % of the variations in EE were explained by the proposed model and only 2.6 % of variations could not be explained by the model. Adjusted R^2 (Adj-R^2) is also a measure of the goodness of a fit and it is more suitable for comparing models with different numbers of independent variables. If there are many terms in a model and not a very large sample size, Adj-R^2 may be visibly smaller than R^2. The value of Adj-R^2 was found to be 0.9505, which was very close to the corresponding R^2 value. The final equation in terms of actual factors is shown in Table 5.

The resulted regression coefficients showed that all the main terms had a positive effect on encapsulation efficiency. It means that at low drug weight, ε -CL weight, and sonication time, the encapsulation efficiency increased by an increase in all the mentioned independent variables and

Table 5. Final equation in terms of actual factors.

Encapsulation Efficiency (EE)	=
+29.02	
+7.23	DOX and Chr weight
+16.63	ε -CL weight
+0.23	sonication time
-0.36	DOX and Chr weight * ε -CL weight
-0.0097	DOX and Chr weight * sonication time
+0.084	ε -CL weight * sonication time
-0.265	DOX and Chr weight ^{2}
-3.317	ε -CL weight ^{2}
-0.0031	sonication time^2

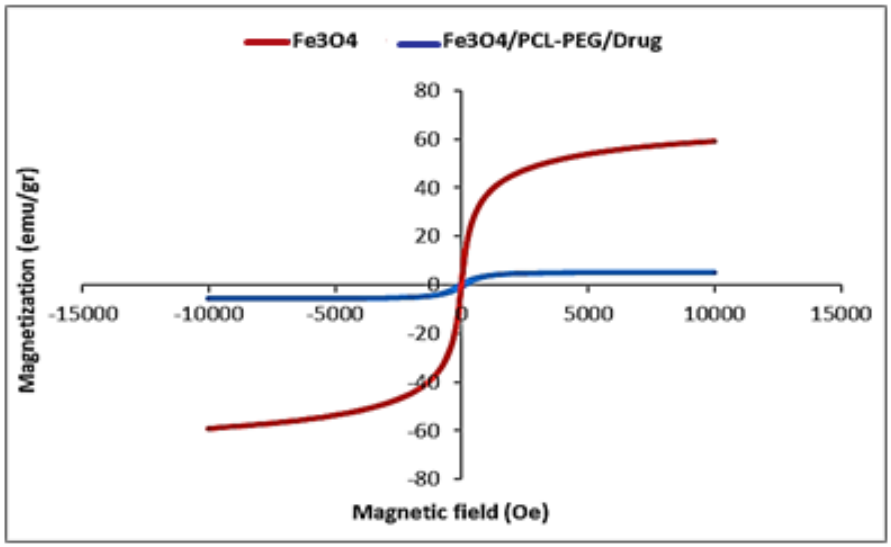

Fig. 7. Magnetic nature of nanoparticles.

vice versa. The opposite results were obtained for the effects of all the independent variables at their high level on the encapsulation efficiency (Table 5).

As clearly observed in Table 4, the interaction of drug weight and ε -CL weight had a significant effect on encapsulation efficiency. While other interactions had non-significant effects on encapsulation efficiency. Figs. 8 and 9 show the interactive effect of DOX and Chr weight and ε-CL weight on encapsulation efficiency in sonication time of 45s. As clearly observed in these figures, at constant CL weight, by increasing drug weight the encapsulation efficiency was increased in the low amount of DOX and Chr weight (A<11) and then in the high amount of DOX and Chr weight, the encapsulation efficiency was decreased. The results obtained in the present study were in agreement with the findings of Manjili *et al*. [30]. They found that with increasing CL/EG feed from 0.5 to 2, the degree of polymerization was increased. Also in their work, the drug loading and encapsulation efficiencies increased with the increase in drug/PCL–PEG–PCL mass ratios (from 0.05 to 1), but the stability of the micelles decreased due to aggregation. So the middle value was chosen for further investigation [30].

Optimization and verification of encapsulation conditions

The amount of ε -CL weight, DOX and Chr weight, and sonication time would be considered optimum if encapsulation efficiency attained the largest possible values. Numerical optimization was used to find the exact optimum levels of the studied variables. The optimum encapsulation conditions were: 3.75 g ε -CL, 11.2 mg DOX and

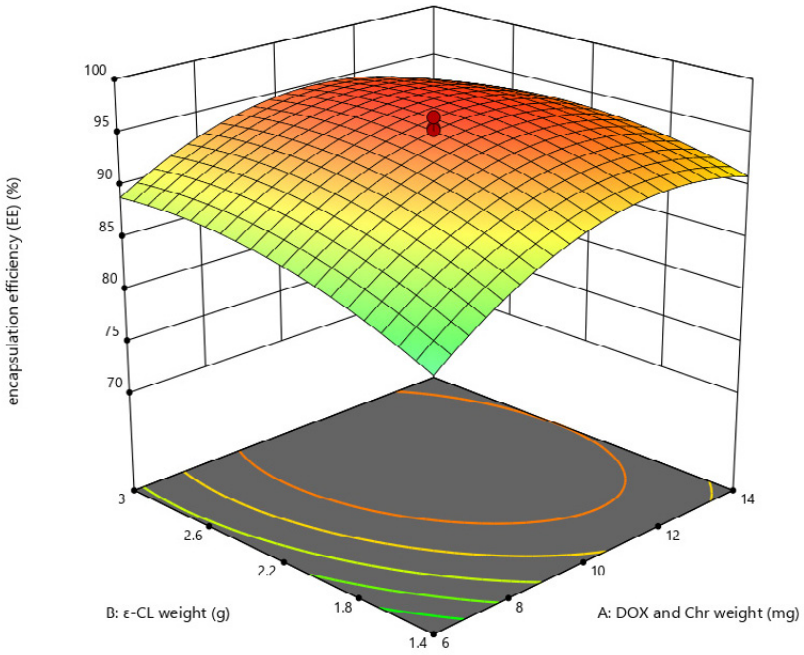

Fig. 8. Response surface plot of encapsulation efficiency versus ε -CL weight and drug weight.

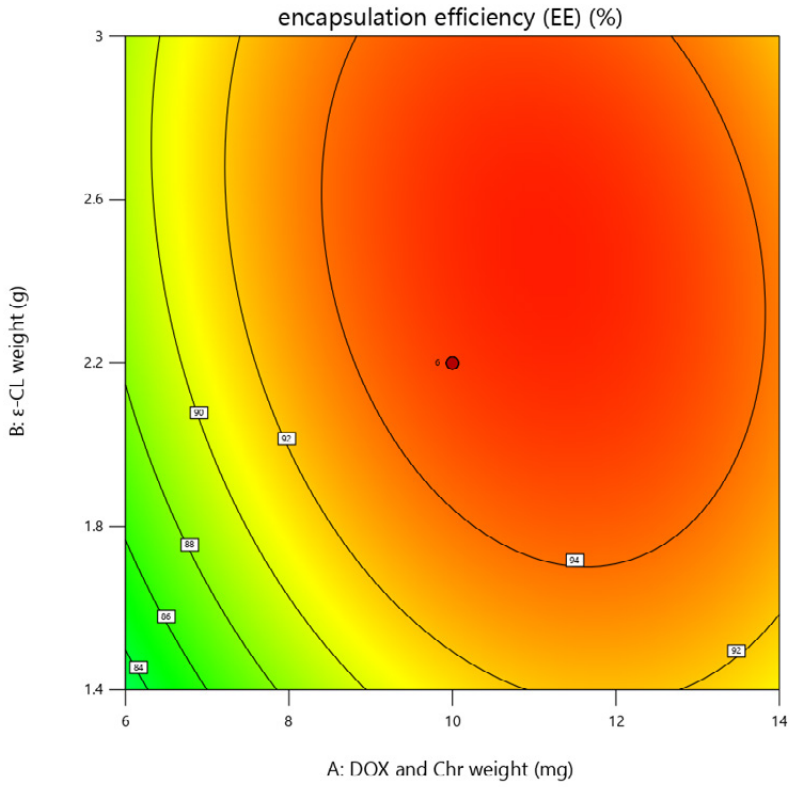

Fig. 9. Contour plot of encapsulation efficiency versus ε -CL weight and drug weight.

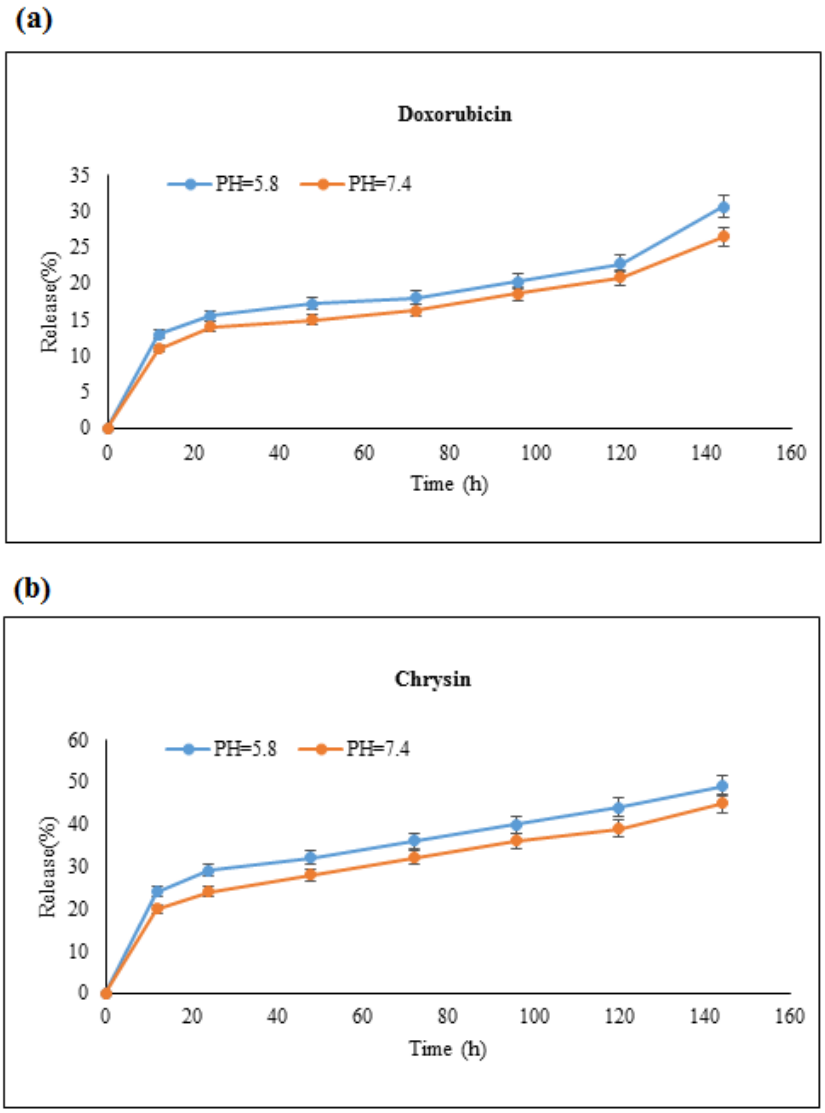

Fig. 10. a) In vitro release experiment of Doxorubicin, b) In vitro release experiment of Chrysin.

Chr weight, and 48. 15 s for sonication time. The highest encapsulation efficiency in these conditions predicted by the equation was 95.68%. The optimal conditions were verified using an experimental test and were in good agreement with the experimental result (95.5%), implying that the model derived from RSM could be used to adequately describe the relationship between the factors and response in encapsulation. The obtained optimum values approximately were closer to the center point and also the condition of row 12 in Table 3. The drug loading was 9.65% in optimum condition.

Process of the in vitro drug release

The release outlines of drugs were attained by the percentage of the drug release to the total quantity of loaded drug. Two phases were observed for the release of drugs from the prepared nanoparticles. So that, the burst release in the initial 12 h followed by the linear sustained release in the second phase. The main release of Doxorubicin (Fig. 10a) and Chrysin (Fig. 10b) from magnetic PCL–PEG–PCL nanoparticles was achieved within 12 h. The full release of the drug was over 7 days. The rate of drug release from the nanoparticles was also pH-dependent and

improved at pH 5.8. As a result, the release of doxorubicin and chrysin was performed at pH 5.8 more than pH 7.4. Therefore, it could be expected that the release rate of the drug in the acidic medium of the extracellular fluid of the tumor was better than that of the other cells [21]. The faster degradation of hydrophobic PCL core in acidic pH and the protonation of the amino group of drugs at lower pH which could lead to an increase in drug solubility in the acidic pH were the reasons for this pH-dependent releasing behavior [31].

CONCLUSION

In this study, duel drug encapsulation of DOX and Chr on the biodegradable PCL–PEG–PCL-coated magnetic nanoparticles were reported. Location and capacity of drug release were organized by magnetic nanoparticles and pH. Various factors were effective on encapsulation efficiency, such as concentration of copolymer in organic solution, the concentration of DOX and Chr in the inner aqueous phase, and the time and speed of homogenization [30]. The optimum encapsulation conditions using the RSM method were obtained: 3.75 g ε -CL weight, 11.2 mg DOX and Chr weight, and 48. 15 s for sonication time. The highest encapsulation efficiency in these conditions predicted by the equation was 95.68%. The results showed the positive effects of PCL nanoparticles on drug delivery of two drugs and loading these compounds into PCL-PEG-PCL nanoparticles improves the solubility of these compounds, the gradual and continuous release of the drug, and ultimately improving their anti-cancerous effects. The results also showed that the combination of the two drugs created a higher lethality than the single and free form of drugs and reduced the process of tumor growth. Finally, the combined effect of the two drugs greatly reduced the resistance of cancer cells to treatment [32, 33]. So the magnetic modified PCL–PEG–PCL nanoparticles with pH-sensitive properties could be used as an effective carrier for anticancer drug delivery.

CONFLICT OF INTERESTS

There is no conflict of interest.

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