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RESEARCH ARTICLE

Nanoparticles-in-Microspheres based Dual Drug Delivery System for Topical Delivery of Anti-acne Drugs

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Abstract

The efficacy of combination drug therapy in the treatment of acne vulgaris, propels the need for development of a delivery system which could selectively deliver combination of drugs at the site of infection in the same formulation. Hence the present study aims at providing spatial localization of combination of drugs at the epidermal site for effective treatment of acne vulgaris. Chitosan nanoparticles (Ch-NP) were prepared by ionotropic gelation method and further used to prepare nanoparticles-in-microspheres dual system (NMDS) and optimized. The prepared system was characterized in terms of size, polydispersity index, morphology, nanoparticles loading efficiency and drug entrapment efficiency. *In vitro*, the system showed a biphasic release characterized by initial burst followed by controlled and prolonged release of the drugs. The stability studies suggested markedly improved stability of drugs in sunlight, UV light and at various storage temperature conditions upon encapsulation. Further the *in-vivo* studies showed NMDS to undergo minimum systemic penetration, provide high epidermal localization and significantly reduce skin irritation. The present study thus explores the potential of this newer novel carrier in treatment of acne vulgaris with interception of minimal side effects.

Introduction

Acne is a dermatologic disorder of pilosebaceous units with its precise etiology still being unknown. The pathogenesis is multifactorial, but is perhaps the consequence of four key pathogenic events viz increased sebum production, occlusion of the pilosebaceous canal due to hyperkeratosis, proliferation of *Propionibacterium acnes* (*P. acne*) and lesions mediated by chemotactic factors and hormone fluctuations [1,2]. Although the disease lacks the urgency of a life threatening conditions, its long-term ramification can be significant due to impaired psychosocial development, reduced self-esteem and emotional distress [3-5]. Acne vulgaris is the most common inflammatory skin disorder that plagues approximately 85% of population at the age between 12 and 24 yrs. Further, the disease is also reported in 8% and 3% of adults aged 25 to 34 yrs and 35 to 44 yrs respectively. The prevalence of the disease results in 20% of all visits to dermatologists being for acne [6].

An array of both topical and systemic drug therapies is available for the treatment of acne vulgaris [7-9]. In this context, when compared with single agent the combination therapy has been assessed to be more efficacious and safe for use in acne patients. This may be attributed to utilization of complementary mechanism of action of drug demonstrating greater and faster results [6,10]. For the management of acne, the agents most commonly used in combination include topical retinoids and antibiotics. Retinoids are comedolytic and anti-inflamatory, while antibiotics besides being antimicrobial are also anti-inflammatory and mild comedolytic and this explains their augmented and faster effect [6,11,12]. Also the combination is better tolerated than tretinoin alone which is the leading drug of topical retinoid category,

possibly because the antibiotics are believed to decrease the irritant effects of tretinoin, its major side effect [13]. However, the basic problem lies in selective localization of drugs at the site of acne. Moreover limited penetration of drug through skin is a great drawback that also affects therapeutic efficacy of a bioactive. At the same time the absorbed drug enters systemic circulation and may further lead to side effects. Thus a delivery system that can penetrate the skin and selectively deliver the drug effectively at the site of *P. acne* infection is needed.

In last half a decade, the potential of various delivery strategies such as liposomes [14-16], niosomes [17], inclusion complexes [18-19], microspheres [1], solid lipid nanoparticles [20], nanostructure lipid particles, etc. in resolving the problem associated with topical delivery of antiacne drugs has been widely explored. Microspheres represent a stable system with minimum cutaneous irritation, yet acceptably effective and have shown potential for site-specific delivery to the hair follicles [1,21]. Also charged niosomes showed good drug stability and improved cutaneous delivery of vesicle entrapped drug [17,22,23]. Among the various novel delivery systems investigated, liposomes showed considerably good results in topical delivery along with reducing side effects associated with topical drug therapy but they suffers from their inherent system instability [24]. Solid lipid nanoparticles emerged as an improved, physically stable system with potential in epidermal targeting [20]. But still, all these novel approaches have their own limitations in delivery of combination drugs which is the best treatment strategy for acne vulgaris.

Attempt has been made to formulate combination of drugs, utilizing the complementary effect of drugs, in which one drug is delivered in a colloidal form and the other in the ointment base, but till date no efforts have been endeavored to deliver both of these drugs for acne in a single delivery system [25]. All these factors warrant the need to develop an alternative drug carrier, which may provide an effective, stable and controlled delivery of the drugs. Hence in an attempt for epidermal targeting, improving delivery and providing stability for combination therapy, it was proposed to prepare nanoparticles-in-microspheres, a dual system (NMDS) for the effective treatment of acne vulgaris. Such delivery system was also expected to reduce the dose of the active agents as the drugs were tried to be targeted to the specific site. Additionally the hair follicles can act as long-term reservoir, a beneficial condition when topical delivery is intended.

Chitosan nanoparticles (Ch-NP) have the potential to be used as an efficient, viable, safe, and cost effective system on account of their biocompatibility, biodegradability, antimicrobial property, low toxicity, high entrapment efficiency of hydrophilic drugs [26-28]. Besides, the PLGA microspheres are hydrophobic in nature, biocompatible, biodegradable, resorbable through natural pathways, have controllable degradation property (using appropriate proportion of lactic acid and glycolic acid units present in the polymer chain) and its end products are non-toxic which further augments its suitability [29-32]. Hence in the present investigation, we attempted to develop a delivery system which may allow a combination drug therapy to be delivered from, within the same system, providing spatial delivery to the acne site and improving stability of the drug with the interception of minimal side effects. In the first part of the experiment the optimized NMDS having a size range below 7 µm was prepared. This size has been postulated as an optimum size of delivery system for pilosebaceous delivery of the therapeutic agents [33,34]. The latter part includes *in vitro* release study, stability testing and *in-vivo* studies to evaluate the performance of the prepared system.

MATERIALS AND METHODS

Materials

Tretinoin (Tre) was received as a generous gift sample from Shalaks Pharmaceuticals Pvt. Ltd, New Delhi and Clindamycin (Clin) from Galderma India Pvt. Ltd, Mumbai. Chitosan (Ch) was generously provided by Central Institute of Fisheries Technology, Cochin (India). PLGA was purchased from Sigma Aldrich Pvt. Ltd. (New Delhi), Sodium cholate (SC) and Sodium tripolyphosphate (TPP) were procured from Otto Chemicals, Mumbai. All other chemicals and reagents were of analytical grade and used as received. Double-distilled deionized water was used in all the experiments.

Preparation of Chitosan Nanoparticles (Ch-NP)

Ch-NP were prepared by ionotropic gelation method previously reported by Calvo *et al*., (1997) with slight modifications [35]. Briefly, chitosan solution (1.0 mg/mL) was prepared in 3% v/v acetic acid and its pH was adjusted to 5.5 with 0.6 M NaOH. 0.1% TPP solution (3.0 mL) was added to chitosan solution (18.0 mL) containing clindamycin/ Rodamine-6, set under mechanical stirrer (Remi Instruments, India) with constant stirring for 45 min at room temperature. The nanoparticles were formed spontaneously upon addition of an aqueous TPP solution to chitosan solution under mechanical stirring. The prepared Ch-NP were purified by ultracentrifugation (Remi, Mumbai) at 16000 g for 30 min at 4˚C on a glucose (33% w/w) bed followed by redispersion in water. The resultant nanoparticles were freeze dried using freeze dryer (Heto Drywiner–Denmark).

Preparation of Nanoparticles-in-Microspheres Dual System (NMDS)

NMDS were prepared by the technique developed in lab. To summarize Ch-NP (10-40 mg) dispersion in water (500 µL) was added to PLGA solution (1.0-2.5%) containing tretinoin/ FITC in dichloro methane (DCM; 2.0 mL) and then sonicated at 35 W/sec for 90 sec. Subsequently, sodium cholate solution (0.01-0.3% w/v, 10-25mL) was added and sonicated (30-120 sec). The resulting dispersion was magnetically stirred at 1000 rpm for 3 hrs. The NMDS so formed were collected by centrifugation at 5000 rpm for 10 min, washed with deionized distilled water to remove sodium cholate, redispersed in water and finally freeze dried [36].

Characterization of Ch-NP and NMDS

Particle size analysis

The average particle size and size distribution of Ch-NP were measured by photon correlation spectroscopy (ZS 90 Zeta Sizer Malvern Instruments, UK) at 25°C. The samples were kept in polystyrene cuvettes and observations were made at fixed angle of 90°. Samples were adequately diluted with 1mM NaCl solution before measurement. Zeta potential (Z) was measured by Laser Doppler Anemometry (LDA) on the same instrument at 25^0C without sample dilution or any salt addition. Similarly, NMDS were also characterized for size and size distribution.

Particle shape and Surface morphology

The freeze dried Ch-NP and NMDS were separately adhered onto double sided carbon tabs on aluminium stubs and were sputter coated with gold-palladium to minimize surface charging. The sputter coated samples were then observed under a scanning electron microscope at an acceleration voltage of 15 kV.

Determination of percentage yield

Yield was calculated as the percent ratio between the Ch-NP/ NMDS weight and the total component amount used in the sample preparation, using the equation:

$$\text{Percentage yield of Ch-NP} = \frac{\text{Ch-NP weight}}{\text{total solids (Ch + TPP + Clin) weight}} \times 100 \quad (1)$$

$$\text{Percentage yield of NMDS} = \frac{\text{NMDS weight}}{\text{total solids (PLGA + SC + Tre + NP) weight}} \times 100 \quad (2)$$

Determination of entrapment efficiency

The clindamycin loaded Ch-NP were dissolved in acidic buffer (pH=3.0) and then subjected to ultracentrifugation (16 000 x g for 30 min at 4°C). The amount of clindamycin [Cr] recovered in the supernatent, from the nanoparticles was estimated by HPLC method based on reverse-phase liquid chromatography developed by Orwa *et al.* (1999) with slight modifications [37]. The column used was Phenomenex (UK) Luna 5µ C-18 (2) 100R, and the mobile phase comprised of acetonitrile:phosphate buffer (1.35 % v/v phosphoric acid, adjusted to pH 6.0 with ammonium hydroxide):water in the ratio 35:40:25 at a flow rate of 1.5 mL/min. UV detection was performed at 214 nm. The entrapment efficiency was calculated from the amount of clindamycin originally added in the nanoparticles suspension [C_0] using the equation

$$\mathbf{E\% = [Cr]/[C_0] \times 100} \quad (3)$$

To determine the drug entrapment efficiency of NMDS, the tretinoin loaded NMDS were dissolved in DCM and centrifuged at 5000 rpm for 10 min. The remaining system i.e. the Ch-NP were treated as above. The amount of tretinoin [Tr] and Clindamycin [Cr] recovered, from the NMDS were estimated by HPLC method based on reverse phase separation and photodiode-array detection method as developed by Ye *et al.*, 2004, with slight modification [38]. A simple gradient with aqueous-acetonitrile (4:1) and aqueous-methanol (1:4) was used as mobile phases at a flow rate of 1.2 mL/min. The injection volume was 20 µl. For quantification, the chromatograph was obtained at 214 nm for clindamycin phosphate and 344 nm for tretinoin. The entrapment efficiency was calculated from the amount of tretinoin originally added in the NMDS suspension [T_0] using the equation:

$$\mathbf{E\% = [Tr]/[T_0] \times 100} \quad (4)$$

Determination of Nanoparticles (Ch-NP) Loading Efficiency in NMDS

The content of nanoparticles entrapped within the NMDS was estimated by lysing the system followed by determination of the clindamycin content by HPLC method as discussed in previous section.

$$\text{Loading capacity} = \frac{\text{Clindamycin recovered from NMDS}}{\text{Total Clindamycin entrapped in the added Ch-NP}} \quad (5)$$

***In vitro* drug release**

The *in-vitro* drug release from Ch-NP and NMDS was determined by dialysis tube diffusion technique. 100 mg of Ch-NP/ NMDS free of any unentrapped

drug were separately dispersed in 2 mL of PBS (pH 5.6) and taken in dialysis bag (MWCO 13 kDa, Himedia, India). These were dialyzed against 50 mL of PBS (pH 5.6) containing 0.1% (w/v) Tween 80, with constant stirring at 100 rpm and the temperature of the assembly was maintained at 37±0.1˚C throughout the study. Samples were withdrawn at predetermined time intervals while replacing equal volume of fresh dissolution medium. The withdrawn samples were diluted and assayed for drug content by HPLC method as described previously.

Photostability Study

The photostability of tretinoin entrapped in NMDS was evaluated for its stability as compared to its methanolic solution (MS) both in natural sunlight (SL) and artificial UV light [17,20,24,39].

Stability study in sunlight (SL)

The photostability of tretinoin in methanolic solution and NMDS was determined by exposing 5 mL of methanolic solution of tretinoin and dispersion of NMDS in concentration equilent to 10 µg/mL to natural sunlight for definite time intervals. The initial and final absorbance of the samples after adequate dilution was recorded using UV-Vis spectrophotometer (Cintra-10 UV-Vis spectrophotometer) at 344 nm.

Stability study in UV radiation

The accelerated photostability study of tretinoin was studied using a UV chamber (JSGW UV Chamber) set at 344 nm. 10 mL of tretinoin methanolic solution and NMDS dispersion with initial concentration equivalent to 1.0 mg/mL were exposed to UV radiation by keeping at a fixed distance of 10 cm. Samples were withdrawn at different time intervals for 3 hrs and processed for measuring the absorbance at 344 nm.

Laboratory precaution: To prevent the photodegradation of drug at time other than experimentation, the drug samples were protected from exposure to sunlight by using amberized glasswares.

Stability Study

The selected NMDS was filled in amber colored screw capped bottles and kept at 4±1˚C, 25±1˚C and 45±1˚C for 30 days. The residual drug content was determined at regular intervals of 10 days.

Preparation of Hydrogel

A weighed amount (2g) of Carbopol 934 P (1% w/w) was dispersed in deionized water under constant stirring. The obtained gel was then diluted separately with an appropriate amount (equivant to 1.0% clindamycin and 0.025% tretinoin) of dispersions of the prepared systems- plain NMDS**,** drug loaded NMDS**,** dye loaded NMDS, dye solution and plain drug solution (Clindamycin and tretinoin). Water was added to make up the volume upto 100 mL and the final viscous forms were named as, NMDS-gel, DNMDS-gel, DYNMDS-gel, DYS-gel and DS-gel, respectively [40].

Skin Irritation Study

The skin irritation study using DNMDS-gel, DS-gel and marketed formulation (Mkt F) containing both drugs was performed by carrying out the Draize patch test on rabbits [20,41]. The experiment was carried out in accordance with the CPCSEA guidelines with prior permission from Institutional Animal Ethics Committee of Dr. H. S. Gour University, Sagar, M.P. India. Proper humane care of animals was taken during the study period. Male rabbits (weighing 1.9-2.0 Kg) were used for the experiment and acclimatized before the beginning of the study. The animals were divided into 4-groups with 4 animals in each. The hairs of 2 x 2 cm^2 area of the rabbits back were removed by application of a hair removing cream 24 hrs prior to the experiment. The control group (group 1) received no drug treatment, DS-gel was applied to group 2, DNMDS-gel to group 3 and Mkt F to group 4.

The weighed quantity (0.5 g) of different formulations with dose equivalent to 1% clindamycin and 0.025% tretinoin were topically applied to each treated group of animals except the control group. After 24, 48 and 72 hrs of application, the exposed skin was scored on the basis of degree of erythema (redness) as follows [41].

No erythema	0
Very slight erythema (barely perceptible light pink)	1
Well defined erythema (dark pink)	2
Moderate to severe erythema (light red)	3

***In Vitro* Skin Permeation Study**

In vitro permeation of drugs from DNMDS-gel in comparison to DS-gel and Mkt F was evaluated using hairless abdominal skin samples of Wistar albino rats, excised from animals aged about 3 months and weighing 200-250g [20]. The locally fabricated Franz diffusion cell having an effective permeation area of 1.0 cm^2 and receiver cell volume of 10 mL was used for the study. The skin was mounted on the receptor compartment such that the stratum corneum side faces upward into the donar compartment. The receptor cell contained 10 mL of PBS (pH 5.6) containing 0.1% (w/v) Tween 80 which was

constantly stirred with a magnetic stirrer at 100 rpm (Remi, India). Experiment was carried out for 24 hrs at 32±1˚C with skin surface in the donar compartment of different cell applied with 0.5g of DNMDS-gel, DS-gel and Mkt F in a dose equivalent to 1% w/w clindamycin and 0.025% w/w tretinoin. Samples were withdrawn through the sampling port at predetermined time intervals over 24 hours and analyzed for drug permeated. The receptor compartment after each withdrawal of 200 µL sample was replenished with equal volume of fresh buffer. All the experiments were carried out in triplicate.

Skin Retention Study

In order to determine the amount of drugs (Clindamycin & tretinoin) retained in the skin of hairless wistar rat, skin stripping experiment was performed by using protective tape (3 M) [23,42]. At the end of permeation study, the skin surface was washed 3 times with PBS, removed and subsequently dried with filter paper. Each treated skin was further tape stripped 10 times to remove the stratum corneum layer of skin i.e., about 100 µg/cm^2. The same skin was also subsequently stripped for another 9 times to assure complete removal of stratum corneum. Drugs on each set of 10 tapes were dissolved separately in PBS (pH 5.6) containing 0.1% (w/v) Tween 80. Additionally the drugs in the remaining skin after tape stripping were also determined by homogenizing the skin twice with 3 mL of PBS. The solution was filtered through 0.22µm membrane filter and analyzed for drug content by HPLC method described previously.

Fluorescence Microscopy

The fluorescence microscopy was performed to confirm the deposition of selected drug carrier system into the dermis region. Fluorescent marker loaded DYNMDS-gel and DYS-gel were applied topically to albino rats (Wistar strain, 200-250g). After 3 hrs, an incision was made on the marked area and a small piece of skin was cut off. The pieces of skin were fixed and their microtomy was done. The sections were viewed under fluorescence microscope (NIKON, Japan) and photomicrographs of different areas were taken.

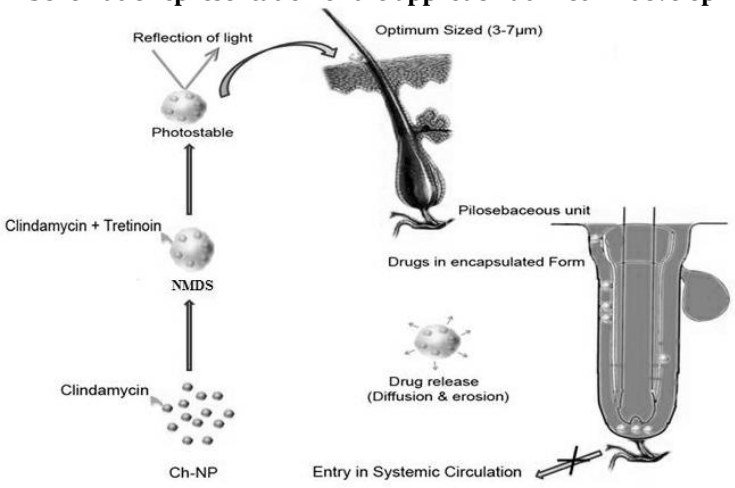
RESULTS AND DISCUSSION

The aim of the present investigation was to prepare and evaluate the potential of a novel dual drug delivery system i.e. nanoparticles-in-microspheres (NMDS) encapsulating two drugs, tretinoin and clindamycin simultaneously for effective management of acne vulgaris (Scheme 1). A considerable stress needs to be laid to develop complex formulations such as the proposed system (NMDS), however a controlled monitoring of various formulation and process variables helps in development of the system with required size range, low PDI, high percentage of drug entrapment and enhanced stability of the drug.

Preparation of Ch-NP and NMDS

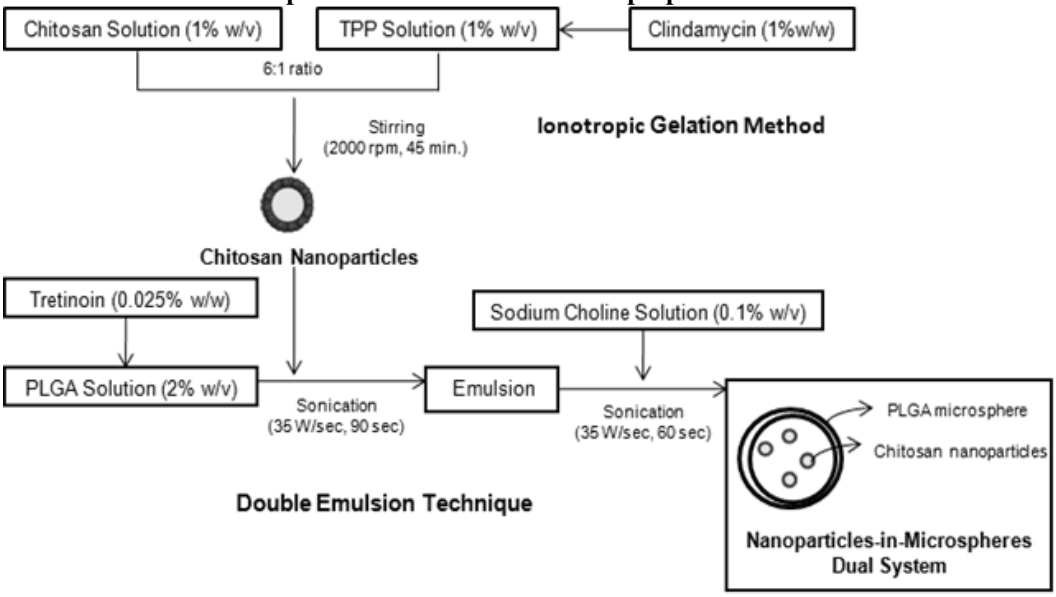
The Chitosan nanoparticles were prepared using ionotropic gelation method described by Calvo *et al.*, 2004 with slight modifications. This is the most common method for preparation of Ch-NP as the method is simple and also excludes the use of organic solvents. The results obtained (Table I) are in accordance to the previous studies. Further, batches of NMDS to obtain discrete particles were prepared using technique developed in lab (Scheme 2). The optimized formulation and process variables are reported in Table I.

Scheme 1- Schematic representation of the approach utilized in development of NMDS.



Scheme 1. Schematic representation of the approach utilized in development of NMDS.

Scheme 2- Schematic representation of the method of preparation of NMDS.



Scheme 2. Schematic representation of the method of preparation of NMDS.

Figure 1- SEM photomicrographs of (A) Ch-NP (B) NMDS.

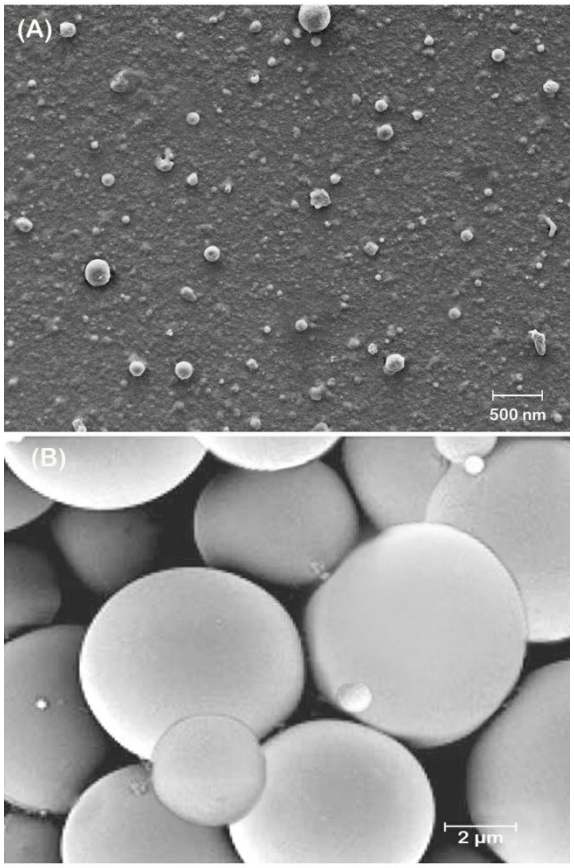


Figure 1. SEM photomicrographs of (A) Ch-NP (B) NMDS.

Figure 2- Cumulative % drug release in PBS (pH 5.6) at 37±0.1˚C.
Data represents mean ± SD (n=3).

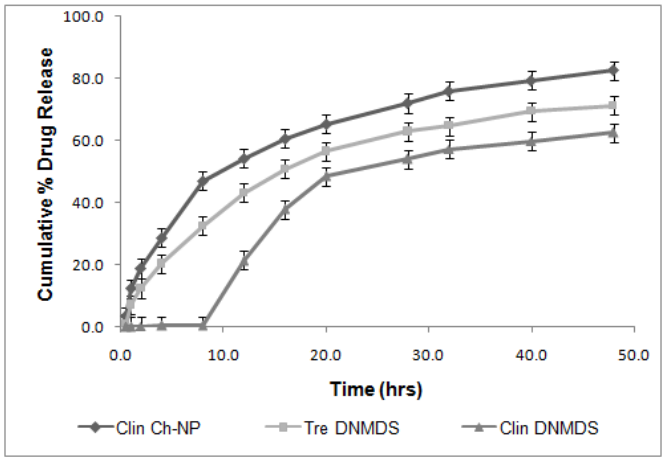


Figure 2. Cumulative % drug release in PBS (pH 5.6) at 37±0.1˚C.
Data represents mean ± SD (n=3).

Figure 3- Effect of sunlight (SL) and UV radiation on degradation of tretinoin.
Data represent mean ± SD (n=3; p≤0.05*). *Significant difference between methanolic solution (MS) and NMDS.

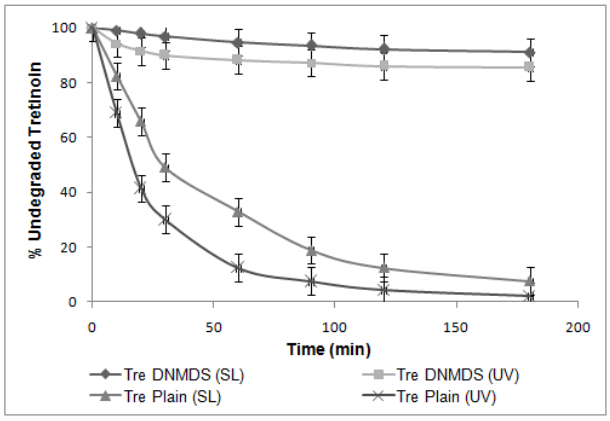


Figure 3. Effect of sunlight (SL) and UV radiation on degradation of tretinoin.
Data represent mean ± SD (n=3; p≤0.05*). *Significant difference between methanolic solution (MS) and NMDS.

Figure 4- Effect of storage temperature on % residual tretinoin and clindamycin content in NMDS.
Data represents mean ± SD (n=3; p≤0.05*, **, *). *Significant difference at 4±1˚C; **Significant difference at 25±1˚C; ***Significant difference at 45±1˚C.**

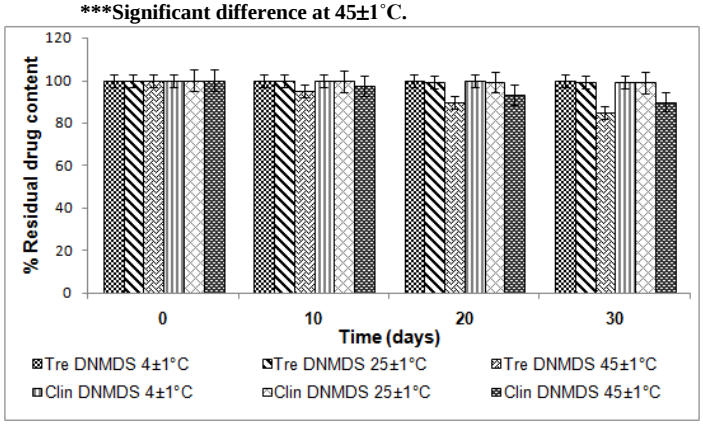


Figure 4. Effect of storage temperature on % residual tretinoin and clindamycin content in NMDS.
Data represents mean ± SD (n=3; p≤0.05*, **, ***). *Significant difference at 4±1˚C; **Significant difference at 25±1˚C; ***Significant difference at 45±1˚C.

Figure 5- Skin irritation study with (A) Control (B) DS-gel (C) DNMDS-gel (D) Mkt F.
(n=4; p≤0.05*, **, *). *Significant difference between DNMDS-gel and Control; **significant difference between DNMDS-gel and DS-gel; ***significant difference between DNMDS-gel and Mkt F.**

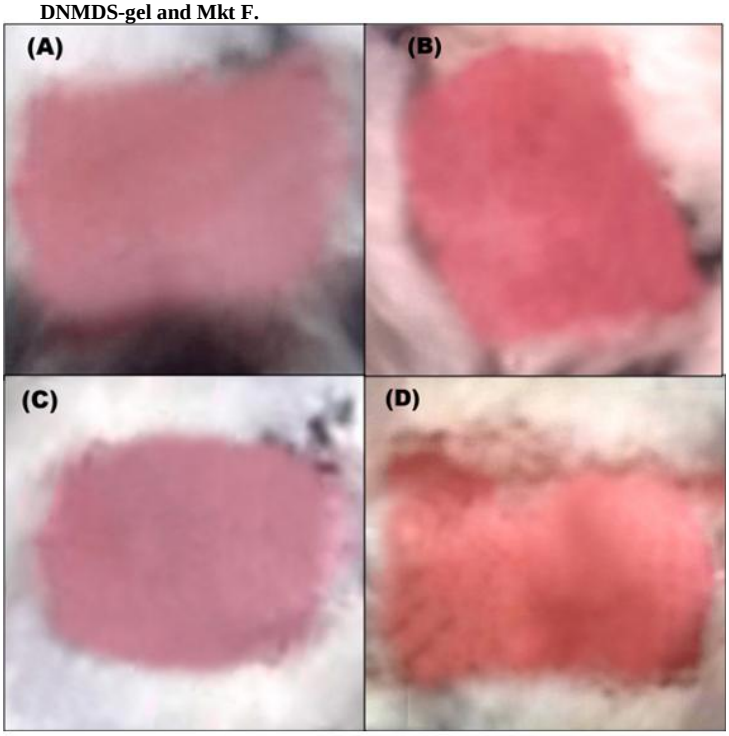


Figure 5. Skin irritation study with (A) Control (B) DS-gel (C) DNMDS-gel (D) Mkt F.
(n=4; p≤0.05*, **, ***). *Significant difference between DNMDS-gel and Control; **significant difference between DNMDS-gel and DS-gel; ***significant difference between DNMDS-gel and Mkt F.

Figure 6- Drug permeation through rat skin.
(n=3; p≤0.05*, **). *Significant difference between DNMDS-gel and DS-gel; **significant difference between DNMDS-gel and Mkt F.

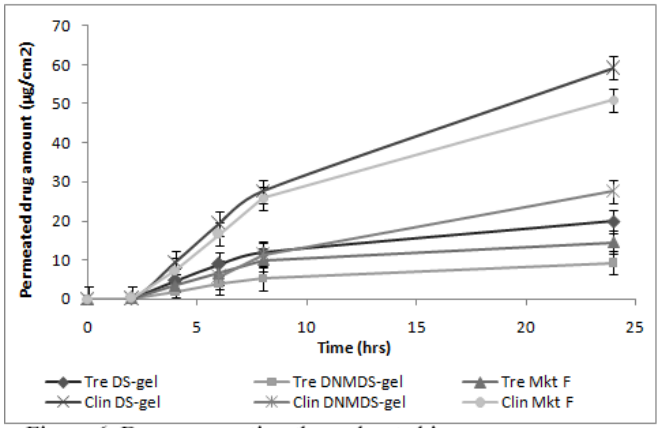


Figure 6. Drug permeation through rat skin.
(n=3; p≤0.05*, **). *Significant difference between DNMDS-gel and DS-gel; **significant difference between DNMDS-gel and Mkt F.

Figure 7- *In vitro* regional distribution of drugs retained in rat skin from different formulations at different time period (A) Tretinoin (B) Clindamycin.
(n=3; p≤0.05*' **). *Significant difference between DNMDS-gel and DS-gel; **significant difference between DNMDS-gel and Mkt F.

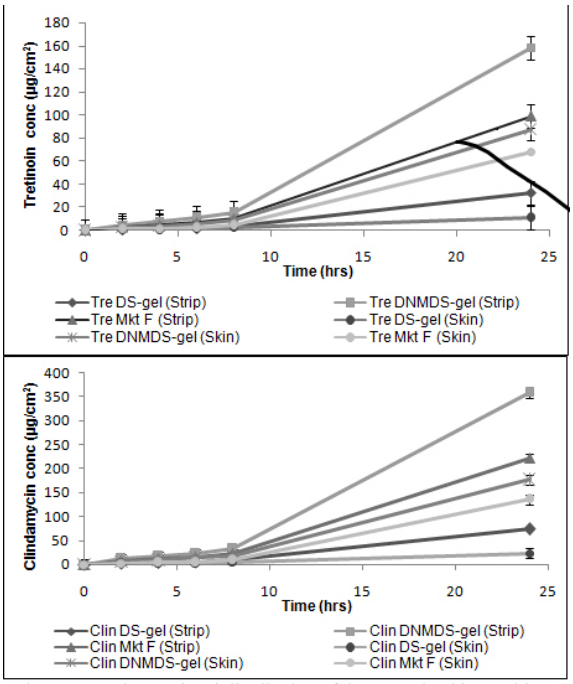


Figure 7. In vitro regional distribution of drugs retained in rat skin from different formulations at different time period (A) Tretinoin (B) Clindamycin.

Characterization of Ch-NP and NMDS

The Ch-NP were produced with a percentage yield of 62.5±1.21%. The size was found to be 253.6±8.23nm with PDI of 0.33±0.04. The zeta potential of the formulation was 28.2 ±2.7mV. The positive zeta potential may be attributed to amino groups present on unmodified chitosan [43]. Also the pH at which the Ch-NP are prepared influences the zeta potential. Clindamycin was successfully encapsulated within the Ch-NP with an entrapment efficiency of 58.2±0.93%. Drug entrapment did not lead to changes in production yield however an increase in size, although not significant, was observed. By careful monitoring of formulation variables, NMDS with average size 5.5±0.43µm and percentage yield 40.8±0.64% were prepared (Table II). The SEM revealed that the developed system were spherical in shape with smooth surface morphology (Figure 1 A-B). The studies clearly indicate the nano size of Ch-NP and an augmented size of NMDS. The results are in accordance with particle size study using Malvern particle size analyzer. SEM studies of dual system showed an increase in size and absence of nanoparticulates which revealed that the nanoparticles were encapsulated within the microspheres thus establishing the formation of dual system.

The prepared formulations were first studied to establish the release profile of the drugs from the Ch-NP as well as NMDS (Figure 2). The *in- vitro* drug release profiles of clindamycin entrapped Ch-NP and, clindamycin and tretinoin entrapped NMDS were studied using dialysis tube in PBS (pH 5.6). In case of Ch-NP, the formulation exhibited biphasic release profile. There was a rapid release of 19.0±0.92% of drug within 1 hour, followed by a slow release leading to 82.5±3.31% clindamycin release form the Ch-NP at the end of 48 hrs of study (Figure 2). The first phase could be attributed to surface erosion and rapid release of clindamycin molecules located at the surface or in the outermost layer of the particles, whereas the second phase might correspond to slow diffusion of drug molecules which are more efficiently entrapped, present in the inner shell and tightly bound to the chitosan molecules [31,44,45]. Drug release from the NMDS encapsulating both the drugs was also studied in similar fashion and was also found to show a biphasic release and cumulative release of 71.3±2.84% for tretinoin and 62.4±2.12% clindamycin was achieved in 48 hrs (Figure 2). Furthermore in contrast with clindamycin release from Ch-NP to that in NMDS, almost no drug release was observed for the initial 8 hrs. This may be attributed to encapsulation of Ch-NP into microspheres of PLGA, a hydrophobic polymer. This possibly inhibits the direct rapid access of surrounding fluid to the Ch-NP located in the inner core of NMDS.

Photostability Study

Tretinoin undergoes degradation when exposed to light, generating different isomers [24,46,47] and is reported to occur within 1-2 hrs after application from the conventional formulations [48]. Therefore the study of photodegradation of tretinoin occurring in the formulation is important. The observations obtained after photostability studies both in sunlight and UV radiation (Figure 3) demonstrated an improved photostability of encapsulated tretinoin as compared to its methanolic solution. Initially a minimal degradation of tretinoin was observed in case of NMDS encapsulated tretinoin which may be due to degradation of drug adsorbed on and nearby to the surface of the system. After 3 hrs exposure to sunlight, 7.1% of tretinoin was found to be remaining in the methanolic solution while 2.1% was left in case of exposure to UV radiation. In case of NMDS dispersion 95.11% of initial tretinoin concentration

was found to be intact after 3 hrs of exposure to sunlight and 90.60% of tretinoin was present after irradiating the system dispersion with UV radiation. This shows that a considerable amount of drug is encapsulated in the system and is therefore shielded from external environment [17]. Subsequently, due to presence of this mechanical barrier and reflection back of the incident light, minimal degradation of encapsulated drug was observed.

Stability Study

Storage stability of formulations based on drug carriers is of a great concern as it is the major restraint in the development of marketed preparations and also an important criterion for dose adjustment and assigning the expiry date. The storage temperature stability study was performed on optimized formulations of NMDS and its effect on percent residual drug content of the formulations was observed. This study indicated that formulations stored at 4±1˚C and 25±1˚C were more stable than those stored at 45±1˚C (Figure 4). Percent residual drug remaining in by assuming the initial drug content to be 100% revealed that no significant percent of drug was lost (1.09% of tretinoin and 1.01% of clindamycin) from the NMDS within 30 days, which were stored at 25±1˚C while only 0.31% of tretinoin and 0.20% of clindamycin was lost from those stored at 4±1˚C. However in case of formulations stored at 45±1˚C a high amount of both the drugs were found to be lost from the NMDS due to disruption of structural integrity.

Preparation of Hydrogel

Carbopol 934 P that was used to prepare hydrogel is hydrophilic, exhibits bioadhesive nature and may possibly be the reason of an increase in residence time of a drug at the site of absorption. In addition, it can also be formulated with an adequate pH value corresponding to physiological conditions and desirable viscosity and thus proved to be compatible with particulate carriers [40].

Skin Irritation Study

One of the major drawbacks accompanied with the tretinoin therapy in topical formulations is skin irritation (erythema) which strongly confines its utility and acceptability by the patients. Hence our aim in preparing such system was to deliver the drugs at the site of *P. acne* infection at the same time diminish or abolish these erythematic episodes. The results clearly indicated that the DNMDS-gel encapsulating the anti-acne drugs resulted in reduced irritation, (Table III and Figure 5) when compared with the DS-gel and Mkt F containing an equivalent amount of both the drugs in the study for 72 hrs. Possibly, this may be attributed to the entrapment of drug in NMDS which could reduce the direct contact of acidic functionalities (-COOH group of tretinoin) the triggering factor for the erythematic events with the stratum corneum [8,49]. This results in reduced erythematic episodes subsequently leading to less skin irritation [1]. The results obtained from the skin irritation studies are listed in table III and the photographs are depicted in Figure 5 (A-D). Thus, the NMDS demonstrates an improved drug delivery system for skin tolerability indicating their potential in topical delivery of anti-acne drugs.

***In vitro* Skin Permeation Study**

In the present investigation the *in vitro* skin permeation study are of vital importance since they help in evaluation of formulation to be retained on the skin and not passing across into the systemic circulation. The *in vitro* permeation of tretinoin and clindamycin through rat skin from the DNMDS-gel, DS-gel and Mkt F were performed in terms of mean cumulative amount diffused at each sampling time point during time period of 24 hrs (Figure 6). It may be predicted that the mechanism involved in permeation of drugs through the skin from dual system based gel is their transport in encapsulated form. The hypothesis is in line with skin irritation studies carried out in rabbits because if the drugs were being released from the system before permeating through skin, then a significant irritation would have been observed as on application of plain drug solution gel [20]. The prepared system showed a 2.1 and 1.6 folds reduction of skin permeation of tretinoin and; 2.1 and 1.8 folds of clindamycin as compared with DS-gel and Mkt F respectively. These results are of real significance since a high epidermal localization and reduced systemic absorption may be achieved using the prepared NMDS which is a desirable property for enhancing the treatment of acne vulgaris.

Skin Retention Study

Effective topical drug therapy requires drug uptake into skin at sufficient concentrations over a particular period of time for expected pharmacological activity. Variation in efficacy may result from inadequate drug delivery to the skin, rapid elimination from the skin, or the inherently weak pharmacological activity of a particular drug within the skin. The skin-stripping study was carried to investigate the drug uptake into skin layers at various time intervals. The amount of drugs retained in the hairless albino rat skin clearly showed that at different and definite time periods, DNMDS-gel containing clindamycin and tretinoin had maximum drug retention in skin as compared to other formulations (DS-gel, Mkt F) (Figure 7). This

increase in skin retention of 7.7 and 1.3 folds in case of tretinoin and; 7.6 and 1.3 folds in case of clindamycin as compared with DS-gel and Mkt F respectively may be attributed to possible effects of hydrophobic nature of the polymer used in formulation and its effect on physiological properties of the skin. The average particle size of NMDS was found to be 5.5µm. This increased its chances to be retained in the skin as the larger sized particles would not be able to enter the follicle while smaller sized would enter the systemic circulation. The study implies that the NMDS and hence the encapsulated drugs are able to enter the skin and at the same time localize at the site of infection exerting an optimal therapeutic action with interception of minimal side effects.

Figure 8- Fluorescence microscopic images of rat skin treated with (A) DYS-gel (B) DYNMDS-gel. (n=3; p≤0.05*). *Significant difference between DYS-gel and DYNMDS-gel

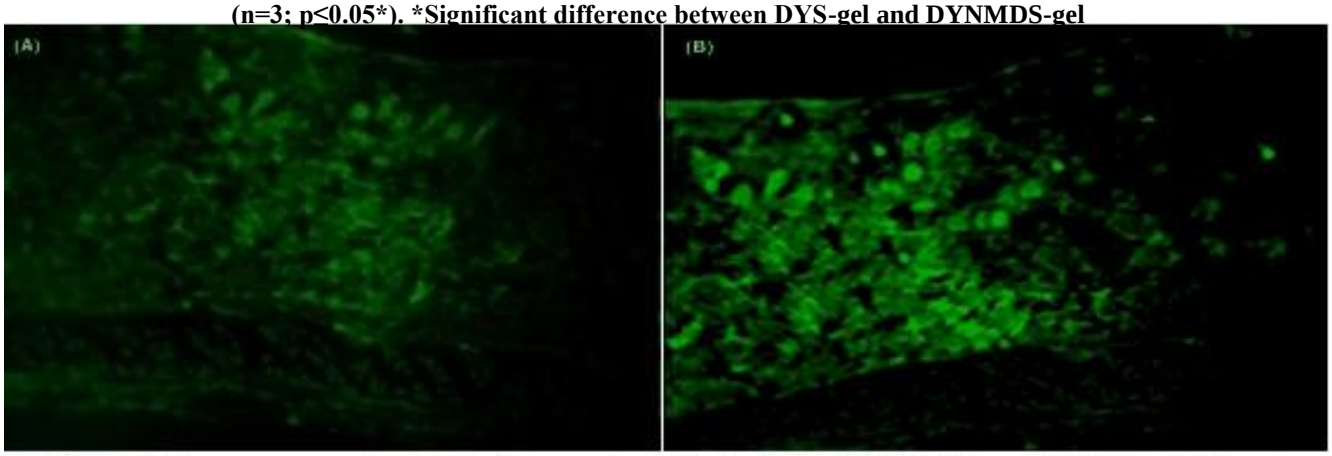


Figure 8- Fluorescence microscopic images of rat skin treated with (A) DYS-gel; (B) DYNMDS-gel. (n=3; p≤0.05*). *Significant difference between DYS-gel and DYNMDS-gel

Table I: Optimized formulation and process variables for preparation of Ch-NP and NMDS. Variables

Ch-NP	Optimized value	NMDS	Optimized value
Chitosan solution	1mg/ mL	PLGA	2% w/v
Chitosan: TPP ratio	6:1	Sodium choline	0.1% w/v
Stirring time	45 min	External Phase Volume	20 mL
Stirring speed	2000 rpm	Sonication time	60 sec

Data represents mean ± SD; (n = 3).

Table II: Physiochemical evaluation of Ch-NP and NMDS

S. No.	Physiochemical Parameter	Ch-NP	NMDS
1.	Particle size	253.6±8.23 nm	5.5±0.43 µm
2.	PDI	0.33±0.04	0.46±0.07
3.	Zeta Potential (mV)	28.2 ±2.7	-
4.	Nanoparticles loading efficiency	-	23.4±0.18 %
5.	Percentage Yield	62.5±1.21%	40.8±0.64 %
6.	Drug entrapment efficiency	58.2±0.93 %	35.4±0.20 %

Data represents mean ± SD; (n = 3).

Table III: Evaluation of skin irritation potency

Formulation	Mean erythemal scores		
	24 hrs	48 hrs	72 hrs
Control	**0**	**0**	**0**
DS-gel	**3**	**3**	**3**
DNMDS-gel	**0**	**1**	**1**
Mkt F	**1**	**2**	**2**

n = 4; $p \leq 0.05$*, **, ***. *Significant difference between DNMDS-gel and Control; **significant difference between DNMDS-gel and DS-gel; ***significant difference between DNMDS-gel and Mkt F.

Fluorescence Microscopy

For studying the extent of skin penetration and qualitative estimation of dye loaded system, the fluorescence microscopy was done as it is an excellent technique to study time profile, penetration pathways and penetration depths of carrier constituents. It can be seen in photomicrograph (Figure 8) that in the absence of carriers, the fluorescent marker fails to penetrate into the highly impermeable stratum corneum. However in case of DYNMDS-gel, the dye is transported extensively into deeper layer of the skin which can be seen by fluorescence intensity. This may be attributed to lipophilic nature of the carrier system, which provided access into the deeper layer of skin. Also the pilosebaceous units may also act as reservoir due to controlled size of the prepared system leading to enhanced flux and targeting deeper layer of skin without transporting the system into systemic circulation which is desirable for treatment of acne vulgaris [33,34].

CONCLUSION

Since last half a decade, extensive studies have been performed to develop an optimized delivery system for effective delivery of anti-acne drugs at site of acne infection. NMDS was successfully prepared and the results indicated the rational of such system to provide combination drug therapy. NMDS provided controlled and prolonged release of an adequate amount of drug in skin and reduced irritation. The present study, explore the possibility for selection of this newer novel carrier utilizing biodegradable polymers for the management of acne vulgaris. In parallel with laboratory evaluation of safety and efficacy, it is hoped that rationally designed clinical trials with this system may ultimately lead to improved treatment of acne vulgaris. Furthermore, studies pertaining to this carrier system may be performed with other antiacne drugs.

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