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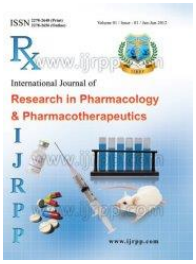

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Research article ***Open Access***

Optimization of cytarabine loaded nanocochleates for targeting LEUKEMIA by response surface methodology

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ABSTRACT

The main approach of the Nanocochleates is to target the cancer cells for the effective treatment against leukemia. Nanocochleate is more stable than any other drug carriers such as Liposomes, Niosomes, Nanoparticles and Nanoliposomes. Nanocochleate drug delivery is most effective in case of both hydrophilic and hydrophobic drugs. Cytarabine is used for the leukemic treatment since long time and its pharmacological effect is well established for the nanocochleate preparation, as it is suitable for the formulation: by increasing the half-life, to avoid getting converted to inactive metabolite by intestinal enzymes, increases the permeability, reduces the dose etc. The storage and release of the conventional dosage form was affected by environmental factors as it can be overcome by the nanocochleates. Nanocochleates are prepared by using the various cross-linking agent like, calcium chloride, zinc chloride and chitosan. The Nanocochleates is optimized by the Response surface methodology using a Box and Behnken's design, as it allows the determination of various factors on nanocochleates properties with a minimum number of experiments. The drug loaded Nanocochleates was prepared with high entrapment efficiency and prolonged drug release. From the results it can be concluded that the novel Nanocochleates drug delivery system has a promising carrier for treatment of leukemia.

Keywords: Nanocochleates, Cytarabine, Cross-linkers, Controlled drug delivery, Target delivery.

INTRODUCTION

Nanocochleate (NC) delivery vehicles are stable phospholipid-cation precipitates composed of simple, naturally occurring materials, generally phosphatidylserine and calcium. They have a unique multilayered structure consisting of a solid, lipid bilayer sheet rolled up in a spiral or in stacked sheets, with little or no internal aqueous space [1]. Nanocochleates are derived from liposomes by trapping method. The liposomes have the lipid layer with the negative charge, on combination with the positively charged cations forms the Nanocochleates. They have the potential for slow or timed release of the biologic molecule *in-vivo* as nanocochleates slowly unwind or otherwise dissociate.

Cytarabine, a synthetic pyrimidine nucleoside, is converted intracellularly, primarily by deoxycytidine kinase, to active cytarabine arabinoside triphosphate. This metabolite then damages DNA by multiple

mechanisms, including the inhibition of alpha-DNA polymerase, inhibition of DNA repair through an effect on beta-DNA polymerase, and incorporation into DNA [2]. The latter mechanism is probably the most important. Cytarabine is a cell cycle phase-specific antineoplastic agent, affecting cells only during the S-phase of cell division.

Cytarabine when introduced orally, it will get degraded from the rapid deamination of cytarabine into an inactive 1-β-D arabino furanosyl uracil (Ara-U) by cytidine deaminase *in-vivo*, the poor membrane permeability ascribes to the hydrophilicity, the extremely short plasma half-life (10–20 min) and drug multi resistance. Less than 20% of the orally administered drug is absorbed from intestine, and the 13% of the drug is protein bound. The drug is metabolized in the liver and excreted by kidneys. Similarly, the drug reaches to the site of action is reduced considerably in an active form due to hepatic portal system. The drug should be stored protected from the light in an air tight container [3, 4, 5].

Moreover, to overcome this drawback other dosage forms for the parenteral is evolved like liposomes, I.V. infusion of active metabolite of cytarabine(ara-C). But it has its own disadvantages like storage problems etc. The nanocochleate here prepared overcomes all those and gives an ideal solution for the leukemic treatment. The major advantage of the NCs is that it is suitable for the hydrophobic and hydrophilic drugs as reported but cytarabine belongs to the highly soluble in water and poorly permeable as protein binding is only 13%. So, this NC helps to deliver the drug to the site of action and along with the sustained rate of release [6, 7, 8, 9]. As NCs does not allow the external factors to interfere with the drug like oxygen, moisture etc. It has the pH 7-9.5 which suits the anticancer acidic environment for the better delivery of the drug to the target cells [10, 11, 12, 13, 14].

The box behnken's method is used to study the various factors like drug concentration, lipid ratio and cross- linkers for the NCs. The Box-Behnken factorial design was used for the analysis. By varying the cross-linkers; calcium chloride, zinc chloride and chitosan, the size and shape of the NC is altered. The design expert of version 11 is used for this purpose

MATERIALS AND METHODS

Cytarabine and cholesterol were purchased from Sigma Aldrich Chemical Private Ltd, Bangalore, India. Soya Phosphatidyl Choline (SPC) was gifted from Lipoid GmbH Ludwigshafen, Germany. Ethanol, Ethylene Diamine Tetra Acetic Acid (EDTA), calcium chloride and zinc chloride were of analytical grade. Chitosan was purchased Sigma, USA. Water were of HPLC grade and procured from Merck, Mumbai, India. All other solvents used for study were of analytical grade.

Preparation of Cytarabine-Loaded NCs

Cytarabine-loaded small unilamellar vesicles were prepared by Ethanol Injection method. Briefly, specific amount of cholesterol and Soya Phosphatidyl Choline (SPC) were dissolved in 2 ml of ethanol and heated up to the phase transition temperature (40°C) of SPC. This ethanolic solution was rapidly injected into 10 ml of the phosphate buffer (pH 7.4) containing cytarabine, which was kept under stirring at 500 rpm using Teflon coated beads and it was continued until complete evaporation of ethanol under vacuum. Further, phosphate buffer was added to adjust the volume of final lipid vesicles suspension to 10 ml. Now the vesicles were passed through a membrane filter of 0.22 µm to obtain a cytarabine-loaded small unilamellar vesicle suspension with uniform size.

Cytarabine-loaded NCs were prepared by Trapping method. 20µl of calcium chloride solution (0.1 M) or Zinc chloride or chitosan solution were added drop-wise into the prepared Cytarabine-loaded small unilamellar vesicles which were under vortex. The vesicle phase immediately turned turbid because of Nanocochleates formation. Precipitated CYT-NCs were refrigerated at 2-8°C.

Box and Behnken's Model of Theoretical Design

Our preliminary experimental results showed that the preparation and characterization of Nanocochleates are influenced by three factors, including the concentration of drug, ratio of lipids and cross-linking agent. Response surface methodology using a Box and Behnken's design was chosen because it allows the determination of influence of these factors on nanocochleates properties with a minimum number of experiments.

A Box Behnken's design was used to investigate the effect of three factors Lipids (A; X1), Drug concentration (B; X2) and volume of crosslinking agent, calcium chloride (C; X_3); on the response variables Y_1 (entrapment efficiency), Y_2 (Size) and Y_3 (Drug release). The independent factors and the dependent variables used in the design are listed in Table 1. The response surface of the variables inside the experimental domain was analyzed using Stat-Ease design expert software version 11 (version 11, state ease, Inc, USA). Subsequently, three additional confirmatory experiments were conducted to verify the validity of the statistical experimental strategy. The statistical design provides a polynomial describing quadratic effect, as well as the interactions of each study factors on the considered response variable. The general model corresponds to the following equation (1)

$$Y_0 = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 \qquad (1)$$

Where, Y is the measured response associated with each factor level combination; b_0 is an intercept; b_1 to b_{23} are the regression coefficients; and X_1, X_2, and X_3 are the independent variables.

Optical Microscopy Image

The Nanocochleates were viewed under Leica optical microscope. The image which gives the overview idea of the formulation, the uniformity in the particle size, number of the particles formed, the presence of the lipid flakes etc. The sample drop is placed on the glass slide and cover with the glass slip. Then it is focused using the 40x magnification. The images are captured by placing it on a specimen stab. The difference in the structure of the CYT-SUV before the addition of the crosslinking ions and later is found clearly.

Scanning Electron Microscopy (SEM)

The Scanning Electron Microcopy is used to identify the particle size and the surface morphology of the CYT-SUV and NCs. For CYT-SUV, the drop of the liquid sample is placed on the covered glass slide and then dried by applying vacuum, later it was coated with gold to a thickness of 100A using VEGAS TESCAN Vacuum evaporator and the image was captured. The FE-SEM-Field Emission Scanning Electron Microscopy (FEI Quanta) is used for the Nanocochleates. It is prepared by placing a pinch of the lyophilized sample on the carbon tape and stick to the grid for the analysis. The surface morphology of the prepared Nanocochleates is in the cigar shaped/ sticks shape/ Cochleates is obtained.

Transmission Electron Microscopy (TEM)

High-Resolution Transmission Electron Microscopy (HRTEM-Technai G-20) is used to capture the internal morphology. The sample is diluted, and it is placed on the TEM grid, approximately two or three drops is sufficient. Then it is air dried until the water molecule is removed from it. Further it is taken for the analysis.

X-Ray Diffraction Technique (XRD)

X-ray diffraction analysis was performed with a PANalytical Xpert Pro X-ray Diffractometer using Ni filtered Cu kα radiation. The powdered samples to be evaluated was taken on the glass slide and placed on the X-Ray Diffractometry. The scanning rate was 10 minutes over a 2θ range of 10 to 90. The Nanocochleate formed is confirmed through this analysis. The obtained data was then processed and reported as follows. The crystalline or amorphous nature of the sample can be found.

Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared (FT-IR) spectra were recorded using an FTIR spectrometry (FTIR-8400; Shimadzu Corporation, Kyoto, Japan) in the spectral range of 450 to 4000 cm^{-1}. A pinch of the drug added to the potassium bromide and triturated to form a uniform mixture. The pellet is formed by the press pellet technique and then analyzed under the IR, the spectra of these samples is recorded to note any interactions or bond formation between the drug and the excipients.

RESULTS AND DISCUSSION

Box and Behnken's Model of Theoretical Design

The theoretical optimization was used to reduce the number of experiments. The design expert

software of version (11) (Stat-Ease Inc., USA) was used to do this.

Table: 1 List of dependent and independent variables in Box-Behnken's design for CYT entrapped nanocochleates.

			Level Used	
Factor	**Name**	**Units**	**LOW (-1)**	**HIGH (+1)**
A	Lipids	(%w/v)	1:2	1:6
B	Drug	(%w/v)	5	15
C	Cross linker	(%w/v)	0.5	1.5
Response	**Name**	**Units**		
Y_1	EE	%		
Y_2	Size	Nm		
Y_3	Drug Release (Q_{12h})	%		

Table 2: Effect of various process parameters on E.E., Size and Drug release from CYT-NC.

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
Std	**Run**	**A: Lipids**	**B: Drug**	**C: Cross linker**	**EE**	**Size**	**Q12h**
					%	Nm	%
14	1	0	0	0	80	900	96
7	2	-1	0	1	50	650	72
1	3	-1	-1	0	60	760	84
12	4	0	1	1	75	800	80
15	5	0	0	0	80	900	96
8	6	1	0	1	70	800	85
4	7	1	1	0	82	1100	72
17	8	0	0	0	80	900	96
9	9	0	-1	-1	72	800	90
2	10	1	-1	0	85	1000	85
6	11	1	0	-1	76	1170	80
16	12	0	0	0	80	900	96
5	13	-1	0	-1	60	550	99
11	14	0	-1	1	69	600	82
10	15	0	1	-1	65	720	95
3	16	-1	1	0	66	640	90
13	17	0	0	0	80	900	96

Effect of the process parameters on the NC Entrapment Efficiency

The influence of the main and interactive effects of the independent variables on the E.E. was studied using polynomial equation **Eq. (2)**, and it was found to be statistically significant ($P < 0.0001$), as determined using regression analysis and ANOVA **(Table 3 and 4)**:

$EE=+80.00+9.63A+0.2500B-1.13C-2.25AB+1.00AC+3.25BC-6.50A^2-0.2500B^2-9.50C^2$ -Eq.(2)

Table 3: Regression analysis for response Y1 (E.E.)

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0231		0.3936	0.1589	
2FI	0.8011		0.2835	-0.5099	
Quadratic	**0.0009**		**0.8901**	**0.8307**	**Suggested**
Cubic			1.0000		Aliased

Table 4: Summary of ANOVA for response parameters of Y1 (E.E.)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1410.63	9	156.74	15.40	0.0008	Significant
A-Lipids	741.13	1	741.13	72.81	< 0.0001	
B-Drug	0.5000	1	0.5000	0.0491	0.8309	
C-Cross linker	10.13	1	10.13	0.9947	0.3518	
AB	20.25	1	20.25	1.99	0.2013	
AC	4.00	1	4.00	0.3930	0.5506	
BC	42.25	1	42.25	4.15	0.0810	
A^2	177.89	1	177.89	17.48	0.0041	
B^2	0.2632	1	0.2632	0.0259	0.8768	
C^2	380.00	1	380.00	37.33	0.0005	
Residual	71.25	7	10.18			
Lack of Fit	71.25	3	23.75			
Pure Error	0.0000	4	0.0000			
Cor Total	1481.88	16				

The predictive ability of the model was indicated by the calculation of R^2 coefficients, which is a criterion of the model fitting. For all experimented batches, the Y1 (E.E) value showed good R-squared value of 0.8317 (**Table 5**). As the equation shows, independent variables A, B, and C have „p" < 0.05 that has significantly effect on the E.E. It is evident from the results that Lipids are cross linked with calcium Ca^{2+} ion, which can reduce the problem of drug leaching during CYT-NC preparation, as expected.

Table 5: Predicted and Actual value for response Y1 (E.E)

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	80.00	80.00	0.0000	0.200	0.000	0.000	0.000	0.000	14
2	50.00	52.25	-2.25	0.750	-1.410	-1.543	0.597	-2.673[1]	7
3	60.00	61.12	-1.12	0.750	-0.705	-0.677	0.149	-1.173	1
4	75.00	72.63	2.38	0.750	1.489	1.667	0.665	2.888[1]	12
5	80.00	80.00	0.0000	0.200	0.000	0.000	0.000	0.000	15
6	70.00	73.50	-3.50	0.750	-2.194	-3.635	1.444[1]	-6.296[1]	8
7	82.00	80.87	1.13	0.750	0.705	0.677	0.149	1.173	4
8	80.00	80.00	0.0000	0.200	0.000	0.000	0.000	0.000	17
9	72.00	74.38	-2.38	0.750	-1.489	-1.667	0.665	-2.888[1]	9
10	85.00	84.88	0.1250	0.750	0.078	0.073	0.002	0.126	2
11	76.00	73.75	2.25	0.750	1.410	1.543	0.597	2.673[1]	6
12	80.00	80.00	0.0000	0.200	0.000	0.000	0.000	0.000	16
13	60.00	56.50	3.50	0.750	2.194	3.635	1.444[1]	6.296[1]	5
14	69.00	65.63	3.38	0.750	2.116	3.262	1.343[1]	5.650[1]	11
15	65.00	68.38	-3.38	0.750	-2.116	-3.262	1.343[1]	-5.650[1]	10
16	66.00	66.13	-0.1250	0.750	-0.078	-0.073	0.002	-0.126	3
17	80.00	80.00	0.0000	0.200	0.000	0.000	0.000	0.000	13

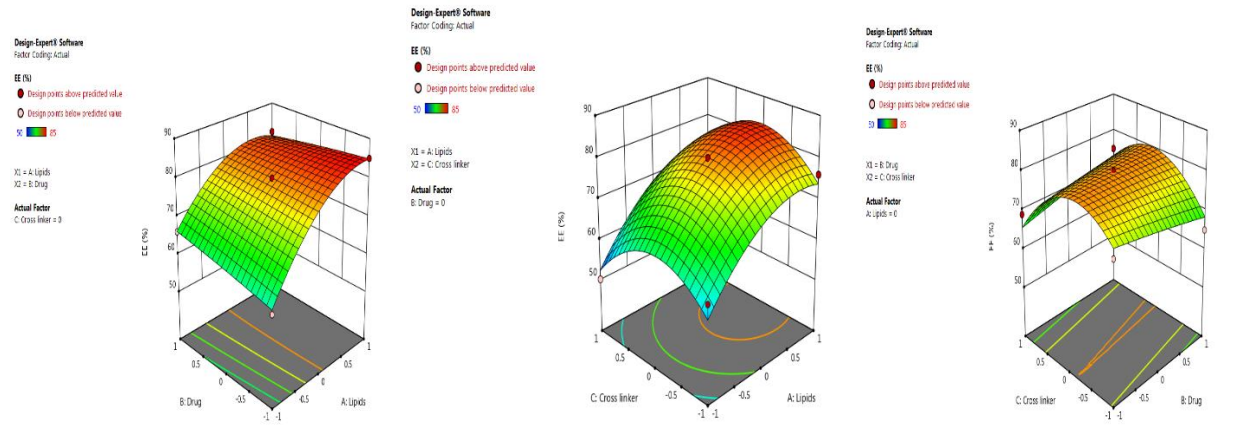

Figure 1. RSM of effect of incorporation of lipid and drug on Entrapment efficiency (EE) of the NC.

The interaction terms (AB, AC, and BC) showed how NC size changes when two independent variables simultaneously changed. The values of coefficients in the equation represent the effect of that term on the E.E. The relationship between the dependent and independent variables that was further elucidated using response surface plots. **Fig. 1** was illustrated RSM of the effect of the incorporation of

lipid on the drug entrapment efficiency of the NC. Figure 1 (A) clearly indicates that EE was found to be increased, while lipid concentration increased. RSM graph confirms that a combination Lipid, Drug and Cross-linker mixture significantly improved the EE.

Effect of the process parameters on the NC size

Uniform and controlled spherical NC size plays an important role in the drug release behavior at the body fluid. Accordingly, the aim of optimizing size was considered to be the minimum value and spherical nature. By considering multiple linear regression analysis, the Y_2 equation is given as below in the Eq.(3).

$$\text{Size} = +900.00+183.75A+12.50B-48.75C+55.00AB-117.50AC+70.00BC+18.75A^2-43.75B^2-126.25C^2 \quad \ldots(3)$$

NCs with low concentration of lipid (−level) were rough and irregular in shape, due to poor molecular packing and cross-linking, in comparison with NCs with medium and high concentration of Lipids (+level), and with more spherical morphology. The response surface plots relating NCs size demonstrated that increasing the lipid concentration led to increasing in NC size **(Fig. 2(A) and (B), Table 6** respectively). The variations in size and morphology of the NC with different lipid concentrations were due to variations in the availability of reacting/binding sites for cross-linking cations (Ca2+). As the cross-linking agent content was increased with lipid, the results were smooth, spherical NC that were well-packed and with discrete structure, Fig. 2(C) reveals that decrease in the average size of these NC was found with the combination of lipid (with increasing concentration). The result could be attributed to the shrinkage of lipid structure by a higher degree of cross-linking between the negative charge of lipid and positivity of calcium ions.

Table 6: Regression analysis for response Y_2 (SIZE)

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0039		0.5438	0.2771	
2FI	0.0597		0.7084	0.2838	
Quadratic	**0.0002**		**0.9709**	**0.7963**	**Suggested**
Cubic			1.0000		Aliased

The influence of the main and interactive effects of the independent variables on the E.E. was studied using polynomial equation, and it was found to be statistically significant ($P < 0.0001$), as determined using ANOVA **(Table 7)**:

Table 7: Summary of ANOVA for response parameters of Y2 (SIZE)

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	4.555E+05	9	50611.27	60.30	< 0.0001	Significant

A-Lipids	2.701E+05	1	2.701E+05	321.84	< 0.0001
B-Drug	1250.00	1	1250.00	1.49	0.2618
C-Cross linker	19012.50	1	19012.50	22.65	0.0021
AB	12100.00	1	12100.00	14.42	0.0067
AC	55225.00	1	55225.00	65.80	< 0.0001
BC	19600.00	1	19600.00	23.35	0.0019
A^2	1480.26	1	1480.26	1.76	0.2258
B^2	8059.21	1	8059.21	9.60	0.0174
C^2	67111.84	1	67111.84	79.96	< 0.0001
Residual	5875.00	7	839.29		
Lack of Fit	5875.00	3	1958.33		
Pure Error	0.0000	4	0.0000		
Cor Total	4.614E+05	16			

The predictive ability of the model was indicated by the calculation of R^2 coefficients, which is a criterion of the model fitting. For all experimented batches, the Y_2 (size) value showed good R-squared value of 0.7963 **(Table 8)**. As the equation shows, independent variables A, B, and C have „p" < 0.05 that has significantly effect on the Y_2 (size).

Table 8: Predicted and Actual value for response Y2 (SIZE)

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	900.00	900.00	0.0000	0.200	0.000	0.000	0.000	0.000	14
2	650.00	677.50	-27.50	0.750	-1.898	-2.524	1.081[1]	-4.371[1]	7
3	760.00	733.75	26.25	0.750	1.812	2.303	0.985	3.988[1]	1
4	800.00	763.75	36.25	0.750	2.503	7.139[2]	1.879[1]	12.366[1]	12
5	900.00	900.00	0.0000	0.200	0.000	0.000	0.000	0.000	15
6	800.00	810.00	-10.00	0.750	-0.690	-0.662	0.143	-1.147	8
7	1100.00	1126.25	-26.25	0.750	-1.812	-2.303	0.985	-3.988[1]	4
8	900.00	900.00	0.0000	0.200	0.000	0.000	0.000	0.000	17
9	800.00	836.25	-36.25	0.750	-2.503	-7.139[2]	1.879[1]	-12.366[1]	9

10	1000.00	991.25	8.75	0.750	0.604	0.574	0.109	0.995	2
11	1170.00	1142.50	27.50	0.750	1.898	2.524	1.081[(1)]	4.371[(1)]	6
12	900.00	900.00	0.0000	0.200	0.000	0.000	0.000	0.000	16
13	550.00	540.00	10.00	0.750	0.690	0.662	0.143	1.147	5
14	600.00	598.75	1.25	0.750	0.086	0.080	0.002	0.138	11
15	720.00	721.25	-1.25	0.750	-0.086	-0.080	0.002	-0.138	10
16	640.00	648.75	-8.75	0.750	-0.604	-0.574	0.109	-0.995	3
17	900.00	900.00	0.0000	0.200	0.000	0.000	0.000	0.000	13

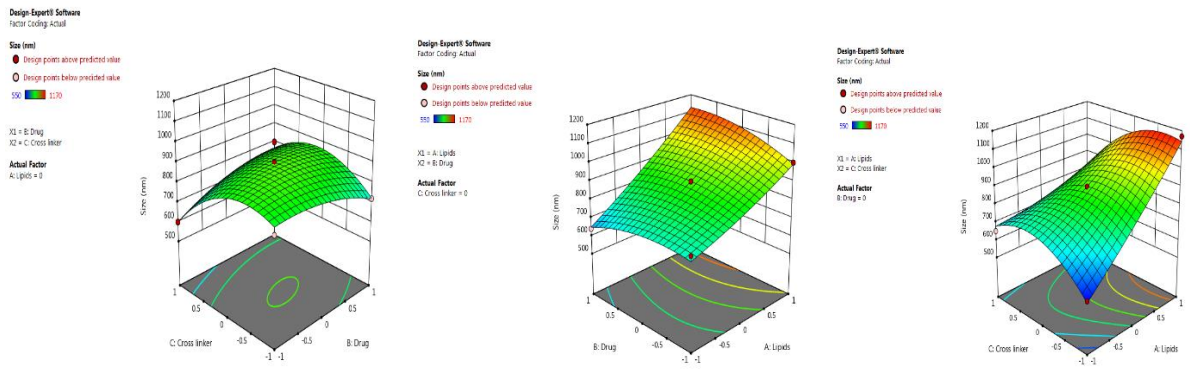

Fig. 2. RSM of effect of incorporation of lipids, drug and cross-linker on NC size.

Effect of the process parameters on NC Drug release

The effect of the variables on drug release is more intricate. The drug release at Q_{12h} was chosen for optimization of the independent variables. Drug release study was simulated in blood pH 7.4 and cancer pH 5.3. The mathematical relationship in the form of a polynomial equation for the measured response, drug release Y_3 is given in the following Eq. (4):

$$\mathbf{Q_{12h} = +96.00-2.88A-0.5000B-5.63C-4.75AB+8.00AC-1.75BC-8.00A^2-5.25B^2-4.00C^2 \ldots(4)}$$

Table 9: Regression analysis for response Y_3 (DRUG RELEASE)

Linear	**0.2441**	**0.0966**	**-0.2734**	
2FI	0.1435	0.3005	-0.1525	
Quadratic	**< 0.0001**	**0.9475**	**0.9324**	**Suggested**
Cubic		1.0000		Aliased

The regression analysis for the response Y_3 was shown in **Table 09**. The influence of the main and interactive effects of the independent variables on the drug release was studied using polynomial equation Eq. (4), and it was found to be statistically significant ($P < 0.0001$), as determined using ANOVA (**Table 10**):

Table 10: Summary of ANOVA for response parameters of Y3 (Q12h)

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	1180.01	9	131.11	33.07	< 0.0001	Significant
A-Lipids	66.13	1	66.13	16.68	0.0047	
B-Drug	2.00	1	2.00	0.5045	0.5005	
C-Cross linker	253.13	1	253.13	63.85	< 0.0001	
AB	90.25	1	90.25	22.77	0.0020	
AC	256.00	1	256.00	64.58	< 0.0001	
BC	12.25	1	12.25	3.09	0.1222	
A^2	269.47	1	269.47	67.98	< 0.0001	
B^2	116.05	1	116.05	29.27	0.0010	
C^2	67.37	1	67.37	16.99	0.0044	
Residual	27.75	7	3.96			
Lack of Fit	27.75	3	9.25			
Pure Error	0.0000	4	0.0000			
Cor Total	1207.76	16				

The predictive ability of the model was indicated by the calculation of R^2 coefficients, which is a criterion of the model fitting. For all experimented batches, the Y_3 (Q_{12h}) value showed good R-squared value of 0.874 **(Table 11)**. As the equation shows, independent variables A, B, and C have „p" < 0.05 that has significantly effect on the Q_{12h}.

Table 11: Predicted and Actual value for response Y_3 (Q_{12h})

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	96.00	96.00	0.0000	0.200	0.000	0.000	0.000	0.000	14
2	72.00	73.25	-1.25	0.750	-1.256	-1.321	0.473	-2.287	7
3	84.00	81.38	2.62	0.750	2.637	29.698(1)	2.086(2)	51.439(2)	1
4	80.00	78.88	1.13	0.750	1.130	1.157	0.383	2.004	12
5	96.00	96.00	0.0000	0.200	0.000	0.000	0.000	0.000	15
6	85.00	83.50	1.50	0.750	1.507	1.697	0.681	2.939(2)	8
7	72.00	74.63	-2.63	0.750	-2.637	-29.698(1)	2.086(2)	-51.439(2)	4
8	96.00	96.00	0.0000	0.200	0.000	0.000	0.000	0.000	17
9	90.00	91.13	-1.13	0.750	-1.130	-1.157	0.383	-2.004	9

10	85.00	85.13	-0.1250	0.750	-0.126	-0.116	0.005	-0.202	2
11	80.00	78.75	1.25	0.750	1.256	1.321	0.473	2.287	6
12	96.00	96.00	0.0000	0.200	0.000	0.000	0.000	0.000	16
13	99.00	100.50	-1.50	0.750	-1.507	-1.697	0.681	-2.939(2)	5
14	82.00	83.38	-1.38	0.750	-1.381	-1.499	0.572	-2.597(2)	11
15	95.00	93.63	1.38	0.750	1.381	1.499	0.572	2.597(2)	10
16	90.00	89.88	0.1250	0.750	0.126	0.116	0.005	0.202	3
17	96.00	96.00	0.0000	0.200	0.000	0.000	0.000	0.000	13

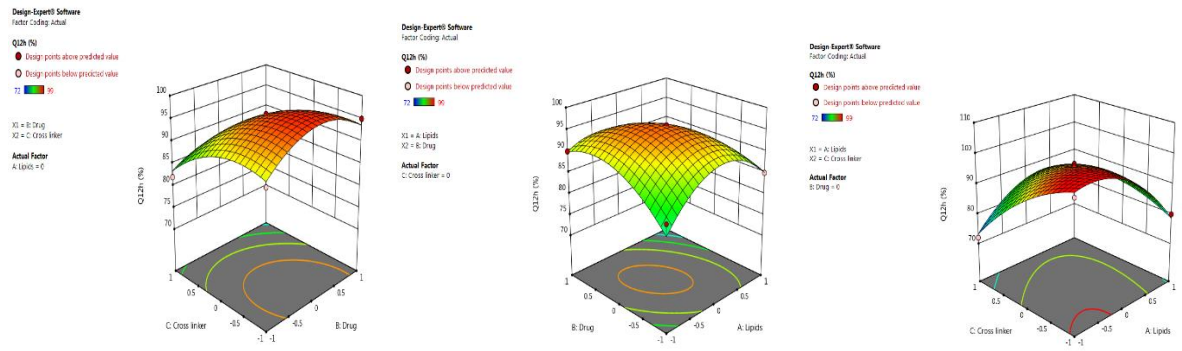

Figure 3. RSM of effect of lipid, drug, cross linker on drug release from the NC.

The relationship between the dependent and independent variables was further elucidated using the perturbation plot (**Fig. 4**), which shows the main effects of A, B, and C on the drug release (Y3) of beads. This figure clearly shows that A has the main and major effect on Y3, followed by C, which has moderate effect on Y3, followed by B, which has little effect on Y3.

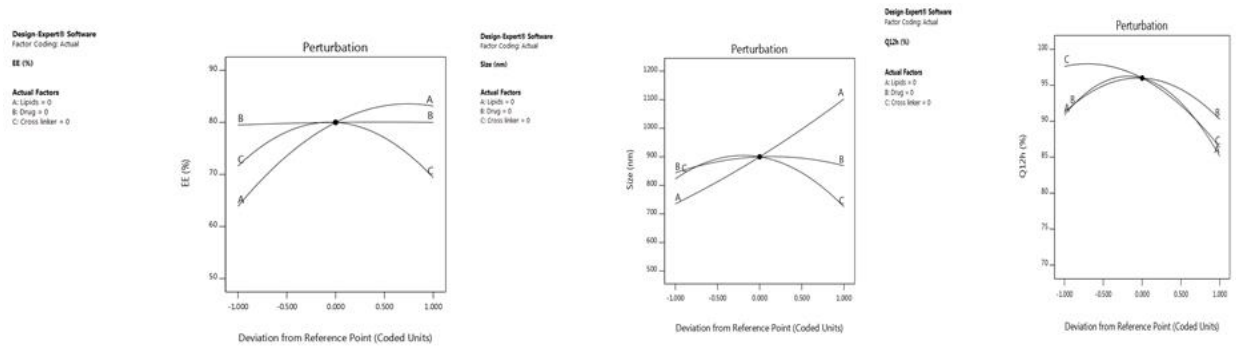

Figure 4. Perturbation plot showing the main effect of Lipid (A), Drug (B) and Cross linker (C) on EE (Y1), Size (Y2) and Drug release (Y3).

Numerical optimization was employed to identify the optimum process level of all the three factors for developing a new targeting delivery system with the desired response, using the desirability approach. A constraint to maximize the EE (%), size (nm) and drug release Q12h (%) was selected to locate the optimum level of independent variables for the optimized formula, by using Design-Expert 10 software version 11 based on the criterion of desirability. To get the desired optimum responses,

independent variables (factors) were restricted to (1:2 ≤ A ≤ 1:6), (5 ≤ B ≤ 15) %, and (0.5 ≤ C ≤ 1.5); whereas the desirable ranges of responses were restricted to (60 ≤ EE ≤ 80) %, (720 ≤ size ≤ 940) %, and (60 ≤ Q12h ≤ 100) %. In order to evaluate the optimization capability of these models generated according to the results of factorial design, optimized Ca2+ ion-induced NC containing CY were prepared using one of the optimal process variable settings proposed by the design. The selected optimal process variable setting used for the formulation of optimized Ca2+ ion induced NC was A = 0.722 %, B = 0.308 %, and C= -0.422 The bead optimization was evaluated for EE (%), size (nm), and Q12h (%). The optimized NC containing CY showed EE of (80.01 ± 3.05) %, Size (900.2 ± 5.08) %, and Q12h of (95.33 ± 4.35) %, with small error values. This reveals that mathematical models obtained from the Box-Behnken design were well fitted. This demonstrates the reliability of the optimized procedure in predicting the operating parameters for the preparation of CY loaded NC for targeting cancer cells. The selected formulation was subjected to characterization study, *in vitro* release and cytotoxicity studies.

Optical image

The Leica optical microscope captures the image of the CYT-SUV and the images of the NCs prepared by various cross-linking agent was shown in Figure no. 4. The SUV image is seen and on addition the lipid layer unwinds and forms the NCs. The shape is uniform round vesicles for the SUVs and no agglomerations or the lipid flakes is prominently found in the sample. The image is captured in 50µm scale. The calcium chloride shows better NCs, which is uniformly distributed than the agglomerated chitosan and the scarcely distributed zinc chloride.

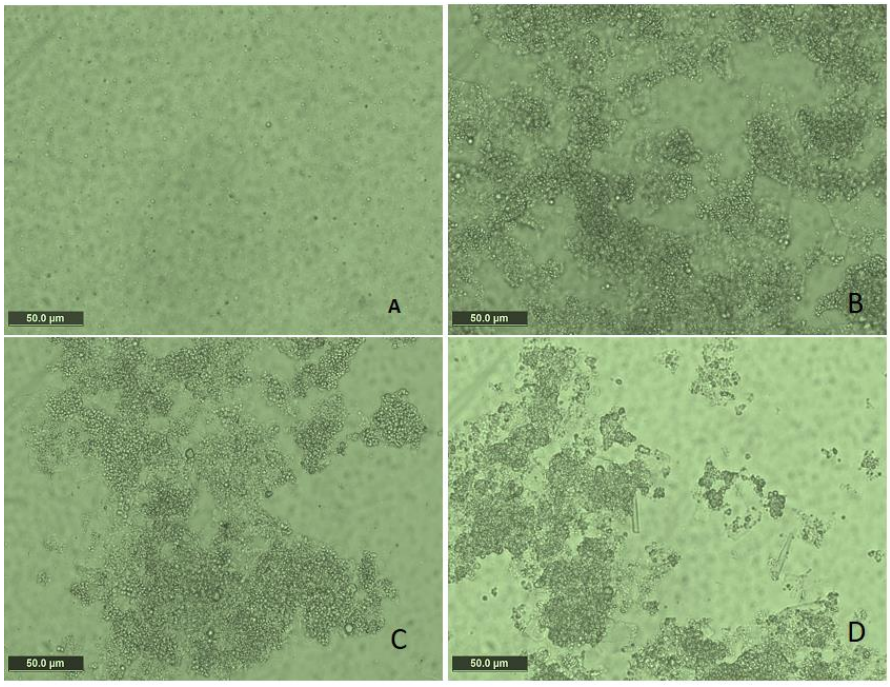

Fig. 4 Optical microscopy images of a) CYT-SUV and CYT entrapped Nanocochleates by (b) Calcium chloride (c) Chitosan and (d) Zinc chloride.

Scanning Electron Microscope (SEM)

The SUVs were subjected to characterization of size and shape. Microscopic examination revealed spherical small unilamellar vesicles of less than 1µm size range. The particle size of the Nanocochleate formulation was shown in the Figure No.5 (a) in the size range of 0.5µm to 1.5µm. The cigar shaped structure is formed and it is uniform in size. The surface morphology of the Nanocochleates is seen clearly as it is embedded within the nano clusters. The SEM image of the CYT-SUV and the NCs using the cross-linking agent are shown below. Of which

the calcium chloride shows the better result out of the others cross- linkers. The scale is 10µm range is taken for the NCs. The Fig.5(b) is seen with the rod shaped NCs. While Fig.5(c) shows the fiber like structure and no prominent difference is found and figure no. 5(d) shows less rods or no visible rods.

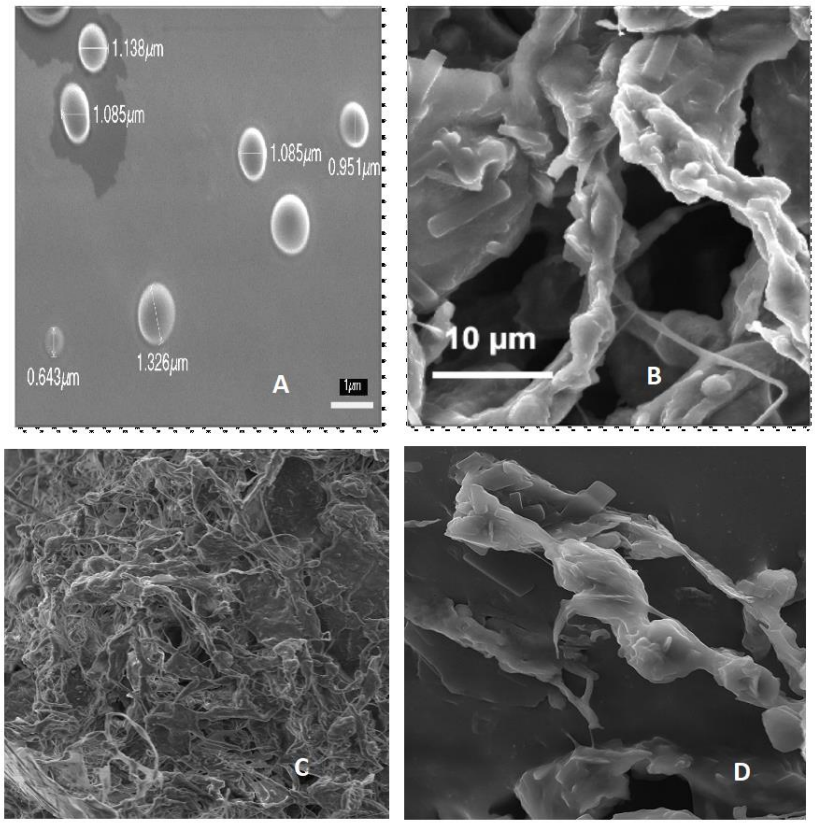

Fig. 5 Scanning Electron microscopy images of a) CYT-SUV and CYT entrapped Nanocochleates by (b) Calcium chloride (c) Chitosan and (d) Zinc chloride.

Transmission Electron Microscope (TEM)

High-Resolution Transmission Electron Microscopy helps to find the internal morphology of the sample. For this the sample is diluted and it is placed on the TEM grid, approximately two or three drops is sufficient. Then it is air dried until the water molecule is removed from it. Further it is taken for the analysis. The scale size is in the 100nm, 0.2µm and 0.5µm range. Figure no. 6 (a) shows that SUV are 100nm range, while the NCs Figure.6 (b, c, d) internal structure is magnified to form the 200nm to 500nm range.

The TEM image of the shown NCs prepared out of calcium chloride shows better result than the other zinc chloride and chitosan out the CYT-SUV. More NCs are prepared from the Calcium than the other cations with the scattered and agglomerated NCs formed.

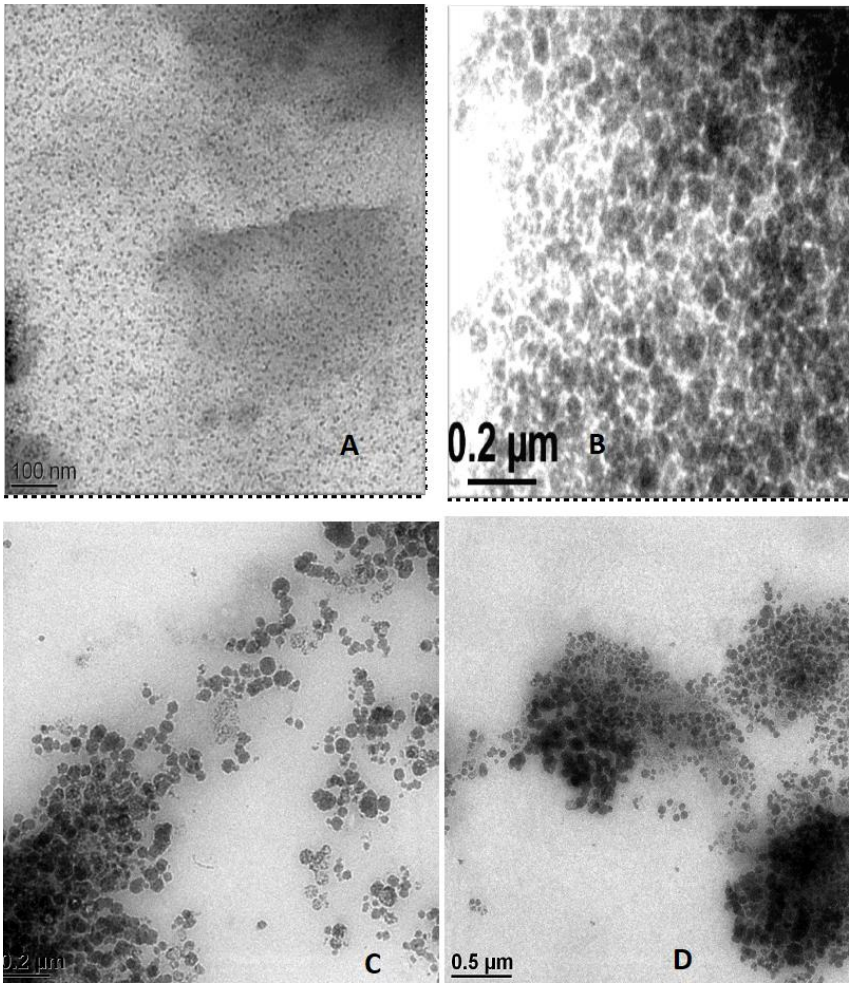

Fig. 6 Transmission Electron microscopy images of a) CYT-SUV and CYT entrapped Nanocochleates by (b) Calcium chloride (c) Zinc chloride and (d) Chitosan.

X-ray diffraction technique (XRD)

The XRD spectrum shows whether the sample is crystalline or amorphous in nature, based on which the drug encochleated or not can be found (**Figure 07**). Cytarabine shows the sharp peaks at 2θ peak position at 16.74º, 23.8º, 26.8º and 34.1º. So, it exhibits in crystalline nature. Cholesterol shows the peaks at 2θ peak position at 15.3º, 17.5º and 21.8º. So, it shows the nature is amorphous. Chitosan shows the sharp peaks at 2θ peak position at 20.1º, thereby it shows the amorphous nature. Calcium chloride shows the peak at 2θ position at 34.5º, 41º, 43.5 and 61º. The peak confirms the crystallinity of the calcium chloride. Zinc chloride shows the peak at 2θ position at 22.7º and it is amorphous in nature. The XRD pattern of the CYT entrapped NCs out of calcium chloride was taken and there are no sharp peaks, as it exists in amorphous state. The result revealed that the drug is encapsulated into vesicles, whereas the plain drug is in crystalline nature.

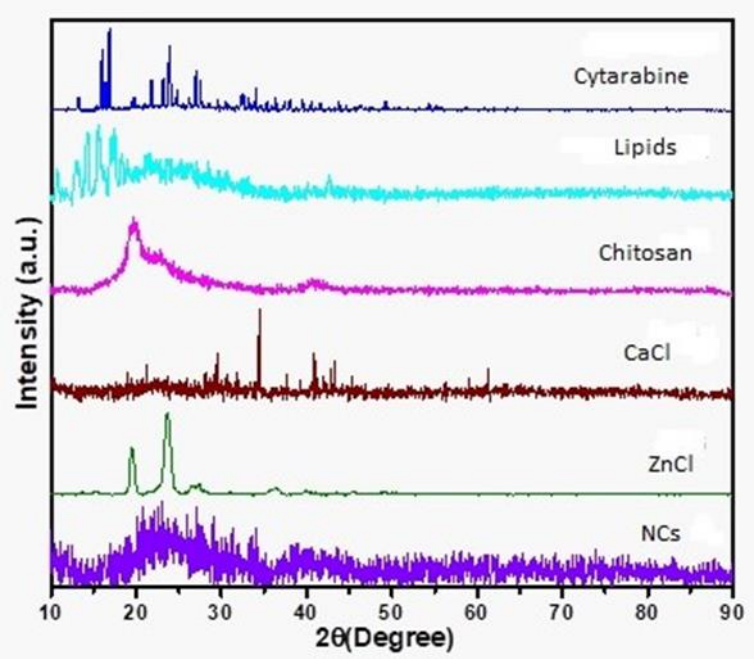

Fig.7 XRD spectrum of Cytarabine, Cholesterol, Chitosan, Calcium Chloride, Zinc Chloride, And Nanocochletes.

Fourier transform infrared spectroscopy

The FTIR studies helps to confirm the formation of the bond between the drug, lipids and the calcium ion source, the FT-IR spectra of the pure drug cytarabine, Cholesterol, Phosphatidylcholine, Calcium chloride and CYT entrapped Nanocochleates was taken for the comparison. By using the press pellet technique, analysis was done. The FTIR spectrum was shown in **Figure no. 8.**

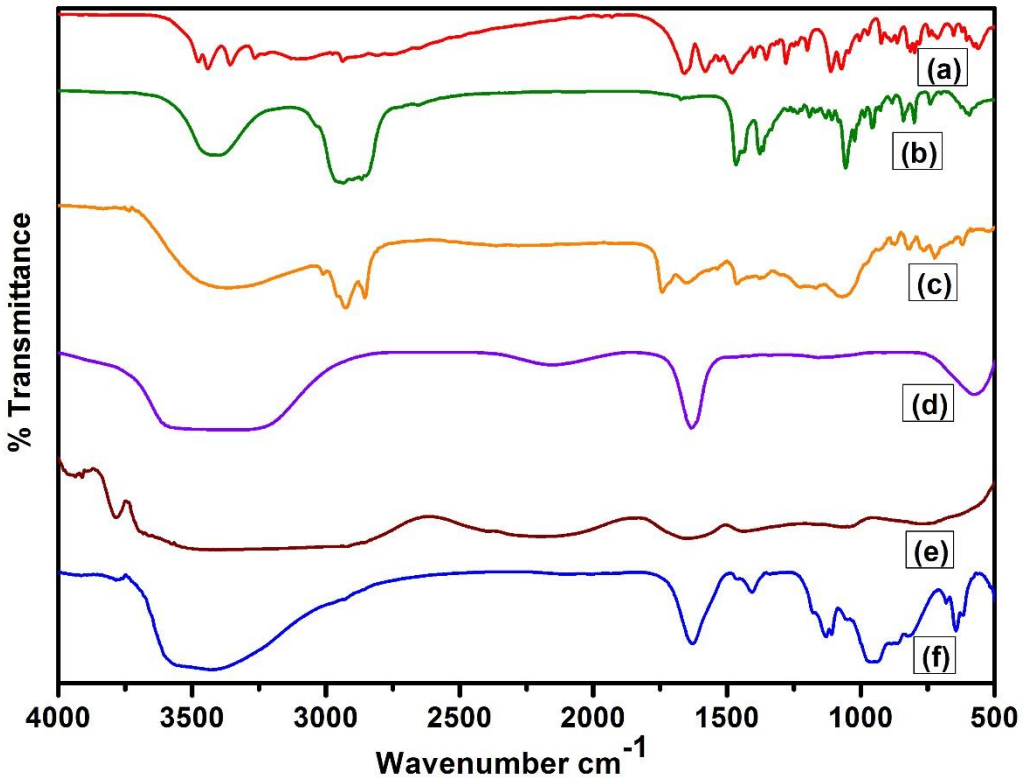

Fig.No. 8 FT-IR graph for (a) Cytarabine, (b) Cholesterol, (c) Phosphatidyl choline, (d) Calcium chloride, (e) CYT-NCs, (f) Zinc Chloride.

From FT-IR spectra of cytarabine, the characteristic peaks indicated at wave number, 3487cm^{-1} O-H stretching, 3357cm^{-1} N-H stretching, 2943cm^{-1} C-H stretching, 1659cm^{-1} C=O stretching,1568cm^{-1} N=O stretching, 1289cm^{-1} C-O bending and 1111cm^{-1} bending vibrations (Fig. (8a)). The characteristics peaks of Cholesterol represented at the wave number 3419cm^{-1} N-H stretching, 2910cm^{-1} C-H stretching, 1467cm^{-1} C=O stretching, 1366 cm^{-1} N-O stretching, 1059cm^{-1} C=O bending and 1025cm^{-1} C-H bending vibrations (Fig. (8b)). From FTIR spectrum analysis of phosphatidyl choline, the peaks obtained at wave number 3433 cm^{-1} N-H stretching, 2928cm^{-1} C-H stretching, 1746cm^{-1} C=O stretching, 1539cm^{-1} C-N stretching and 1059cm^{-1} C=O bending vibrations (Fig. (8c)). The characteristic peaks of Calcium chloride obtained from FT-IR spectra have indicated at the wave number 3210 cm^{-1} OH stretching vibrations and 1629 cm^{-1} carbon chloride stretching (Fig. (8d)). The characteristics peaks of CYT entrapped Nanocochleates shown at the wave number 3785cm^{-1} O-H stretching, 3424 cm^{-1} N-H stretching, 2851 cm^{-1} C-H stretching, 2227 cm^{-1} C=N stretching, 1648 cm^{-1} carbon chloride stretching vibration and 1036 cm^{-1} C=O bending vibrations (Fig. (8e)). By comparing the different spectra, it is inferred that there are no interactions between cytarabine, cholesterol and phosphatidyl choline. The cross-linking agent, $CaCl_2$ between the formulations has made a weaker Vander Waal's interaction which was characterized through the small peak obtained at wave number 1648cm^{-1}.

CONCLUSION

The Nanocochleates are prepared experimentally after optimized by the box and behnken's method. The software helps in reducing the experiments by 17 trails. The various cross-linking agent used in the preparation show the comparison of the formation of the NCs. Characterizations like Optical image, SEM and TEM confirms the nanocochleate is prepared by the crosslinker calcium chloride shows better than the

other zinc chloride and chitosan. The optical image shows the uniformity in the particle size, the SEM image gets the size range of 0.5µm to 1.5µm. FTIR says that the drug is linked to the $CaCl_2$ with the weak wander walls force from the peak formed. In conclusion, ionic crosslinking method was proved to be a successful method to form nanocochleates.

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