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Research Article

NANOLIPOBEADS BASED DRUG DELIVERY SYSTEM FOR EFFECTIVE MANAGEMENT OF PEPTIC ULCER

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ABSTRACT

The objective of the present investigation was to prepare and evaluate hydrogel anchored lipid vesicles system *i.e*. nanolipobeads for the treatment of mucosal ulcer consisting of (i) poly vinyl alcohol (PVA) biocompatible polymer (ii) ranitidine bismuth citrate (RBC), and amoxicillin trihydrate (AMOX) an antisecretory and antimicrobial agent and (iii) phosphotidylethanolamine (PE) liposome on surface of PVA hrdrogel nanoparticles to permit target specific delivery of drugs to *H.Pylori*. The effect of various formulation and process variable in the preparation of PVA hrdrogel nanoparticles *viz* surfactant concentration, stirring speed, stirring time, sonication time, Number of freez thaw cycles and oil: PVA phase ratio were optimized while keeping other variable constant for particle shape, size distribution and % entrapment efficiency. The optimized PVA hrdrogel nanoparticles was found with 268.2 ±3.4 nm size and *In vitro* drug release of RBC from PVA hrdrogel nanoparticles was found 94.3±1.4% up to 144 hr. Surface acetylation accomplished by treating PVA hrdrogel nanoparticles with 1M polymitoyl chloride. Finally nanolipobeads synthesis was carried out by combining equal parts of suspension of acylated PVA hrdrogel nanoparticles to AMOX encapsulated (7:3) PE liposome suspension of 174.8±5.2 nm. The uniformity of supported lipid PE layer on acylated PVA hrdrogel nanoparticles was examined using fluorescent microscopy. The optimized nanolipobeads formulation demonstrated 773.38 ±2.32 nm average size and *In vitro* sustained release of AMOX and RBC 96.3±1.6% and 91.4±1.2 RBC up to 144 hr respectively. The reduction of the absolute alcohol induced ulcerogenic index from 3 ± 0.08 to 0.2 ± 0.01 suggested that nanolipobeads are a useful for the development of dual drug delivery system for effective treatment of peptic ulcer.

Keywords: hydrogel anchored lipid vesicle system; nanolipobeads; sustained release; PE liposomes; peptic ulcer.

INTRODUCTION

Peptic ulcer disease is a major health problem with multifactorial etiology. The development of gastric ulcer occurs with acid and the breakdown of mucosal defence.Local mechanisms implicated in mucosal defence are; mucus-like alkaline secretions, mucosal hydrophilicity, rapid epithelial cell renewal, rich mucosal blood flow, mucosal sulphydryls and increased resistance of gland cells in deep mucosa to acid and peptic activity [1]. The two main etiological factors in peptic ulcer disease are *H. pylori* infection and adherence of pathogenic bacteria to target cells is an important step in the pathogenesis of many bacterial diseases and the use of anti-inflammatory drugs (NSAIDs) including aspirin [2]. Attachments of organisms to gut mucosal surfaces, host tissues are exposed to higher concentrations of bacterial enterotoxins. Adherence is also important for entry of organisms into epithelial cells. Histological examination of biopsy specimens from the antrum of human stomach has revealed the presence of *H. pylori* within gastric mucous and adherent to the apical membranes of gastric epithelial cells [4-5]. *H. pylori was* shown previously to bind to a specific alkylacyl glycerolipid derived from human erythrocytes, HEP2 cells and human antral epithelium [6]. Furthermore cultured human cells with less PE show minimal attachment of *H. pylori* in vitro [7] emphasizing the importance of PE–*H. Pylori* interaction. In bacterial adhesion PE is a predominant lipid in the antrum of the human stomach and functions as a receptor for *H. pylori* adhesion. Correlation of the ability of *H. pylori* to adhere to eukaryotic cells with the detected presence of the PE receptor, however underscores the importance of this lipid as a major receptor in promoting *H. pylori* adhesion to intact cells. PE bacterial adhesin exists as a cell surfaceassociated ligand. On the basis of the above facts, antiadhesion drug delivery system based on PE has been developed as a receptor-mediated drug delivery system for use in blocking adhesion of *Helicobacter* and thereby preventing the sequelae of chronic gastric infections.

A growing amount of literature describes the development and application of novel targeting or release triggering schemes to improve the therapeutic index of drugs encapsulated within PE liposomes [8]. However phosphatidyl ethanolamine liposomes are unstable in acidic pH and in the presence of divalent cations, their loading capacity being limited by the water solubility of the material to be loaded [9]. Polymeric beads although mechanically more stable and having a larger loading capacity than liposomes, lack many of the useful surface properties of a lipid bilayer shell [10].

Artificial particulate systems such as polymeric beads and liposomes are finding a variety of biomedical applications in drug delivery, drug targeting, protein separation, enzyme immobilization and blood cell substitution [11-12]. Liposomes have a flexible, cell-like lipid bilayer surface which acts as a permeability barrier such that compounds can be entrapped in their aqueous interior. However, liposomes can be mechanically unstable and their loading capacity limited by the water solubility of the material to be loaded [13]. Polymeric beads, although mechanically more stable and having a larger loading capacity then liposomes, lack many of the useful surface properties of a lipid bilayer shell. Here we describe the preparation and characterization a new hybrid vesicle system with structural similarity to natural cells that combines complementary advantages of liposomes and polymeric beads this system which we have called 'Lipobeads' consists of a lipid bilayer shell that is anchored on the surface of a hydrogel polymer core. Lipid bilayers supported on various solid surfaces, such as glass plastic and metal as well as modified polymers have previously been shown to provide a stable and well defined cell membrane-like environment that has found a number of basic and applied uses. It was, therefore reasonable to expect that assembly of lipid bilayers on spherical hydrogel surfaces could be a useful approach for preparing an artificial cell analogue. Indeed in 1987, Gao and Huang [14] reported that encapsulation of hydrogel particles into liposomes enhanced the loading capacity and overall mechanical strength of the liposomal structure. However in that system the unanchored bilayer is still somewhat unstable and the system could only be formed with specific lipid mixtures and only with polymer cores of certain sizes and shapes. We have developed a hydrogel anchored liposome in which these limitations have been overcome.

Our approach has been to create hydrophobic anchors for the bilayer by attaching lipid molecules to the surface of pre-formed polyvinyl alcohol (PVA) xerogel (dry hydrogel) beads. When the surface modified xerogel is then hydrated and treated with a liposome suspension the hydrophobic lipid molecules and other intrinsic membrane components of the liposomes associate spontaneously with the hydrophobic fatty acid anchors on the

surface of the hydrated polymer and self-organize into a distributed membrane over that surface through hydrophobic interactions. Thus not only do these anchors add mechanical stability to the bilayer shell but they also promote its self-assembly. In a sense, these acyl anchors and the polymer to which they are attached act as a 'cytoskeleton'. This self-assembly property allows the bilayer coating to be established on cores ranging in shape from sheets to spheres. Lipobeads *in vitro* mimic natural biological membrane and thus provide a model environment for cell-cell interactions for various biotechnological applications and operation of purified and reconstituted transmembrane protein such as ion channels transporters [15-19].Such preparation have many attractive properties and may serve as useful tools for increasing the under standing of processes at and across the membrane surface. Phospholipids supported on a hydrophilic solid substrate are extensively used in the study of the interaction between the membranes and proteins/ polypeptides. In addition lipid coated particles can be exploited as a novel approach to fix the membrane bound enzyme onto the carrier surface [20]. Another potential application of lipobeads is red cell substitute. By combining the structural features of liposomes and hydrogels lipobeads may provide a more realistic red cell replacement while at the same time having much higher encapsulating efficiency than liposomes.In addition to these, fluorescent lipobeads for intracellular oxygen measurement in murine macrophase and fluorescence sensing lipobeads have also been exploited for intracellular pH measurement.

We utilized the concept in developing targeted drug delivery systems based on PE in the treatment of *H. pylori*. Although *H. pylori* is susceptible to many antimicrobial agents, clinical trials with single antimicrobial agent(s) have not shown the complete eradication of *H. pylori*. This is because *H. pylori* exclusively resides on the luminal surface of the gastric mucosa under the mucous gel layer and access of antimicrobial drugs to the site of infection is restricted both from the stomach and from the gastric blood supply. For effective *H. pylori* eradication therapeutic agent(s) must penetrate into the adhesion sites and permit localized release.

MATERIALS AND METHODS

The Ranitidine bismuth citrate (RBC) and amoxicillin trihydrate (AMOX) was generously supplied as a gift sample by M/s Glaxo Smith Kline, Bombay (India) and Torrent Pharmaceutical, Ahmedabed India respectively. Phosphotidylethanolamine (PE) was generously supplied as a gift sample by M/s Lipoid GmBH (Germony). Polyvinyl vinyl alcohol (PVA) was purchased from M/S sigma USA. Polymityl chloride was obtained from Himedia Laboratories Ltd, Mumbai, India. All other chemicals and reagents used were of analytical grade. The *in vivo* study was performed following the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA) of the Ministry of Social Justice and Empowerment, Government of India. The institutional animal ethical committee of Dr. Hari Singh Gour Centre University, Sagar, India approved the study.

Preparation of Pva Hydrogel Nanoparticles

PVA hydrogel nanoparticles bearing RBC were prepared by emulsification followed by freeze thaw cyclization method. Fully hydrolysed PVA with molecular weight of (1,250000) was dissolved in preheated water (70-80°C) at a concentration of 15% and the solution was heated at 90°C for 30min. 50 mg of RBC was added to the PVA solution at room temperature and mixed thoroughly. 5ml of PVA aqueous solution was dispersed in soya oil (PVA: Oil) containing emulsifier Span 80 under continuous mechanical agitation (Remi Instruments, India) at constant speed for 45 min. This leads to the formation water-in-oil (w/o) after sonicated upto 3 min by using Titanium probe ultrasonicator (Imeco, Ultrasonic India).The prepared w/o emulsion were then crosss linked by frozen at -20°C(Sanyo ultra freeze Japan) for 24 hr followed by thaw cycle 4 hr for each. Three such FT cycles were resulted in conversion of the emulsion to a suspension of PVA in soya oil. The PVA hydrogel nanoparticles were centrifuged at 10,000 rpm for 2 min decant the supernatant oil. PVA hydrogel nanoparticles particles were rinsed twice with petroleum ether to remove the trace oil phase. The nanoparticles were than dried and kept in a 4°C refrigerator.

Optimization of Formulation Variables

Optimization of PVA concentration

The optimization of PVA concentration varying concentration PVA *viz* 5%, 10%, 15% and 20% while keeping other variables constant..Optimization was done on the basis of average particle size and poly dispersibity index (PDI) of PVA hydrogel nanoparticles which were determined by using zetasizer. (Malvern ZS 90).

Optimization of drug (RBC) concentration

For the optimization of RBC concentration the formulation (NP-C_3) was selected and different PVA hydrogel nanoparticles were prepared with varying with varying concentration RBC *viz* 10, 25, 50 and 75 mg while keeping the other variables constant. Optimization was done on the basis of average particle size and maximum % drug entrapment.

Optimization of Surfactant concentration

For the optimization of concentration of Span 80 formulation **(**NP-C_3D_3) was selected and different PVA hydrogel nanoparticles were prepared with varying concentration of Span 80 *viz* 1 %, 1.25 % 1.5%, and 2 % w/v in aqueous medium while keeping the other variables constant. The optimization was done on the basis of average particle size and maximum % drug entrapment efficiency.

Optimization of oil: PVA phase ratio

For the optimization of soya oil: PVA phase ratio formulation **(**NP-$C_3D_3E_2$) was selected and different PVA hydrogel nanoparticles were prepared by taking varying oil: PVA phase ratio *viz* 1:1, 3:1, 5:1, 10:1 while keeping the other variables constant. The optimization was done on the basis of average particle size and maximum % drug entrapment efficiency.

Optimization of Stirring speed

For the optimization of stirring speed formulation (NP-$C_3D_3E_2O_4$) was selected and different PVA hydrogel nanoparticles were prepared by taking varying stirring speed i.e. 1000, 2000, 3000 and 4000 rpm (Remi,Moters) keeping the other variables constant. The optimization was done on the basis of average particle size and maximum % drug entrapment efficiency.

Optimization of Stirring time

For the optimization of stirring time formulation (NP-$C_3D_3E_2O_4R_4$) was selected and different PVA hydrogel nanoparticles were prepared by taking varying stirring time i.e.

15, 30, 45, and 60 min while keeping the other variables constant. The optimization was done on the basis of average particle size and maximum % drug entrapment efficiency.

Optimization of Sonication Time

For the optimization of sonication time formulation (NP-$C_3D_3E_2O_4R_4T_3$) was selected and different PVA hydrogel nanoparticles were prepared by taking varying sonication time i.e. 1, 2, 3and 4 min while keeping the other variables constant. The optimization was done on the basis of average particle size and maximum % drug entrapment efficiency.

Optimization of Freeze thaw (FT) cycles

For the optimization of freeze thaw cycles formulation (NP-$C_3D_3E_2O_4R_4T_2$ ST_2) was selected and different PVA hydrogel nanoparticles were prepared by taking varying freeze thaw cycles *i.e.* 1, 2, 3 and 4 cycles by frozen the formulation at -20°C (Sanyo ultra freeze Japan) for 12 hr followed by thaw cycle for each 4 hr. The optimization was done on the basis of average particle size and maximum % drug entrapment efficiency.

Table 1: The various formulations and process variables for preparation of PVA hydrogel nanoparticles

S. No.	Formulation code (S)	PVA Concentration	Particle size (nm)	PDI
1.	NP-C_1	5 %	191.3 ± 3.3	0.552
2.	NP -C_2	10 %	228..8 ± 4.1	0.02
3.	**NP -C_3**	**15 %**	**268.7 ± 2.4**	**0.324**
4.	NP- C_4	20 %	340.2 ± 4.2	0.408
		RBC Conc. (mg)		**% EE**
1.	NP- C_3D_1	10	251.1 ± 2.2	83.1 ± 3.2
2.	NP – C_3D_3	25	257.4± 1.1	87.3 ± 2.2
3.	**NP- C_3D_4**	**50**	**263.5± 5.1**	**94.6 ±3.5**
4.	NP – C_3D_2	75	266.5 ± 6.3	73.2 ± 1.9
		Span 80 Conc. (% W/V)		
1.	NP- $C_3D_3E_1$	1 %	438.3 ± 4.3	78.6 ± 2.2
2.	NP- $C_3D_3E_2$	1.25 %	306.3± 7.2	72.2± 1.9
3.	**NP- $C_3D_3E_3$**	**1.5 %**	**265.7± 5.4**	**84.6 ± 2.5**
4.	NP- $C_3D_3E_4$	2 %	210.2± 5.1	49.2± 1.6
		Oil: PVA phase ratio		
1.	NP- $C_3D_3E_2O_1$	1:1	711.3 ± 4.2	51.3±2.3
2.	NP- $C_3D_3E_2O_2$	3:1	662.4 ± 7.4	64.71.7
3.	NP- $C_3D_3E_2O_3$	5:1	467.5 ± 5.1	82.5±2.3
4.	**NP- $C_3D_3E_2O_4$**	**10:1**	**288.6± 3.4**	**91.7±1.5**
		Stirring Speed (rpm)		
1.	NP-$C_3D_3E_2O_4R_1$	1000	556.2 ± 6.2	74.2 ± 2.6
2.	NP-$C_3D_3E_2O_4R_2$	2000	438.3 ± 7.1	86.1± 2.2
3.	NP-$C_3D_3E_2O_4R_3$	3000	310.6 ± 5.7	84.2± 1.8
4.	**NP-$C_3D_3E_2O_4R_4$**	**4000**	**279.8 ± 4.2**	**93.4 ± 2.7**
		Stirring Time (min)		
1.	NP-$C_3D_3E_2O_4R_4T_1$	15	421.3± 7.1	89.8 ± 3.6
2.	NP-$C_3D_3E_2O_4R_4T_2$	30	382.7 ± 6.4	85.9 ± 2.8
3.	**NP-$C_3D_3E_2O_4R_4T_3$**	**45**	**265.3 ± 5.7**	**88.3 ± 4.3**
4.	NP-$C_3D_3E_2O_4R_4T_4$	60	706.5 ± 4.8	67.5 ± 1.7
		Sonication Time (min)		
1.	NP-$C_3D_3E_2O_4R_4T_3$ ST_1	1	433.3 ± 7.1	83.1 ± 2.4
2.	**NP-$C_3D_3E_2O_4R_4T_3$ ST_2**	**2**	**256.2 ± 8.5**	**85.2 ± 3.1**
3.	NP-$C_3D_3E_2O_4R_4T_3$ ST_3	3	180.3± 6.2	69.4 ± 1.3
		Number of (FT) cycles		
1.	NP-$C_3D_3E_2O_4R_4T_2$ ST_2	1	223.3 ± 6.3	73. 7 ± 2.7
2.	NP-$C_3D_3E_2O_4R_4T_2$ ST_2	2	250.2 ± 2.1	78.2 ± 3.9
3.	**NP-$C_3D_3E_2O_4R_4T_2$ ST_2**	**3**	**273.6 ± 4.4**	**89.6 ± 4.5**
4.	NP-$C_3D_3E_2O_4R_4T_2$ ST_2	4	371.3 ±6..2	71.3 ± 3.6

S.D. ± Mean (n=3)

Table 2: The optimized variable for PVA hydrogel nanoparticles selected were as follows

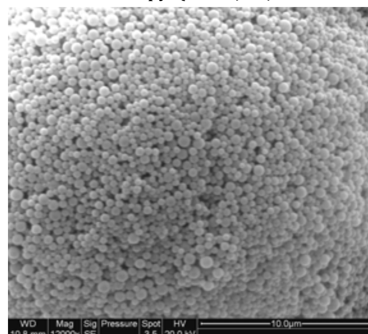
Parameter (s)	Optimized Variables
PVA concentration	15 %w/v
RBC	50 mg
Span 80	1.5 % w/v
Oil : PVA phase ratio	10 : 1
Stirring time	45 min
Stirring Speed	4000 rpm
Sonication time	45 sec
Freeze thaw cycles	3

On the basis of optimization studies formulation NP-$C_3D_3E_2O_4R_4T_2$ ST_2 FT_3 was selected for further studies.

Characterization of Pva Hydrogel Nanoparticles

Shape and surface morphology

Shape of the optimized PVA hydrogel nanoparticles formulation was investigated by scanning electron microscopy (SEM, JealJX 840-A, Tokyo, Japan). A thin film of aqueous dispersion was applied on double stick tape over an aluminium stub and air dried to get uniform layer of particles. These particles were coated with gold using sputter gold coater. The sample were scanned photographmicrograph were taken are shown in Fig 1 (a) and (b).



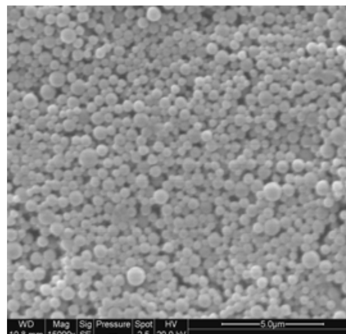


Fig. 1: (a) SEM photomicrographs of optimized PVA hydrogel nanoparticles formulation; (b). SEM photomicrographs of acetylated PVA hydrogel nanoparticles formulation.

Average particle size, zeta potential, polydispersity index of optimized PVA hydrogel nanoparticles

The particle size distributions of different formulations were measured by Zetasizer (Malvern ZS 90). For determination of zeta potential of a voltage is applied across a pair of electrodes at either end of a cell containing the particle dispersion. Charged particles are attached to the oppositely charged electrode. This method uses the auto correlation function of light scattered by the particle is collected by the photomultiplier and the measured autocorrection function is first converted using a fourier transform into a frequency spectrum. The frequencies are than converted successively to velocities, electrophoretic mobilities and finally zeta potential. The measurements of the zeta potential of optimized PVA hydrogel nanoparticles were performed using 0.1M KCl buffer in demineralized water at 25 °C.

The poly dispersity index optimized PVA hydrogel nanoparticles was calculated using following formula

$$\text{Polydispersity index} = \frac{\text{Standard deviation}}{\text{Average particle size}} \quad (1)$$

Theoretically polydispersity index is zero for a monodisperse colloidal suspension. However the standard latex particles with a PDI of about 0.05 are considered as monodispersed and with PDI greater than 0.5 are assumed to have a broad size distribution [21].

% Entrapment efficiency of RBC in different PVA hydrogel nanoparticles

The percentage entrapment efficiency of added drug that is dissolved, dispersed or encapsulated in any formulation can be calculated on the basis of ratio of actual amount of drug in the formulation and theoretically amount of drug added to the formulation during preparation.

100 mg dried PVA hydrogel nanoparticles were accurately weighed and dissolved in small quantity of hot water and the digested hydrogenate was centrifuged at 3000 rpm for 3 min. The solution was than filtered with membrane filter (1 µm) the filtrate was diluted approximately with SGF (pH 1.2) and analyzed spectrophotomatrically for RBC at λ_{max} 272 nm. The % entrapment efficiency was calculated by using following equation.

$$\text{Entrapment Efficiency (\%)} = \frac{M_{actual}}{M_{theoretical}} \times 100 \quad (2)$$

Were M_{actual} is the actual amount of RBC entrapped PVA hydrogel nanoparticles which were determined by above experiment and $M_{theoretical}$ is the amount of drug added during preparations.

Table 3: Characteristic of optimized PVA hydrogel nanoparticles (NP-$_3D_3E_2O_4R_4T_2$ ST_2 FT_3) *In vitro* RBC release from optimized PVA hydrogel nanoparticles.

S.No.	Formulation	Particle size (nm)	Zeta Potential (mV)	Polydispersity Index (PDI)	% Entrapment efficiency
1.	NP-$C_3D_3E_2O_4R_4T_2$ ST_2 FT_3	268.2 ±3.4	-18.38±0.97	0.183	86. 2 ± 2.2 %

S.D. ± Mean (n=3)

The release behavior of optimized PVA hydrogel nanoparticles formulation was determined by *in vitro* drug release studies in simulated gastric fluids SGF (pH 1.2), simulated intestinal fluids SIF (pH 6.8) and phosphate buffer saline PBS (pH 7.4) was studied by dialysis cell membrane method.

500 mg of optimized PVA hydrogel nanoparticles (NP-$C_3D_3E_2O_4R_4T_2ST_2FT_3$) formulation were taken in dialysis membrane (cellulose dialysis membrane, 2.4nm porosity, Himedia Labs, Mumbai, India) against 100 mL of medium and the solution was continuously stirred using magnetic stirrer at 37±2°C. After appropriate time intervals 1mL of sample was withdrawn and analysed for drug content. Simultaneously equal volume was fresh media was added to replenish the withdrawn sample. RBC release was meseaured at suitable wavelength by spectrophotometerically (Shimadzu 1601 UV/Visible Spectrophotometer) are shown in Fig. 2.

Statistical analysis

Statistical analysis was performed with Graph Pad Instat Software (Version 3.00 Graph Pad Software, San Diego California USA) using one way ANOVA followed by Tukey-Kramer multiple comparison test. Difference with P< 0.05 was considered statistically significant.

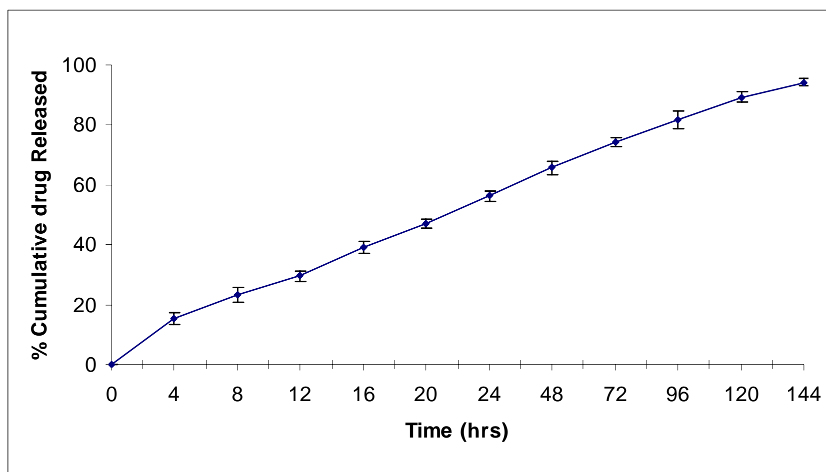


Fig. 2: % Cumulative RBC release from optimized PVA hydrogel nanoparticles in PBS (pH 7.4)

Preparation of Acylated Pva Hydrogel Nanoparticles

Surface acetylation with fatty acid chain was accomplished by treating PVA hydrogel nanoparticles with 1M polymitoyl chloride in hexane at room temperature for 2-3 days and evaporating the solvent. The observation shown in Fig. 3 (a) and (b)

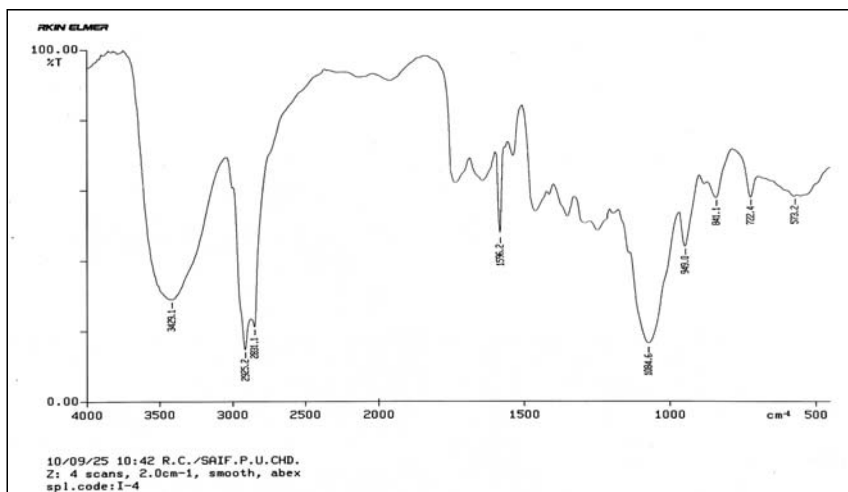


Fig. 3: (a) FTIR spectrum of PVA hydrogel nanoparticles

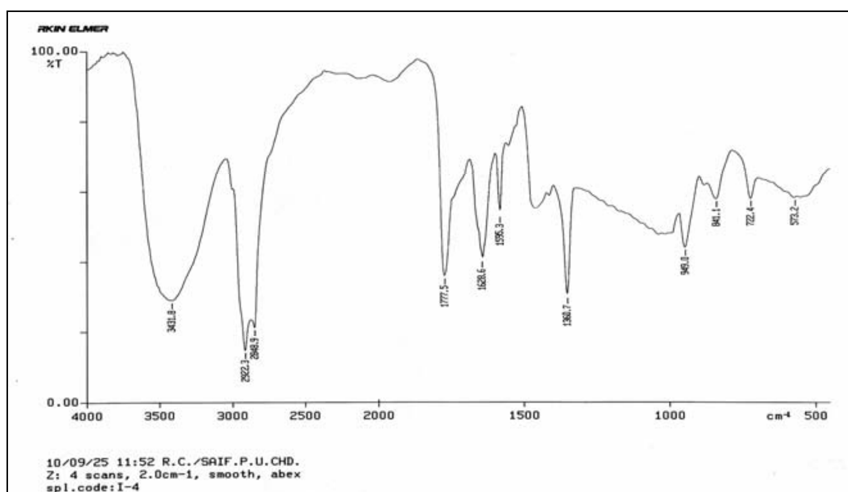


Fig. 3: (b) FTIR spectrum of acetylated PVA hydrogel nanoparticles

Preparation of Nanolipobeads by Vesicle Fusion Method

Nanolipobeads synthesis was carried out by combining equal parts of suspension of acylated PVA hydrogel nanoparticles to PE liposome suspension. PE liposome prepared by reverse phase evaporation method in which PE and cholesterol (7:3 molar ratio) were dissolved in 5 ml diethyl ether. To the above solution 10 mL of distilled water containing 10 mg amoxicillin trihydrate (AMOX) was added. The mixture was sonicated at 40W and 40% output frequency (Titanium probe ultrasonicator, Imeco Ultrasonic India) for 10min. The thick emulsion so formed was vortexed (Superfit, India) for 15 min to remove any residual ether. The liposomes were passed through sephadex G-50 minicolumn followed by eluting the column with 5 mL distilled water to separate free drug and lipid debris. Liposomes were than extruded through polycarbonate membrane (Millipore, USA) of 0.2 µm pore size to obtain vesicles of uniform size distribution. Suspension of acetylated PVA hydrogel nanoparticles was combined with equal part of PE liposomal suspension. Fusion of liposome on the acylated PVA hydrogel nanoparticles was than accomplished by lowering the pH below 6.5 of the mixture by addition of 0.1N NaOH. AMOX

Characterization of Nanolipobeads

Surface morphology

Surface morphology and the uniformity of supported lipid layer on acylated PVA hydrogel nanoparticles was examined using fluorescent microscopy (Vanox-S, Olympus Germany). For Fluorescent Microscopy study PE: rhodamine: cholesterol in chloroform at molar ratio of 7: 0.1:3 were used to prepare the liposome. Liposomes were formed by rotating flask evaporating method. Fluorescent labelled liposomes and acylated PVA hydrogel nanoparticles were combined and the pH was lowered below 6.5 by addition of 0.1N NaOH to induce vesicle fusion. The prepared nanoplipobeads were examined using fluorescent microscopy and photomicrographs are shown in Fig 4.

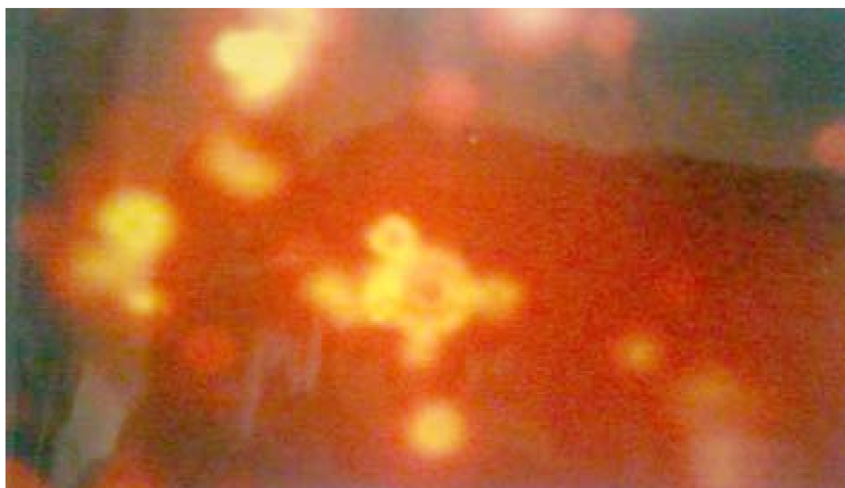


Fig. 4: Fluorescent photomicrograph of rhodamine labeled nanolipobeads formulation

Average particle size, polydispersing index, and Zeta Potential of nanolipobeads formulations

The average particle size, polydispersing index, and Zeta Potential of nanolipobeads formulations were determined by a particle size analyzer (Malvern ZS 90)

Table 4: Particle size, shape, Zeta Potential and Polydispersity index of different nanolipobeads formulations

S.No.	Formulations code (s)	PE:CH ratio in nanolipobeads formation	Particle size (nm)	Particle shape	Zeta Potential (mv)	Polydispersity Index (PDI)
1.	LB1	9:1	832.78 ±5.17	Deformed	+ 5.62±0.39	0.434
2.	LB2	8:2	779. 22±4.43	Nearly spherical	+3.19±0.25	0.337
3.	LB3	7:3	773.38 ±2.32	Spherical	+1.26±0.25	0.098

S.D. ± Mean (n=3)

% Entrapment efficiency of AMOX and RBC in nanolipobeads formulations

% Entrapment efficiency was determined by treating the nanolipobeads suspension with a few drops of Triton-X-100 (1% v/v) to induce complete disruption of lipid layer. Add 5 ml of hot water and the digested hydrogenate was centrifuged at 3000 rpm for 3 min. The solution was than filtered with membrane filter (1 µm). The filtrate was diluted approximately with SGF (pH 1.2) and analyzed for drugs RBC & AMOX spectrophotometrically at 223 and 272 nm respectively by using simultaneous equation [30]. The drug content of each sample was determined in triplicate and values are shown in shown in Fig.5.

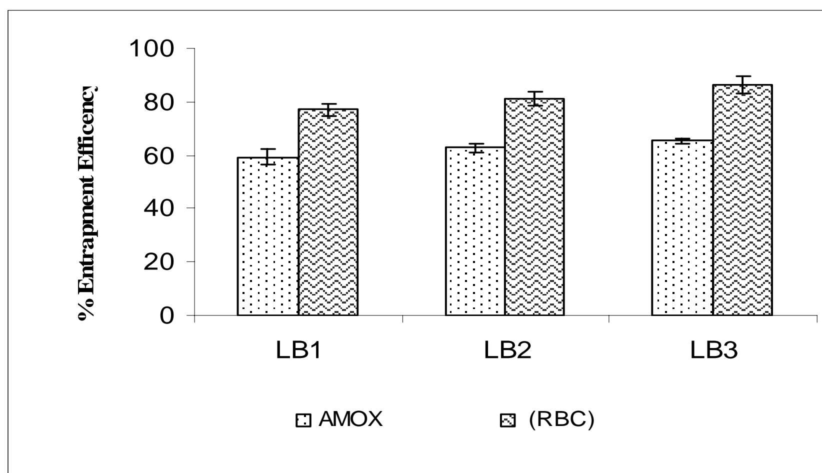


Fig. 5: % Entrapment Efficiency of AMOX and RBC in different nanoipobeads formulations

***In vitro* drug release study of Optimised nanolipobeads formulation**

Optimised nanolipobeads formulation (LB3) with maximum entrapment efficiency of RBC and AMOX were selected for *in vitro* drug released studies in simulated gastric fluids PBS (pH 7.4).

500 mg of nanolipobeads formulation were taken in dialysis membrane (cellulose dialysis membrane, 2.4nm porosity, Himedia Labs, Mumbai, India) against 100 mL of medium and the solution was continuously stirred using magnetic stirrer at 37±2°C. After appropriate time intervals 1mL of sample was withdrawn and analysed for drug content. Simultaneously equal volume was fresh media was added to replenish the withdrawn sample. After appropriate dilution the sample were analysed at 223 and 272nm for RBC and AMOX respectively in PBS (pH 7.4) spectrophotometerically (Shimadzu 1601 UV/Visible Spectrophotometer). The amount of drug was calculated by using simultaneous equations.

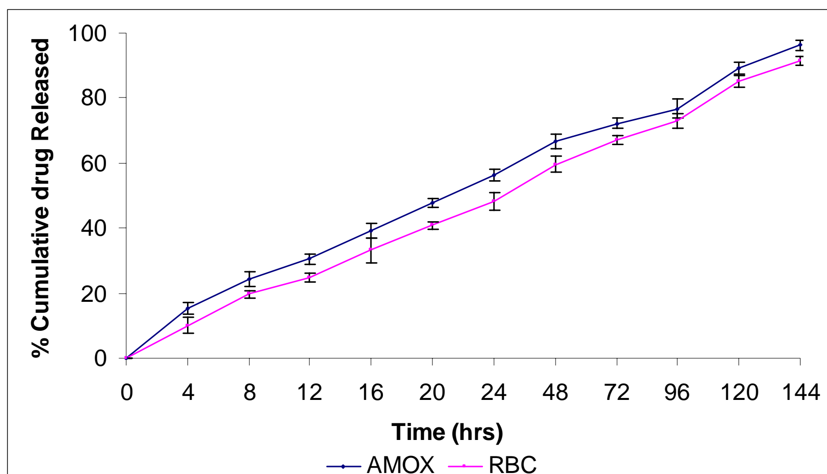


Fig. 6: % Cumulative amount of drug released from optimized nanoipobeads formulation (LB3) in PBS (pH 7.4)

Antisecretory and Ulcer protective activity of optimized Nanolipobeads (LB3) formulation

The albino rats were randomly distributed into three groups containing 3 rats in each group and they kept under standard laboratory housing conditions. The animal weight range 100 ± 20 gm of either sex. They were fasted for 24hr before drug treatment. The animals were deprived of food and water during the experiment.Rats of group I were administered absolute alcohol (2ml/kg p.o). The groups II were administered 10 ml solution of plain drugs (equivalent to 100mg/kg Ranitidine bismuth citrate+50 mg/kg Amoxicillin trihydrate p.o) 30min before oral dose of absolute alcohol (2ml/kg p.o).The group III were administered 10 ml suspension of optimized nanolipobeads (equivalent to 100mg/kg Ranitidine bismuth citrate+50 mg/kg Amoxicillin trihydrate p.o) 30min before oral dose of absolute alcohol (2ml/kg p.o). After 4 hr of pyloric-ligation the animals of each group were sacrifice by decapitation. The abdomen was opened and the oesophageal end (cardiac end) of the stomach was tied. The entire stomach was cut and removed from the body of the animal. A cut was given to the pyloric region just above the knot and the contents of the stomach were collected in a graduated centrifuge tube and the stomach was opened along the greater curvature. Stomach mucosa was washed with 1ml-distilled water and the washing was added to the gastric juice. Each stomach was examined by 10-X magnification glass and the ulcers were graded using the following scoring system suggested by Kunchandy [22].

0 = normal mucosa
0.5 = red colouration
1.0 = spot ulcers.
1.5 = haemorrhagic streaks
2.0 = ulcers> 3 but <5
2.5 = Ulcers > 5

The gastric contents were centrifuged at 3000 rpm for 10 min and 1ml of the supernatant was diluted to 9 ml of distilled water. The solution was titrated against 0.01N NaOH using Topfer's reagent till the solution turns to orange colour. The volume of sodium hydroxide required to neutralize the acid corresponding to the total acidity, was determined from the optimized formulation. Acidity (meq/l/100g) can be expressed as:

$$\text{Acidity} = \frac{\text{Volume of NaOH} \times \text{normality} \times 100}{0.1} (\text{mEq}/1/100\text{g}) \quad (3)$$

The acidity and ulcer index of control (absolute alcohol taken) and formulation treated animals are reported in Table 5.

Table 5: Anti-secretary and ulcer protective index of plain drug combination and optimized nanolipobeads (OL2IL4) formulations

S. No.	Formulation code (s)	Dose	Volume of gastric juice (mL/100g)	pH	Acidity (meq/L/100g)		Ulcer index (mm)
					Free	Total	
1	Control (absolute alcohol)	2 mL/kg	4.4 ± 0.3	2.1±0.4	70.1±0.9	163.4 ±1.3	3.01 ± 0.25
2	Plain (RBC+AMOX)	10ml (equivalent to 20mg/kg ranitidine bismuth citrate +5 mg/kg amoxicillin trihydrate).	2.9±0.5	3.9±0.2	39.5±0.6	101.3±1.9	1.94 ± 0.19
4	OL2IL4	10ml (equivalent to 20mg/kg ranitidine bismuth citrate +5 mg/kg amoxicillin trihydrate).	1.9±0. 2	4.1±0.4	19.4±0.2	58.9±1.3	0.51 ± 0.08

RESULT AND DISCUSSION

The development of dual drug delivery system that combines complimentary properties of liposomes and polymeric nanoparticles i.e. nanolipobeads represent a new trend in the application of site specific targeted drug delivery. Preparation of nanolipobeads involves three steps (a) Preparation of PVA hydrogel nanopartices by emulsification followed by freeze thaw cyclization method (b) Surface acylation of PVA nanoparticles by treatment with polmityl chloride and (c) Preparation of nanolipobeads by formation of PE liposome on acylated PVA hydrogel nanoparticles by vesicles fusion method. The Prepared of PVA hydrogel nanopartices was optimized by various formulation and process variables variables e.g. polymer concentrations, drug concentration, emulsifier concentrations, number of freez thaw cycles, stirring speed, stirring time and sonication time to obtain nanosized PVA hydrogel nanoparticles with maximum entrapment efficiency.

PVA employed in the production of PVA hydrogel nanoparticles was first subjected to optimization by varying the concentration of PVA *viz* 5 %, 10 % 15 % and 20 % w/v ect. The prepared formulation NP-C_3 was optimized on the basis of particle size and PDI. Formulation showed an optimum size of 268.7 ± 2.4 and a PDI of 0.324 (Table1) therefore formulation NP -C_3 was selected as optimum for further optimization.

Amount of drug was also optimized on the basis of particle size and maximum percentage drug entrapment by varying the amount of RBC viz 10, 25 50 and 75mg. it was observed that on increasing the amount of drug the entrapment efficiency increased upto 50 mg while on further increasing drug concentration the entrapment efficiency gradually decreased might be attributed to limited solubility of drug in PVA. In Particle size does not significantly change was observed (Table1). Sothat formulation NP-C_3D_3 was selected for further optimization process.

The effect of span 80 was also observed on particle size and entrapment efficiency. The particle size found to be decreased upon decreased upon increasing the concentration of span 80. This might be due to the decrease the surface tension between oily phase and aqueous phase which ultimately seem to allow formulation of nano range particles. Particles of optimum size 265.7± 5.4 nm with maximum the drug entrapment were obtained at 1.5 % v/v surfactant concentration (Table1). However on further increasing surfactant concentration although the particle size decrease because of the formation of micelles but entrapment efficiency also decrease because of the leaching out of the drug. So formulation NP- $C_3D_3E_3$ was selected for further optimization process.

The effect of oil: PVA phase ratio viz 1:1, 3:1, 5:1 and 10:1 was observed on particle size and entrapment efficiency. Study revealed that 10:1 oil: PVA phase ratio yielded the smallest and more evenly distrusted 288.6± 3.4 nm PVA hydrogel nanoparticles with 91.74±1.5% of entrapment efficiency. Sothat formulation NP- $C_3D_3E_2O_4$ was selected for further optimization process (Table1).

The stirring speed viz 1000, 2000, 3000 and 1000 rpm on average particle size and drug entrapment efficency was also optimized. In these experiments results was concluded that increase in agitation speed decrease an average particle size of PVA hydrogel nanoparticles. At 4000 rpm formulation NP-$C_3D_3E_2O_4R_4$ produced narrowest size distribution 279.8 ± 4.2 nm with spherical shape and 93.45 ± 2.7 % drug entrapment efficiency (Table1).

Average particle size of PVA hydrogel nanoparticles reduces with increased in stirring time and narrow size distribution 265.3± 5.7nm with 88.3± 4.3 % entrapment efficiency as found formulation NP-$C_3D_3E_2O_4R_4T_3$ after 45 min. Stirring time for more than 45 min causes the oil: PVA phase to separate due to agglomeration of the PVA (Table1).

On increasing the sonication time upto 2 min formulation NP-$C_3D_3E_2O_4R_4T_3ST_2$ decreasing the average particle size as well as % entrapment efficiency. Decreased in entrapment efficiency might be due to higher extrusion of polymer (Table1).

For optimization of physical method of cross linking number of the freez thaw cycles (FT cycles) were optimized in term of the average particle size and % entrapment efficiency of PVA hydrogel nanoparticles. Study revealed that average particle size increased with increase number of freez thaw cycles. Formulation NP-$C_3D_3E_2O_4R_4T_2ST_2$ Show 273.6 ± 4 nm particle size with 89.6 ± 4.5% entrapment efficiency after three cycles due to increased crosslinking density. More than three freez thaw cycles due to formation of rubbery gel decreases the entrapment efficiency (Table1). On the basis of optimization studies formulation NP-$C_3D_3E_2O_4R_4T_2$ ST_2 FT_3 (Table 2) was selected for surface acetylation.

In vitro RBC release from optimized PVA hydrogel nanoparticles were carried out in PBS (pH 7.4) by dialysis cell membrane method. After 144 hr 94.3± 1.4 % cumulated release was observed from PVA hydrogel nanoparticles.

Surface acetylation with fatty acid chain was accomplished by treating PVA hydrogel nanoparticles with 1M polmitoyl chloride in hexane at room temperature for 2-3 days and evaporating the solvent. The palmitic species became anchored on the surface via esterification with surface hydroxyls groups. The completion of this reaction was characterized by Fourier Transform Infra Red spectroscopy. FT-IR spectra of PVA hydrogel nanoparticles Fig. 3 (a) and acetylated hydrogel nanoparticles Fig. 3 (b) were assigned and compared. In PVA hydrogel nanoparticles spectra peak due to O-H streching appeared at 3429.8 cm^{-1} which was significantly suppressed in acylated PVA hydrogel nanoparticles spectra. The new peak due to ester bond formation at 1777.5 cm^{-1} was observed in acylated PVA hydrogel nanoparticles spectra. This finding suggested the estrification of hydroxyl groups of PVA hydrogel nanoparticles with palmitic species. The peak due to ester C=O strech (1628.6 cm^{-1}) was prominent in acylated PVA hydrogel nanoparticles spectra further influence the esterification of PVA hydroxyl groups. Peak due to C-H strech (2831.1 $cm^{-1}$and 2925.2 cm^{-1}) in PVA hydrogel nanoparticles spectra become sharp in acylated PVA hydrogel nanoparticles spectra (2848.9 cm^{-1} and 2922.3 cm^{-1}). No noticeable change observed in other peaks of both acylated PVA hydrogel nanoparticles spectra and nonacylated PVA hydrogel nanoparticles spectra. Other characterization was based on contact angle due to acylation of the PVA beads. When PVA beads and dried acylated beads were placed in water. Acylated beads formed aggregate and float on the water surface while PVA beads sink to the bottom of water swelled to gel like structure. These observations indicate the hydrophobic surface of the acylated bead due to anchoring of palmitic species to hydroxyl group of PVA.

Final formation of nanolipobeads was accomplished by phosphotidylethanolamine (PE) liposome bearing RBC by vesicle fusion method. Effectiveness of method used for nanolipobead preparation and final properties of lipobeads was evaluated by fluorescent microscopy. Rhodamine labelled nanolipobeads were prepared by vesicle fusion method and photomicrograph Fig. 4 were taken using Vanox-S and Olympus. The photomicrograph of fluorescent nanolipobeads formed by vesicle fusion method showed lipid bilayer fusion on acetylated PVA hydrogel nanoparticles.

Nanolipobeads prepared with varying concentration of phosphotidylethanolamine PE *viz.* LB1 LB2 and, LB3. Maximum entrapment efficiency of RBC (65.3 ± 1.2%) and AMOX (86.1± 3.2 %) was formed with formulation LB3 which might be due to the multiplayer formation of lipid on polymer support.

In vitro drug releases of AMOX and RBC were carried out with optimized nanolipobeads (LB3) formulation in PBS (pH 7.4) by dialysis cell membrane method. Both the drugs AMOX and RBC were released simultaneously from nanolipobeads. The amount of drug released were calculated by using simultaneous equations.

The % Cumulative amount of AMOX and RBC released from optimized nanolipobeads (LB3) was found to be 96.3±1.4 % and 91.4±1.2% respectively. The release RBC from nanolipobeads is slower as compared to PVA hydrogel nanoparticles. This might be due to PE lipid layer on PVA support is acting as a barrier for diffusion of drug from PVA hydrogel nanoparticles .This finding

influences the fact that release of drugs from nanolipobeds occur in sustained and controlled manner.

The study of anti secretary and ulcer protective effect of combination of drug solution and optimized nanolipobeads formulation (LB3) in absolute alcohol induced ulcer in rats were found to protect gastric mucosa from ulcer. The nanolipobeads formulation reduced the volume of gastric juice 4.4 ± 0.3 to 1.9±0 mL/100g. The total acidity was reduced from 163.4± 1.3 to 58.9 ± 1.3 meq/L/100g and the free acid registered change with 70.1±0.9 to 19.4±0.2 (meq/L/100g) by the suspension of LB3. The ulcer index reduced from 3± 0.08 to 0.2 ± 0.01.Therefore it is clear from this study shown in Table 4 that the formulation nanolipobeads prevented the ulceration completely.

CONCLUSION

The study was undertaken with an objective to develop and design effective drug delivery system for sustained and targeted delivery of AMOX and RBC to the *H.Pylori* infected mucosal site so as to completely eradicate the *H.Pylori* infections. AMOX /RBC reside in the stomach for longer period of time when it was administered in the form of nanolipobeads formulation than it was administered as plain drug solution. Furthermore there is amply evidence that the topical action of AMOX /RBC on the gastric mucosa played an important role in the clearance of *H.Pylori*.

The drug AMOX and RBC released from the system are remarkly act on *H.Pylori*. the event stated above leads to a higher drug concentration at the site of action this being a result of superior cellular uptake of the drug vehicle thus inhibiting the surface associated urease activity and maximizing therapeutic index.

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