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Development of Curcumin nanoniosomes for skin cancer chemoprevention

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Abstract: The study is aimed to deliver curcumin nanoniosome topically to treat skin carcinogenesis. Nanoniosomes were prepared by reverse phase evaporation method using Brij S10, Tween 65, Tween 60 and Glycerylmonostearate as surfactant. The morphology of prepared nanoniosomes was characterized by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). The nanoniosomes were incorporated into gel formulations and the drug permeation study was performed across excised rat abdominal skin. The optimized niosomal gel was evaluated for anti-cancer efficacy in 7,12-dimethylbenz [a] anthracene (DMBA)-induced skin carcinogenicity in Swiss albino mice and the histopathology of skin was studied microscopically. Curcumin was entrapped with encapsulation efficiency ranging between 40.35 ± 1.49 and 78.32 ± 0.91 . Average particle size was found between 91.9 nm to 1996.1 nm. The surface of the vesicle was smooth with uniform drug distribution within the vesicle. The drug release from nanoniosome dispersions and nanoniosome gels followed zero order and Higuchi kinetics and release of drug followed non-Fickian diffusion. The histopathological study revealed a suppressed abnormal skin cell proliferation during DMBA-induced skin carcinogenesis. The curcumin nanoniosome may prove to be a potential nanovesicle for skin cancer chemoprevention upon dermal application.

Key words: Curcumin, Carcinogenesis, Nanoniosome, Chemoprevention, Skin cancer

Introduction

Numerous investigations on curcumin have established that curcumin possess a wide range of pharmacological activities that include antitumor, anti-inflammatory, antioxidant properties, beside this curcumin acts on skin cancerous cell by down regulation of cell proliferative controls, involving thymine dimer, apoptosis, transcription factors NF- κ B and of inflammatory responses involving COX-2, PGE2, and NO, while upregulation of p53 and p21/Cip1 to prevent DNA damage and facilitate DNA repair¹. Despite curcumin's superior properties as an anti-cancer agent its applications are limited due to its low solubility and physico-chemical stability, rapid systemic clearance and low cellular uptake. The development of curcumin nanoniosome formulation will improve its therapeutic index through enhanced cellular uptake, localization to targeted areas and improved bioavailability. Previous reports reveal that a good transdermic and sustained release behavior of curcumin was obtained from nano-liposome formulation in inflammatory conditions² and skin melanoma³. The sustained release behaviour may due to the accumulation of nano-liposome within the skin. Proniosomal formulation of curcumin showed lower anti-inflammatory and anti-arthritis effects than the

marketed indomethacin products⁴. It may be related to the higher size of proniosome in micrometer range having less skin permeability. However, the micro-niosome had enhanced the skin permeation of curcuminoids through shed snake skin⁵. The present work was an attempt for the development and evaluation of curcumin loaded nano-niosomes for its topical delivery in skin cancer chemoprevention.

Experimental

Materials

Curcumin, Tween 60 and Cholesterol were purchased from HiMedia Laboratories Pvt. Ltd. Mumbai, India. Tween 65 and Brij S10 were purchased from Sigma Aldrich Chemicals Pvt. Ltd. Kolkata, India. Glycerylmonostearate (GMS), ButylatedHydroxy Toluene (BHT) and Carbopol 934 were obtained from Yarrow Chem Products, Mumbai, India. Other reagents were of analytical grade and obtained from Merck India Ltd, Mumbai.

Methods

Preparation of nanoniosomes

Nanoniosomes were prepared by reverse phase evaporation method with slight modification^{6,7}. The compositions of different nanoniosomal formulation are listed in Table 1. Weighed amounts of nonionic surfactant, Cholesterol and BHT (2% w/w of nonionic surfactant) were dissolved in 10 ml of chloroform by magnetic stirrer in 30 ml closed vial to which 30 mg of curcumin was added and dissolved. Further 5 ml of distilled water was injected rapidly in to organic phase through 23-gauge needle from a 5 ml syringe. This prepared solution was transferred in homogenization tube and homogenized (High-speed Homogenizer IKA®T25 digital ULTRA-TURRAX, Germany) for 1 minute resulted in milky water-in-oil emulsion. It was then attached directly to rotary vacuum evaporator (Buchi rotary evaporator, rotavapor R-210, Switzerland) and rotated at 125 rpm at 37°C in a water bath under vacuum until a gel was formed, vacuum was released and the round bottom flask was removed from the evaporator and subjected to vigorous mechanical shaking until the gel collapsed to fluid consisting of suspended niosomes. It was again fitted to rotary evaporator and rotated at 100 rpm at 60°C for 1 hour for complete removal of organic solvent. The dispersion was sonicated in a bath type sonicator (Ultrasonic cleaning bath, Model UCB 30, Spectralab Instruments Pvt. Ltd, Navi Mumbai, India) at a frequency of about 40 KHz at 45°C temperature for 30 minute. Prepared nanoniosomal dispersion was kept at room temperature for one hour for swelling of vesicle and then the preparation was stored at 4°C in a closed vial.

Nanoniosomes size and size distribution

Particle size and polydispersity index (PDI) was determined by dynamic light scattering (DLS) technique using Brook Haven 90 Plus particle size analyzer.

Zeta Potential Study

The zeta potential of freeze dried nano-niosomes was measured by using the Zetasizer 2000 (Malvern Instruments, UK).

Scanning Electron Microscopy (SEM)

The morphological observation of nano-niosomes was studied by scanning electron microscope (JEOL-JSM-6360 JAPAN). One drop of sample was placed on a slide attached with copper grid, excess water was left to dry at room temperature. The dried sample was gold coated under vacuum using a sputter coater (Model JFC-1100, Jeol, JAPAN) for 10 minute and investigated at 20kV.

Transmission Electron Microscopy

TEM was used to characterize lipid bilayer of nano-niosomes. 1-3 µl of the niosome suspension was placed over Formvar-Carbon coated grids and left it on for 30 seconds then drew off from the edge of the grid with filter paper. Before sample was dried immediately applied 6-10 µl of 2% aqueous Ammonium molybdate (pH adjusted to 7.3 using 1N NaOH) and left for 30 seconds, and drew off from the edge of the grid with filter paper. The grid was directly placed into grid box and allowed them air-dry at room temperature before observation.

Drug entrapment efficiency and drug loading determination

A volume of 10 ml prepared drug-loaded niosomal suspension was cold centrifuged at 12500 rpm for 45 min to separate the nano-niosomes and aqueous phase. 1ml clear supernatant was diluted to 10 ml with methanol and analysed spectrophotometrically (UV-1800 Shimadzu, Japan) at 425 nm for untrapped drug. The same procedure was used for the batch without drug. The absorbance due to drug was the difference between the readings obtained from the respective preparations with drug and without drug. The drug content was then determined from the standard curve^{8,9}. The entrapment efficacy (%EE) and drug loading (%DL) of nano-niosome was calculated as follows:

$$\%EE = (\text{Amount of curcumin in niosomes} / \text{Amount of curcumin used in the formulation}) \times 100$$

$$\%DL = (\text{Amount of curcumin in niosomes} / \text{Amount of niosomes obtained}) \times 100$$

In-vitro drug release studies of nanoniosomes dispersion

In vitro drug release from isolated nanovesicles was carried out by dialysis method in aqueous PEG400 (50% v/v) at 37±1°C. The samples at specified time intervals were analyzed by UV spectrophotometer at a wavelength of 437.50 nm. With the help of the standard curve prepared earlier, drug concentration was measured^{10,11}.

Preparation of gel

Based on the previously mentioned characterizations (particle size, entrapment efficiency and *in vitro* release profile) of nano-niosome dispersion, nano-niosome formulation with optimum physicochemical properties were selected and gel formulation with different polymers were prepared. Polymers like carbopol, hydroxy propyl methyl cellulose and combination of both the polymers were selected for preparing the gel formulation. The gels were evaluated in terms of the spreadability, occlusion and pH.

In vitro skin permeation of nano-niosomal gel through rat abdominal epidermis

For *in vitro* permeation studies, skin were allowed to hydrate for 1h before being mounted on the Keshary-Chien diffusion cell with the stratum corneum facing the donor compartment with an effective diffusion area of (3.14 c.m²). The receptor compartment cell were filled with PEG400 50% (v/v) in distilled water and the bathing solution were stirred at 500 rpm by Teflon coated magnetic bar to keep them well mixed. The permeation media were maintained at 37±0.5°C thermostatically with the help of water bath and pump (Remi Instruments Ltd. Mumbai). Aliquots of 1ml from the receptor solution were withdrawn periodically for 24h and replaced with fresh PEG400 50% (v/v) in distilled water. The aliquots withdrawn were diluted with respective solvent and estimated for drug content spectrophotometrically^{12,13}.

In vivo studies on anti-cancer activity: DMBA induced skin cancer in Swiss male albino mice

The protocol of the study in rats was approved by the Institutional Animal Ethical Committee (IAEC), Department of Pharmaceutical Sciences, Dibrugarh University (Approval No. IAEC/DU/40, Regd.No.1576/GO/a/11/CPCSEA). Male, Swiss Albino mice 4-6 weeks old, weighing 15-20g were selected for the experiment. The animals were housed in polypropylene cages and provided standard pellet diet and water ad libitum and maintained under controlled conditions of temperature and humidity, with a 12 h light/ dark cycle. The animals were maintained as per the principles and guidelines of the ethical committee for animal care in accordance with Indian National Law on animal care and use. A total number of 24 male Swiss albino mice were divided into four groups of 6 each, like, normal group, Cancer control group, Placebo control group and Test group. Skin carcinogenesis was developed in Swiss albino mice according to the method of Azuine and Bhide 1992¹⁴. Three days before the commencement of experiment, depilatory cream was applied to remove hair from the back of each mouse 3 × 3 cm² area and the mice to be left untreated for two days. Mice having no hair growth after two days were selected for the experimental study. The depilated back of mice of every group except normal group were painted with DMBA (25µg in 0.1 ml acetone/mouse) twice weekly for 7 weeks. The chemopreventive potential of Curcumin was assessed by measuring the tumor incidence, tumor volume, tumor burden and body weight.

Results

Particle size and Particle size Distribution

Particle size and polydispersity index was determined by dynamic light scattering technique using Brook Haven 90 Plus particle size analyzer. The mean particle size for all formulation (N1-N16) varied in the size range from 91.9 nm to 1996.1 nm (Table 2).

Zeta Potential (ζ)

The zeta potential value of all prepared niosomes was in the range of -25.4 mV to -59.1 mV. The surface charge values were negative for all the optimized formulation. The zeta potential value was affected by nature of surfactant and cholesterol content.

Scanning Electron Microscopy (SEM) for surface Morphology

Information about particle shape and surface morphology of nanoparticles was obtained by SEM (Figure 1). Spherical and disc like particle with a size in the nanometer range were observed for the nanoniosome formulation, which was in agreement with the size data determined by light scattering technique .

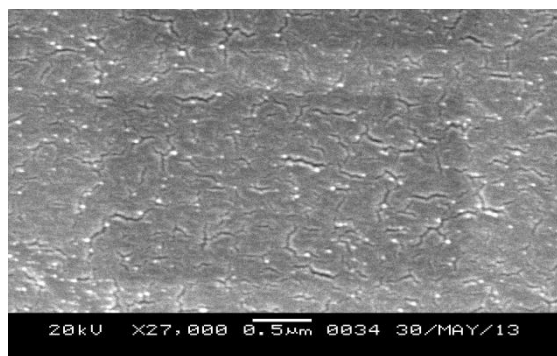


Figure 1 SEM photomicrograph of N 14 formulation.

Transmission Electron Microscopy

External morphology of nano-niosomes in suspension was determined using transmission electron microscopy (TEM) (Figure 2). TEM image revealed the smooth and spherical surfaces of the nano-niosomes with distinct membrane bilayer. Magnification of the vesicle showed the uniform distribution of curcumin within the niosome vesicles.

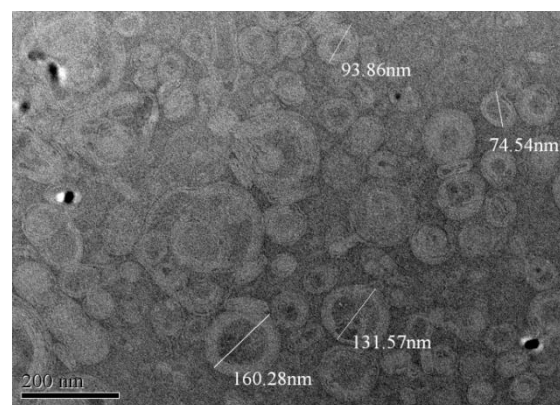


Figure 2 TEM micrograph of N14 formulation.

Determination of drug entrapment efficiency and drug loading

The drug entrapment efficiency and drug loading were found maximum in case of formulation prepared by using GMS followed by Tween 60, and the ratio of cholesterol to GMS having highest drug loading was found to be at 1:1. The values were tabulated for all the formulation in Table 1.

In vitro drug release studies of niosome dispersion

The cumulative percentage release of curcumin from different niosomal dispersion was investigated for a period of 12 hours. Each sample was analyzed in triplicate. The in-vitro release of the drug from nanoniosomal suspension was found to be biphasic with the initial burst effect, followed by slower gradual release of the drug. The initial burst release may be due to the presence of untrapped drug in niosomal suspension. It is found to be of advantageous since it will provide for the achievement of initial therapeutic concentration serving as a loading dose, burst release as well as sustained release both is of interest for dermal application. Burst release can be useful to improve the penetration of drug while sustained release supplied the drug over a prolonged period of time.

In vitro release study of nanoniosomal gel

In vitro studies were performed to compare the release rate of the drug from the various niosomal gels (NHG, NCHG, NCG and PCG). On the basis of cumulative percentage release of curcumin from different nanoniosome dispersion were investigated for a period of 24 hours (Figure 3).

Histopathological study for the evaluation of anti-cancer efficacy

The anticancer potential of the optimized formulation was evaluated in 7, 12-dimethylbenz[a]anthracene induced skin carcinogenesis. The chemopreventive potential of Curcumin was assessed by measuring the tumor incidence, tumor volume, tumor burden, body weight and histopathological study. Repeated topical applications of DMBA (3 times per week for 7 weeks) induced skin carcinogenesis in Swiss albino mice. DMBA alone treated animals showed 40% tumor incidence and the tumor was histopathologically confirmed as well differentiated squamous cell carcinoma (Figure 4).

Table 1 Nanoniosome formulation and characterization

Formulation Code		Surfactant: Cholesterol (μ M ratio)	Mean size (nm)	Polydispersity Index (PI)	Entrapment Efficiency (%)	Drug loading (%)
Tween 60	N1	75:150	1996.1 \pm 0.9	0.344 \pm 0.003	77.39 \pm 0.26	2.90 \pm 0.10
	N2	75:100	660.4 \pm 0.6	0.275 \pm 0.002	75.82 \pm 0.64	2.67 \pm 0.13
	N3	75:75	643.8 \pm 0.7	0.317 \pm 0.011	74.8 \pm 0.21	2.45 \pm 0.21
	N4	50:50	600.8 \pm 0.4	0.289 \pm 0.005	74.0 \pm 0.19	1.84 \pm 0.11
Brij S10	N5	100:200	316.8 \pm 0.4	0.259 \pm 0.002	65.91 \pm 1.70	2.16 \pm 0.087
	N6	100:150	359.0 \pm 0.5	0.245 \pm 0.004	64.01 \pm 0.89	1.97 \pm 0.16
	N7	100:100	391.6 \pm 0.7	0.256 \pm 0.008	46.12 \pm 2.02	1.88 \pm 0.12
	N8	90:75	299.1 \pm 0.3	0.354 \pm 0.003	40.35 \pm 1.49	1.60 \pm 0.09
Tween 65	N9	75:150	512.5 \pm 0.4	0.357 \pm 0.012	65.07 \pm 0.26	1.92 \pm 0.075
	N10	75:100	284.2 \pm 0.3	0.335 \pm 0.002	67.46 \pm 0.89	1.75 \pm 0.055
	N11	75:75	495.8 \pm 0.8	0.398 \pm 0.004	54.35 \pm 1.49	1.31 \pm 0.1
	N12	50:50	494.5 \pm 0.6	0.292 \pm 0.006	54.11 \pm 1.33	1.29 \pm 0.09
GMS	N13	300:300	154.6 \pm 0.3	0.250 \pm 0.001	78.01 \pm 0.54	3.11 \pm 0.084
	N14	300:250	156.2 \pm 0.2	0.254 \pm 0.003	78.32 \pm 0.91	3.15 \pm 0.086
	N15	300:200	091.9 \pm 0.086	0.390 \pm 0.005	74.8 \pm 0.64	2.65 \pm 0.09
	N16	175:150	202.4 \pm 0.4	0.278 \pm 0.002	65.46 \pm 0.78	2.01 \pm 0.088

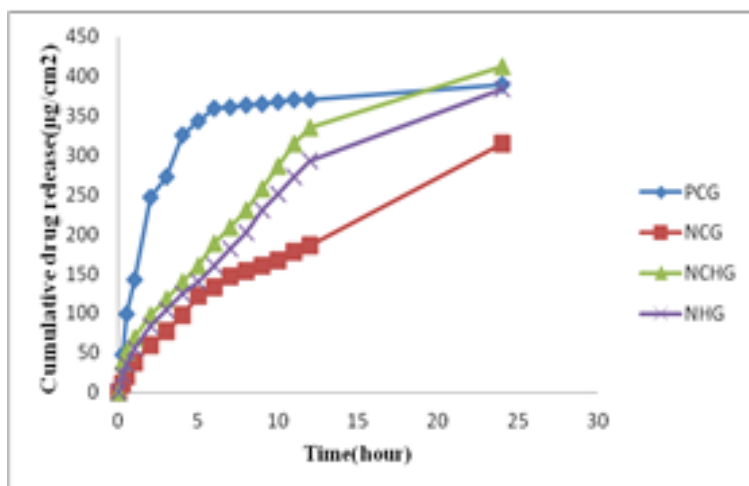


Figure 3 Drug release profile of curcumin from plain carbopol gel (PCG), niosomal carbopol gel (NCG), niosomal HPMC gel (NHG), niosomal carbopol-HPMC gel (NHCG)

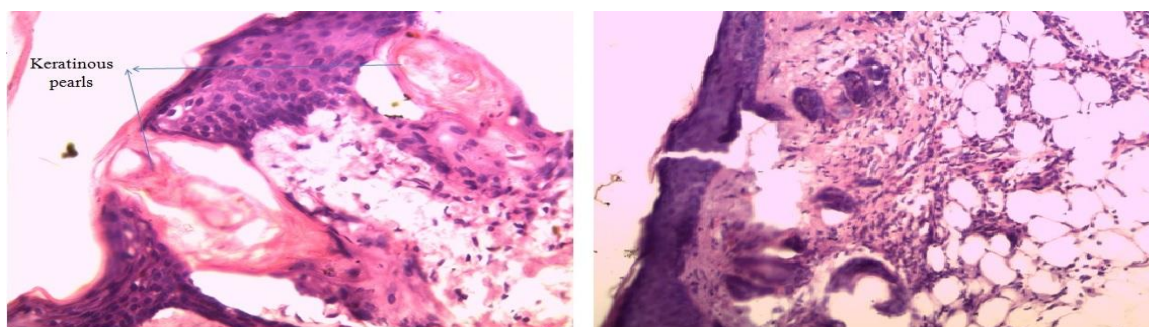


Figure 4 Microscopy of DMBA treated mice skin showing tumor cells with keratinous pearl (left), niosome treated mice skin showing intact epithelial layer with hyperkeratosis

Discussion

The particle size data suggest that increasing the HLB value of surfactant particle size was increased due to high entrapment of aqueous phase. It was also observed that by increasing cholesterol concentration particle size was increased. Cholesterol makes surfactant layer rigid, higher rigidity of surfactant membrane hindered particle size reduction. The smallest size was observed in the case of Glycerylmonostearate surfactant which has smallest HLB value (3.2) amongst all used surfactants and highest phase transition temperature (65°C)¹⁵. The result of the Zeta potential study suggests that there was less variation in zeta potential due to presence of similar functional group (Hydroxy group, Ester group and Alkyl group) in all surfactant.

The SEM photomicrograph suggested uniform particle distribution in the dispersion and the particle size data complied with that of data generated by particle size analyser. The TEM study revealed the distribution of drug within the vesicle was uniform and the particles were having distinct membrane bilayers.

The percentage of drug entrapment was high in GMS, and Tween 60 formulations followed by Tween 65 and Brij S10 formulations (Table 1). Hydrophilic nonionic surfactants form vesicles when cholesterol is included in the bilayer. Incorporation of cholesterol into niosomes at ratios up to 1:1 increased the encapsulation efficiency of curcumin. Inclusion of cholesterol increases the viscosity of the formulation indicating more rigidity of the bilayer membrane. Moreover, drug partitioning will occur more easily in highly ordered systems of surfactant and cholesterol. The ability of the lamellar surfactant phase to accommodate drug, depends upon the structure of the surfactant phase and phase transition temperature. It was observed that concentrations of nonionic surfactant affected the percentage entrapment efficiency of formulation. The mean size of niosomes increases proportionally with increase in the HLB of surfactants like Span 85 (HLB 1.8) to Span 20 (HLB 8.6) because the surface free energy decreases with an increase in hydrophobicity of surfactant

The drug release profile of nanoniosomal dispersion followed the zero order and Higuchi model kinetics. The best model with the highest correlation coefficient was shown in the Higuchi model followed by a zero order equation. The obtained correlation coefficient values indicated that drug release followed the diffusion control mechanism from the nanoniosomes. The data supported the findings that the drug incorporated in the nanoniosomes was mainly released by a diffusion mechanism. The data obtained were inserted in the Korsmeyer-Peppas model in order to find out the n value. The release showed a high correlation with the Korsmeyer-Peppas model. The n value ranged from 0.582 to 0.682, which suggested that drug release from the nanoniosomes was non-Fickian diffusion controlled. By comparing the drug release pattern from nanoniosomal gel and nanoniosomal dispersion burst release rate was observed less in nanoniosomal gel in comparison to the nanoniosomal dispersion^{17,18}.

The *in vivo* anticancer efficacy study suggested the DMBA alone treated animals showed 40 % tumor incidence and the tumor was histopathologically confirmed as well differentiated squamous cell carcinoma. The tumor cells have pleiomorphic hyperchromatic nuclei with epithelial pearl formation. Topical application of curcumin nanoniosome gel completely prevented the formation of well differentiated squamous cell carcinoma in DMBA treated animals. We have however observed hyperkeratosis in nanoniosome treated mice. Our results thus suggested that curcumin nanoniosome suppressed abnormal skin cell proliferation during DMBA-induced skin carcinogenesis^{19,20}.

Conclusion

Curcumin nanoniosome was prepared by reverse phase evaporation method using non-ionic surfactant Glyceryl monostearate, Tween 65, Brij S10, Tween 60 and Cholesterol. The nanoniosome gel was prepared by incorporation of isolated vesicle after centrifugation in carbopol, HPMC and carbopol-HPMC combination polymer base. Nanoniosomes were smaller in size ranging from 91.9 nm to 299.6 nm and with polydispersity index of 0.250-0.390 and entrapment efficiency of 65.46%-78.01% was obtained. The TEM and SEM analysis for morphological study revealed that the size of the nanoparticles was in uniformity with the size range obtained by dynamic light scattering technique. It was observed that particles were spherical with smooth surface and drug was dispersed homogeneously in the surfactant bilayer. The zeta potential value of the nanoniosome was found to be -25.4mV to -59.1 mV indicating stable formulation. The *in vitro* drug release pattern of nanoniosome dispersion and gel showed burst and controlled release. Burst release as well as sustained release both are of interest for dermal application. Burst release can be useful to improve the penetration of drug while sustained release supplied the drug over a prolonged period of time. The optimized N14 formulation potentially suppressed abnormal skin cell proliferation during DMBA-induced skin carcinogenesis.

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