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Design and Development of Multi drug loaded and Dual Drug loaded Nanostructured Drug Delivery Carriers (NSDDS)

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ABSTRACT

The objective of this research work was to formulate and develop a nanosponge delivery system comprised of the polymers viz. β- Cyclodextrin, Chitosan and Hyaluronic acid using Ultrasound Assisted Synthesis. The prepared blank nanosponges were optimized by Box Behnken Design. The optimized batch of the Blank β- Cyclodextrin -Chitosan crosslinked Hyaluronic acid Nanosponges (CCHNSs) had the particle size of 0.326 nm, PDI 0.474 and Zeta Potential of 30.8mV. The prepared Blank CCHNS were co-loaded with antitubercular drugs viz. RIF, PYZ and IBU to obtain the Single and Multi drug loaded CCHNS. Additionally, the Blank CCHNS were also loaded for anticancer drugs viz. Curcumin and DOX. The prepared CCHNS were evaluated for loading and entrapment efficiency. The optimized formulation was found to be stable for 6 months at real time stability conditions of 4°C±2°C and 25°C±2°C /60%±5% RH respectively. This engineered dry powder could be used as an enhanced therapeutic alternative of the standard oral antitubercular and anticancer regimen.

Keywords: Nanosponges, Anticancer, Antitubercular, Pulmonary, Dry powder Inhaler

INTRODUCTION

It has been reported that Tuberculosis has become the second deadliest disease affecting 9 million people worldwide. The disease is caused by the most virulent strain Mycobacterium tuberculosis which attacks the alveolar macrophages and produces 2 substances ammonia and sulfatides. Due to this the fusion of phagosomes is prevented and the Mycobacteria survives inside the macrophages. The current treatment patterns is very complex and it involves high dose combinations of antitubercular drugs several times a week for 6 month. This causes non compliance in TB patients. Due the underdosing of medications mycobacteria acquire resistance. This causes the failure of the chemotherapy incase of TB. Though, the need for intracellular chemotherapy has been recognized for many years but, the challenge to design the means of carrying an antibiotic in a form that is able to be endocytosed by phagocytic cells and then released into these cells has not been met. Hence, there is an urgent requirement of the low dose antitubercular drug therapy which can be less toxic, completely kill the Mycobacterium tuberculae and administered easily by TB patients with ease.

Targeting drug delivery into the lungs has become one of the most important aspects of systemic or local drug delivery systems. Lungs are an attractive target for the pulmonary administration of active pharmaceutical ingredients (APIs) in the form of various drug delivery systems (Zumla et al 2014). Additionally, this route offers

many advantages over conventional per oral administration, such as a high surface area with rapid absorption due to high vascularization and circumvention of the first pass effect. This selectivity allows targeted drug delivery and, hence, reduces the side effects (Gupta et al 2013). Colloidal drug delivery systems have extensively been investigated as drug carrier systems for the application of different drugs via different routes of administration. Pulmonary drug delivery is a technology in which medicines are inhaled through the lungs and enters the bloodstream through the alveolar epithelium. It provides a noninvasive, alternative method to subcutaneous injection, and also intravenous injection. The delivery by inhalation uses the extensive surface area of the alveoli, avoiding hepatic first-pass metabolism and enabling noninvasive administration of larger doses to the lungs, leading to greater therapeutic efficacy without increasing toxicity (Parikh et al 2013). Pulmonary drug delivery is the preferred route of administration of aerosolized drugs in the treatment of respiratory diseases including asthma and cystic fibrosis, infectious diseases, in particular tuberculosis, and some non respiratory diseases such as type I diabetes. (Gupta et al 2013)

Tuberculosis

Incidence

TB has been ranked as the ninth most devastating disease worldwide, which is above AIDS. Millions of deaths and new cases every year have compelled the World Health Organization and United Nations to include this issue in the Sustainable Development Goals, the ultimate being to end the global TB epidemic by 2035. Pulmonary tuberculosis is the most common form of tuberculosis; involving alveolar macrophages infected with Mycobacterium tuberculosis (Deol et al., 1997). TB causes substantial mortality and morbidity.

Pathogenesis

Tuberculosis is spread by airborne droplet nuclei, which are particles of 1–5 μm in diameter that contain Mycobacterium tuberculosis. Because of their small size, the particles can remain airborne for minutes to hours after expectoration by people with pulmonary or laryngeal tuberculosis during coughing, sneezing, singing, or talking (Wells et al., 1934, Louden et al., 1966). The infectious droplet nuclei are inhaled and lodge in the alveoli in the distal airways.

Current Therapy of Tuberculosis

The WHO recently released its fourth edition of "Treatment of tuberculosis: guidelines" (WHO Guidelines 2012). The treatment of TB involves long-term treatment with a combination of drugs. The treatment should never start with a single drug or a single drug should not be added to the failing regimen [1]. The treatment of new patients starts with first line drugs, the second line drugs are later included in the regimen if patients develop resistance to the first line drugs since the second line drugs are usually more toxic and less effective. According to the WHO, the standard regimen (Table 1) for treating new patients with drug-susceptible pulmonary TB includes daily intake of first line drugs, isoniazid, rifampicin, pyrazinamide and ethambutol for two months, followed by isoniazid and rifampicin three times a week for four months (WHO World Global tuberculosis report. 2017; Geneva, Switzerland).

First-line therapy consists with Rifampicin (RIF), Isoniazid (INH), Pyrazinamide (PZA), Ethambutol (ETH) and Streptomycin because of their efficacy and acceptable degree of toxicity.

An initially intensive phase consist of three-four drugs (RIF, INH, PZA and ETH) daily

Challenges for the development of DPI for TB Patients

Although considerable research has involved a large number of drugs to develop dry powder inhalers for treating TB patients, there is no marketed anti-TB DPI as yet. In general, DPI has shown limited promise in clinical studies. The reasons for this have been summarised by Yadav et al. 2011.

1. One of the main challenges in inhalation therapy for CF is to selectively deliver drugs in the heavily obstructed regions of the lung, rather than the better ventilated healthier regions where drugs are more likely to deposit.

1. The drugs fail to penetrate the lesion after pulmonary delivery since lesions are poorly aerated

2. Moreover, MTB can produce biofilms with subsequent difficulty in drug penetration through the biofilm.

3. A very high dose in the lung that can produce a large concentration gradient on the surface of the biofilm was proposed to be useful in overcoming this problem. This requires the development in both formulations and devices.

4. There are challenges associated with delivery devices. For example, inconsistent delivery from the device may result in variability in available drugs at the site of infection that may lead to resistance.

5. The greatest challenge for testing the inhalers in biological model is the requirement of a sophisticated biosafety laboratory.

6. In human trial, the delivering of the high amount of drugs to the lung may cause irritation, coughing, bronchospasm or poses osmotic challenge in the lung since it contains only a little amount of fluid (Yadav et al 2011) more time consuming (Changsan et al 2008).

Table 1: Deposition of particles in different parts of lungs according to particle size and mechanism

Location in lung	Size	Mechanism
Primary Bronchi	5–10 μm	Impaction
Secondary Bronchi	1–5 μm	Sedimentation
Bronchioles	1–3 μm	Sedimentation
Alveoli	0.5–1 μm	Brownian motion

Nanosponges as Drug Delivery Systems

An advance version of nano-particulate systems is entitled to nanosponges. These are designed with a hyper cross-linked polymer-based colloidal structure. Nanosponges are spongy polymeric delivery systems that are minor sphere-shaped particles with great porous external surfaces.

Nanosponges are discrete solid porous nanoparticles composed of hyper-cross-linked polymers with superior drug absorption/complexation properties (D'souza et al 2008). Their molecular architecture generally contains different polymer chains which can form specific microdomains suitable for the co-encapsulation of two drugs with a different chemical structure. These are designed with a hyper cross-linked polymer-based colloidal structure. Nanosponges are spongy polymeric delivery systems that are minor sphere-shaped particles with great porous external.

These are not really sponge-like structured and shape, but more like a network of molecules in three-dimensional structures. Nanosponges are very small, with a size of about a virus having a diameter below 1 μm. By varying the proportion of cross-linker to polymer, the nanosponge particles can be made larger or smaller (Sharma et al 2011). Nanosponge technology helps to reduce drug associated side effects, improve stability, increase elegance and improve the flexibility of formulations, administered orally, parenterally and topically. Among nanosponges, cyclodextrin-based nanosponges (CDNS) are smart versatile carriers studied widely for drug delivery applications (Kumar et al 2019). Polymers used for architecting the NS are include polyvinyl alcohol (PVA), ethyl cellulose, polymethylmethacrylate, hyper connected polystyrenes, cyclodextrins and their derivatives like methyl beta cyclodextrins, alkyloxy carbonyl cyclodextrins [45]. Among these, cyclodextrins (CDs) have been the most popularly employed for fabrication of nanosponges.

LITERATURE REVIEW

Literature Review on Rifampicin

Khalluru et al 2013 fabricated NPs of PLGA containing either a high concentration of rifampicin or detectable levels of the green fluorescent dye, coumarin-6. Their aim was twofold: first to resolve the controversial issue of whether, after phagocytic uptake, PLGA NPs remain membrane-bound or whether they escape into the cytoplasm, as has been widely claimed. Second, to make NPs that enclosed sufficient rifampicin to efficiently clear macrophages of infection with Mycobacterium bovis BCG. Using fluorescence microscopy and immuno-electron microscopy, in combination with markers for lysosomes, they showed that BCG bacteria, as expected, localized to early phagosomes, but that at least 90% of PLGA particles were targeted to, an Hakkimane et al 2018 developed nanoparticles of RIF-loaded poly lactic-co-glycolic acid (PLGA) and INH modified as INH benz-hydrazone (IH2) which gives the same therapeutic effect as INH but is more stable and enhances the drug loading in PLGA NPs by 15-fold compared to INH. The drug release from NPs and stability of drug were tested in different pH conditions. It was found that RIF and IH2 loaded in NPs release in a slow and sustained manner over a period of 1 month and they are more stable in NPs formulation compared to the free form. RIF- and IH2-loaded NPs were tested for antimicrobial susceptibility against Mycobacterium tuberculosis H37Rv strain. RIF loaded in PLGA NPs consistently inhibited the growth at 70% of the minimum inhibitory concentration (MIC) of pure RIF (MIC level 1 μg/mL), and pure IH2 and IH2-loaded NPs showed inhibition at MIC equivalent to the MIC of INH (0.1 μg/mL).

Literature Review on Isoniazid

Omar et al 2019 prepared INH-loaded chitosan microparticles (Cs-Mps-1-3) as an inhalable carrier for the previously prepared INH-loaded polyvinylpyrrolidone/polyitaconic acid nanoparticles (NPs) using spray-drying technique. Here, Cs-Mps-1-3 are composed of Cs: INH-loaded NPs: Free INH at w/w ratios (1:1:0), (1: 0:1), and (1:1:1), respectively. Subsequently, the prepared Cs-Mps-1-3 characterizations were studied. Cs-Mps-1-3 showed a spherical, smooth, positively charged surface (ζ-potential values +20.2, +28.7, and +22.6) and a size range 1.52–3.12 μm. In addition, Carr's compressibility indices of Cs-Mps-1-3 were 32.5%, 24.8%, and 28.02%, respectively. The in vitro INH released showed good correlation with first-order pattern, with predominance of the diffusion-controlled mechanism. In vitro aerodynamic deposition of Cs-MPs-3 possessed 56.81% effective fine particle fraction with lower impaction loss and device

Literature Review on Pyrazinamide

Verma R et al 2015 formulated polymeric nanoparticles based drug delivery system to sustain the release profile and reduce the dosing frequency of pyrazinamide. These polymeric nanoparticles were prepared by simultaneous double-emulsion (W/O/W) solvent evaporation/diffusion technique. The prepared dispersions were characterized for various biopharmaceutical parameters such as particle size, zeta potential, polydispersity index, drug loading capacity, entrapment efficiency and targeting to alveolar macrophages. The formulated polymeric nanoparticles were in the particle size range of 45.51 to 300.4 nm with a maximum drug entrapment efficiency of 80.9%.

Literature Review Curcumin

Pushpalatha et al 2018 prepared Curcumin (CUR) is a poorly water-soluble and photoreactive drug having potent anticancer activity. The purpose of the study is to fabricate cyclodextrin nanosponges, for the delivery of curcumin using two crosslinkers, diphenyl carbonate (DPC) and pyromellitic dianhydride (PMDA) and to study the influence on drug solubility, stability and cytotoxicity. Solubility studies were performed with assorted cyclodextrin to crosslinker ratio and with selected drug nanosponge stoichiometric complex. The drug-loadednanosponges were characterized using DSC, FTIR, XRD and **SEM**. Pore size and surface topology of nanosponges confirmed the nanochannels available for the drug to get entrapped. In vitro cytotoxicity study revealed increased toxicity of drug nanosponge complex to MCF-7 cells at a lower concentration. IC50 value of the drug (22.51 µg/ml) was reduced by 2.2 fold (10.44 µg/ml) by CUR-PMDA-CDNS as against 1.4 fold (15.92 µg/ml) decrease by CUR-DPC-CDNS. In conclusion, PMDA-CDNS was found to be a potential nanocarrier compared to DPC-CDNS for curcumin.

Literature Review on DOX

Naderinezhad et al 2017 studied the Codelivery of doxorubicin and curcumin with lipid nanoparticles results in improved efficacy of chemotherapy in liver cancer. The proposed formulation provided potential benefits, including pH-sensitive sustained release, smooth globular surface morphology, high entrapment efficiency (80% for both therapeutic agents) and small diameter (42 nm). Exposure of cancer cells to LipoNiosome-doxorubicin–curcumin has shown an excellent performance of specific cellular internalization and synergistic toxic effect (>40%; as compared to free drugs and >23% when compared to single doxorubicin delivery) against Saos-2, MG-63 and KG-1 cell lines. A new cationic formulation (zeta potential: +35.26 mV; diameter: 52.2 nm) was also designed for co-delivery of abovementioned drugs and gene as well.

Rationale

Inhalation proves to be one of the most non invasive approaches for pulmonary delivery where drug can directly the target site. Inorder to achieve higher drug levels, nanoparticles offer the best solution as they provide sustained action due to their increased circulation times. Additionally, nanoparticles reduce the dose significantly due to their targeted delivery.

Polymeric nanoparticles have achieved a potential in controlled release of therapeutic agents. The co-delivery of anti tubercular drugs is the need of the hour. Hence we require drug delivery systems like Nanosponges which not only show high bioavailabilty but also site targeted delivery.

Dry Powder Inhalers are the most preferred inhalation option due to their greater physiochemical stability over liquid or suspension based formulations. Dry powder inhalers (DPIs) are easy to handle and appropriate for high-dose formulations.

The aim of the present research work is to prepare the nanoparticles of Gellan Gum and load the antitubercular drugs and deliver them by dry powder inhalers to achieve the targeted pulmonary delivery. The front line ATDs were selected for the same as we wanted to explore the possibilities of nanoparticulate drug dose on the bacterial resistance.

DISCUSSION

We prepared Blank β-Cyclodextrins- Chitosan Crosslinked Hyaluronic Acid Nanosponges (CCHNS) using Ultrasound Assisted Synthesis. The blank batch was optimized using a Quality by Design Approach by Box-Behnken Design. In this design the factors selected were Hyaluronic acid concentration, β-Cyclodextrins: Chitosan, Sonication Pulse, while the responses were Particle size, Zeta Potential and PDI. The optimized batch composition was as follows: Hyaluronic acid 4.50 µg, β-Cyclodextrins- Chitosan 33.2 µg and Sonication pulse was 60 amplitude. The optimized batch had the particle size of 0.326 nm, PDI 0.474 and Zeta Potential of 30.8. The prepared batch was validated by the Check point batches and Overlay plots.

Optimization of Drug Loading and Entrapment Efficiency

For the purpose of drug loading, 2 different concentrations of drugs were selected from literature review. The drugs were co- loaded as described in Section 9.4.2. and their entrapment efficiencies were recorded. The optimization was performed using 23 Full factorial Design. The factors and responses were as shown in Table 2 and 3.

Table 2: Drug loading and Entrapment Efficiencies

Runs	X1= Concentration of Rifampicin	X2= Concentration of Pyrazinamide	X3= Concentration of Ibuprofen	Y1 Particle Size	Y2 % LE of Rifampicin	Y3 % LE of Pyrazinamide	Y4 % LE of Ibuprofen
1	25.00	50.00	100.00	396	16	40	52
2	12.50	25.00	50.00	362	20	32	42
3	12.50	50.00	50.00	345	21	35	56
4	12.50	50.00	100.00	375	22	42	54
5	25.00	25.00	50.00	350	15	38	48
6	25.00	25.00	100.00	354	18	42	50
7	25.00	50.00	50.00	330	20	36	55
8	12.50	25.00	100.00	342	22	45	58

Table 3: Check Point Batch Composition for Drug loading

Check Point Batch	Composition
Rifampicin Concentration	12.59 µM
Pyrazinamide Concentration	36.72 µM
Ibuprofen Concentration	61.44 µM

Table 4: Loading Efficiency, Entrapment Efficiency and %Yield

Formulation	Drugs	Loading Efficiency (%)	Entrapment Efficiency (%)	%Yield
Blank CCHNS	----	---	----	45.11± 1.72
Multi Drug CCHNS	Rifampicin	23.25±1.3	87.39 ± 1.8	62.5 ± 1.29
	Pyrizinamide	45± 2.1	94.27 ±2.2	
	Ibuprofen	52.32±1.7	91.53 ± 1.4	

It was seen from Table 4 that highest loading is achieved for hydrophobic drug Ibuprofen. On the contrary among the lipophilic drugs drugs, Rifampicin and Ibuprofen, Ibuprofen is better loaded. Same trend was observed for the entrapment efficiencies of the drugs on the CCHNS Matrix, since, the drugs were loaded onto the blank hypercrosslinked matrix, some of the drug was lost during loading in the centrifuging process. Still a decent entrapment efficiencies are seen ranging from 87 to 94%. These results, shows that the 3 drugs were successfully incorporated into the nanosponges to a good extent. Similar results were reported by Clemente et al 2019. Similar results were reported by Argenziano et al 2020 who loaded DOX on cyclodextrin based nanosponges.

Invitro release Studies

The drug release was performed at different time points viz. 0, 0.5, 1, 3, 5, 10, 12, 24 , 48 hrs. Drug release for single drugs was used for CCHNS was found to be as follows: RIF showed Cumulative Drug Release of 72%, PYZ- 85% and IBU- 68% respectively after 48 hrs. While the Multi drug loaded CCHNS showed a slower release with % CDR of 62%.

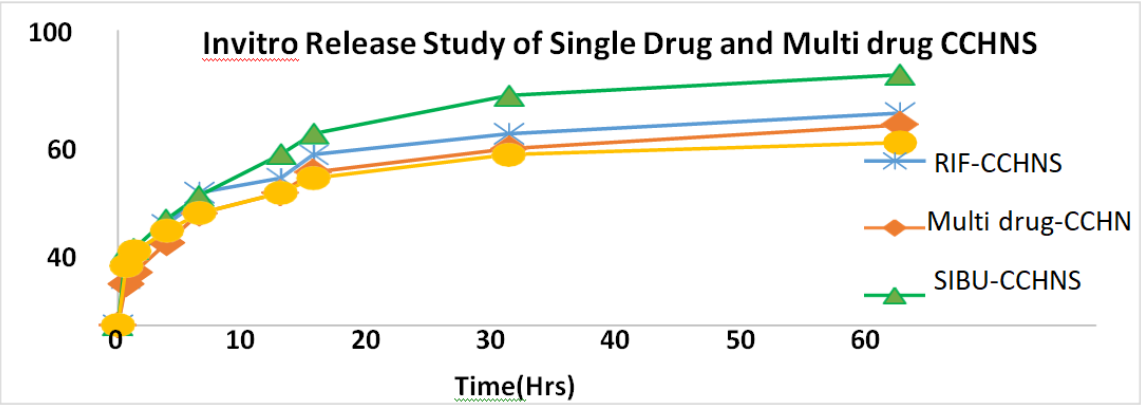

Fig 1: Invitro release Studies oF Multi drug CCHNS

Release Kinetic Studies

The data obtained from in-vitro release studies of Singlely loaded CCHNS and Multi drug loaded CCHNS was fitted into release kinetic models like zero order, first order, Higuchi, Hixson – Crowell and KorsemeyerPeppas. These values are depicted in Table 5. Similarly, for singlely loaded nanosponges, higher r2 values, were seen for Higuchi model which showed matrix erosion mechanism of drug release. While, the Multi drug loaded CCHNS followed a KorsemeyerPeppas release with anomalous non-fickian transport indicating diffusion controlled release. The n-value obtained after the Korsmeyer plot was in the range of 0.57 indicating that release followed anomalous non Fickian transport showing the release mechanism by diffusion.

Table 5: Release Kinetics Studies

Formulation	r^2 Values					nvalues
	zeroorder	firstorder	Higuchi	HixsonCrowell	Korsemeyer-Peppas	
RCIN-CCHNS	0.9262	0.9753	0.9979	0.9516	0.9420	----
PYZ-CCHNS	0.9528	0.9635	0.9814	0.9407	0.9531	-----
IBU-CCHNS	0.9733	0.9458	0.9926	0.9640	0.9268	----
Multi drug loaded CCHNS	0.9409	0.9245	0.9814	0.9726	0.9865	0.7

Invitro Permeation Studies

The diffusion cell model ensure sauniform permeation and distribution of pulmonary formulations by the continuous stirring of the receptor phase and there by maintaining temperature throughout the experiment. Drugs was estimated to permeate through the area of75 µg/cm2 over the time of 2 h from CCHNS Formulations. Fig. shows that the order of release obtained was in the following order: IBU-CCHNS>RIF-CCHNS>Multidrug CCHNS>PYZ-CCHNS>. SinceIBU is a BCS ClassII Drug, it shows the highest permeability followed by RIF which is a borderline ClassII drug with high permeability. PYZ being a Class III drug with moderate permeability. These results were evidently in correlation with the results of in-vitro release studies. As the drug was entrapped in then an a carrier system in the amorphous form, there by facilitating the drug diffusion overtime

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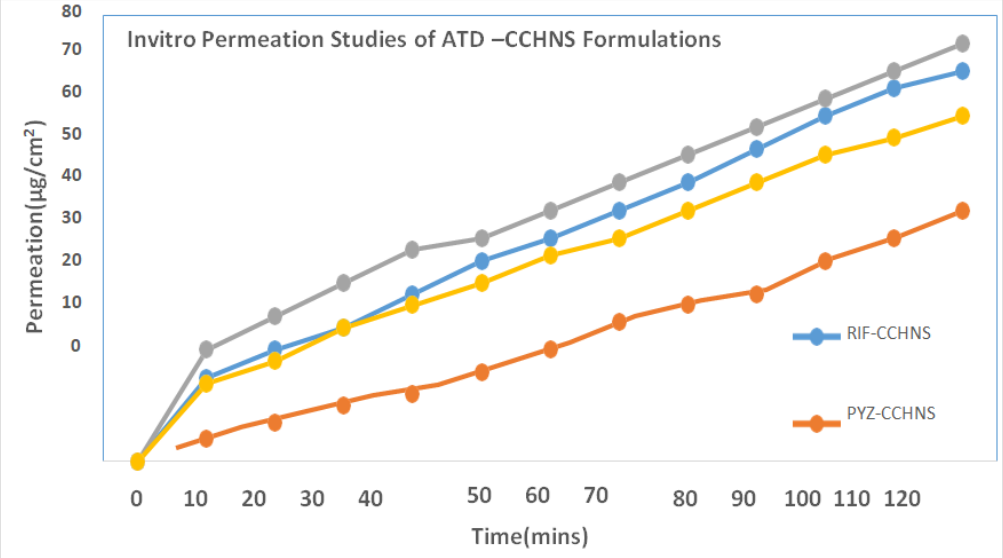

Fig 2: Invitro Permeation Studies of Multidrug CCHNS

Cytotoxicity Studies

The cell viabilities of A549 cells were determined by MTT Assay after the exposure of 24 h with different concentrations of free RIF, PYZ, IBU and Multi drug CCHNS. Untreated cells were used as a positive control to maintain the accuracy of the assay. Ic 50 is the maximal concentration of the drug / formulations required to cause 50% inhibition of the biological activity of mycobacterium cells as shown in Table 6.

Table 6: IC50Values of the free and Dual Drug CCHNS on Cell line A549

Formulation	IC50	IC50 free RIF	IC50 free PYZ	IC50 free IBU	R^2
BlankCCHNS	1.8±0.4	8.64	24.7	11.2	0.9928
Multi Drug CCHNS	2.5±0.5				0.9982
RIF-CCHNS	6.72±0.8				0.9726
PYZ-CCHNS	15.82±0.3				0.9905
IBU-CCHNS	8.59±0.72				

Multi drug loaded CCHNSs induced cell viability above 90% at all concentrations ranging from 50 to 200 µM. (Fig. 9). It is worth mentioning that the results of Multi drug CCHNS loaded NPs were very similar to blank NPs, giving evidence of the absence of toxicity of INH itself. The responses are correlated with literature review that IC50 of free RIF is 8.64, IC 50ofPYZis 24.7, IC50 OF IBUis 11.2 for pulmonary cells (Singh etal 2010).

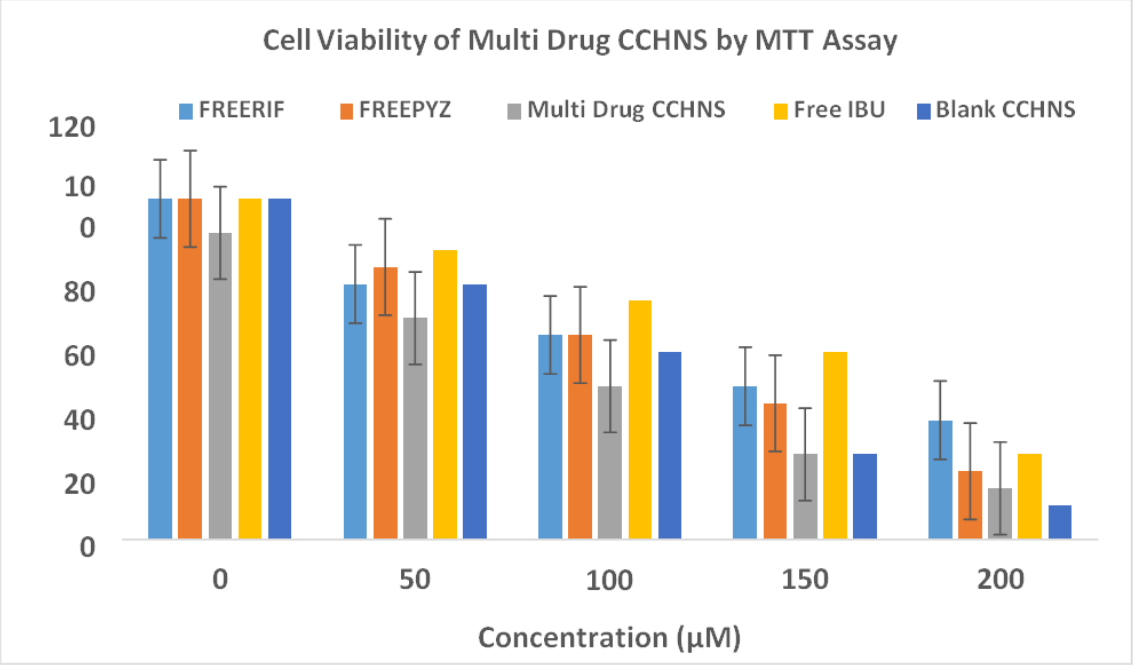

Fig 3: Cell Viability Studies of Multi drug CCHNS

Moreover, the results highlighted that NPs were compatible with the cells. One of the key properties of chitosan and chitosan derivatives is that they are non-toxic and do not elicit animmune response. Similarly, many investigations have proven in the past that hyaluronic acid nanoparticles showed 100% viability for A549 cells which comply with the results obtained here (Zheng et al 2014,) Although a slight toxicity was reported by Sakul wech et al 2020, we did not find cytoxicity of β-cyclodextrins and chitosan in our formulations.

Cell Up take Studies

Then extstep was to determine whether the Drug loaded CCHNS could traffic to mycobacteria residing in macrophages. Firstly, we checked that the Drug loaded CCHNS were not toxic to healthy macrophages (using the MTT assay). For this purpose, we made use of U937 human macrophage cell line (Passmoreetal 2001). The CCHNS and drug stested did not significantly affect cell viability ($P > 0.05$) (Fig.). We used fluorescence imaging to demonstrate up take of both mycobacteria and drug loaded CCHNS and to determine if they co-localised to the same intracellular location of the cell. Macrophages were seeded (5 × 104 cells/well) and infected with mycobacteria at an MOI of 5:1, 10:1, and 30:1,then treated with drug loaded CCHNS. Control experiments included macrophages only, macrophage streated with nontoxic, fluorescent Nanosponges (FITC-CCHNS), and macrophages infected with mycobacteria only (Fig.A). Washing removed extracellular mycobacteria/ free drug

loaded CCHNS prior to imaging. Fig. B illustrates the uptake of fluorescent versions Multi drug CCHNS by macrophages. The Multi drug CCHNS, being larger particles, appear to be agglomerated (consistent with the DLS data) with limited uptake by cells observed. Overall, it appears that CCHNS remained as full, intact particles once engulfed by macrophages.

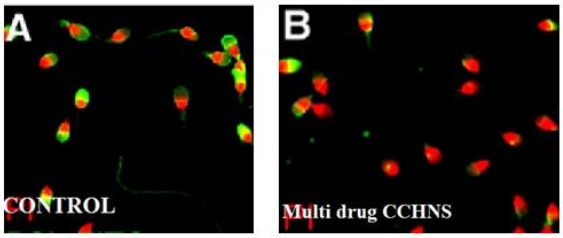

Fig 4: Celluptake studies

Fig 5 shows the uptake kinetics of neutral liposomes qualitatively and quantitatively over a period of 8 h. An increase in fluorescence intensity was observed with increasing time depicting increased uptake of CCHNS Formulations suggesting saturation of uptake after 4 h. Interestingly, The Multi CCHNS Formulations showed the slowest uptake kinetics while the hydrophobic drugs showed the fastest uptake kinetics. This was confirmed by the mean fluorescence intensity shown in Figure 4 by the macrophages for efficient saturation in targeting the mycobacterium. Studies on similar lines were performed by Donnellan et al 2017.

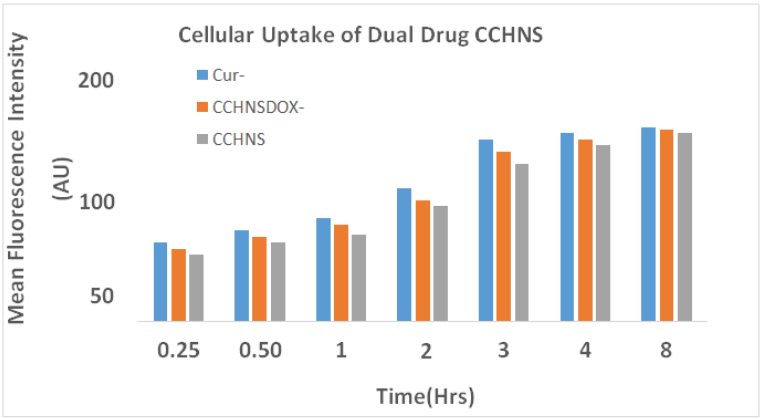

Fig 5: Cellular Uptake of Dual Drug CCHNS

SEM

The surface morphology of Blank nanosponges and Drug loaded were analyzed by SEM analysisas shown in Fig. The CCHNS nanoparticles showed flaky and clusters like morphology. Moreover, it can be seen from the SEM photomicrographs of the sponge-liketexture of the Blank and Drug loaded nanosponges and the average particle sizes may bevaried from 300 to 400 nm (Figs. (A) and (B)). The particles are very closely attached withother, which looks like the water adsorbed sponge-like morphology.

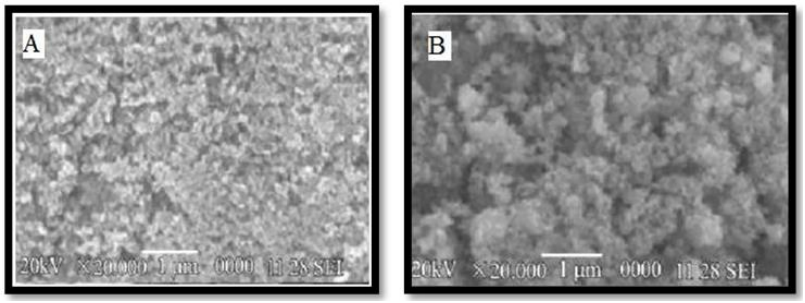

Fig 6: SEM of Blank and Multi drug CCHNS

The stability of a nanoformulation tested under particular conditions is very crucial for its efficacy, shelf-life and bioavailability. Hence, the formulations developed were investigated that 2 different temperatures viz.25°C±2°C /60% ±5% RH and 4°C±2°C. The optimized formulation was found to be stable for 6 months at real time stability conditions of 4°C±2°Cand 25°C±2°C /60%±5% RH respectively. Based on this results a shelf life of 6 months can be proposed for the optimized formulation (Kalam et al 2016 and Son et al 2012).

Drug Loading and Entrapment efficiency (%EE) in the Nanosponges

For targeted delivery of anti-cancer drugs viz Curcumin and Doxorubicin to lung tumor, Dual Drug loaded β-Cyclodextrin-Chitosan–HA Nanosponges (CCHNS)were prepared by the method reported by Wang T. et.al. 2017 and Boiteauetal 2019. It was observed from Fig8. That as the concentration of hyaluronicacid increases there is increase in particle size and entrapment efficiency. Formation of HA cross linked chitosan nanoparticles is due to the strong electrostatic interaction between the cationic amino group of Chitosan (CS) and the anionic carboxyl group of Hyaluronic acid (HA), HA was conjugated inside the surface of the β-CD-CS Nanosponges by charge adsorption to obtain Rifampicin-loaded HA-coated CSNSs. Similar studies were performed by Dhamane et al 2020.

Table 7. Loading Efficiency, Entrapment Efficiency, Drug Content and %Yield of Dual Drug CCHNS

BATCH	Drugs(µg)		Loading Efficiency(%)		Entrapment Efficiency(%)		%Yield	Drug Content
	Curcumin	DOX	Curcumin	DOX	Curcumin	DOX		
D-CCHNS1	50	25	15.2±1.3	18.5±1.5	80.37±0.92	72.29 ±1.5	45.11±1.72	85.27±1.4
D-CCHNS2	100	50	28.7 ±2.7	22.1±0.4	94.27±2.2	82.14±0.72	62.8±1.36	92.36±1.7
D-CCHNS3	50	50	21.3± 2.1	24.7±1.9	86.42±1.4	65.33±2.4	51.4±1.75	74.48±0.29
D-CCHNS4	100	25	25.11±0.84	16.3±1.6	90.53±1.8	78.29 ±1.5	58.26±1.36	89.69±1.63

In the previous studies, it was discussed that as the concentration of Curcumin and DOX increased, the Loading Efficiency also increased. The loading efficiency of D-CCHNS2 batch obtained 22.1% for hydrophilic drug DOX while the loading efficiency of lipophIllic Drug Curcumin was 28.7% since, the drugs especially lipophilic drugs compete for the hydrophobic binding core of the cyclodextrin. In this process, they get more loaded onto the CCHNS Matrix.

The drug content of the formulations were in the range of 74-92% which showed an increase trend with increase in the drug concentration. It was observed that the % yield of Dual drug loaded CCHNS ranged from 45.11-62% which is quite decent and shows that the spray drying conditions were optimized fully.

Table 8: Particle Size, PDI and Zeta Potential of Optimized Dual drug loaded CCHNS

Formulation	Avg Particle Size (nm)	PDI	Zeta Potential (mV)
Blank CCHNS	326.6	0.472	30.8
Dual Drug loaded CCHNS	415.8	0.750	28.3

Invitro release Studies

The drug release was performed at different time points viz. 0, 0.5, 1, 3, 5, 10, 12, 24 , 48 hrs. Drug release for single drugs was used for CCHNS was found to be as follows: Curcumin showed Cumulative Drug Release of 65%,and DOX-78% respectively after 48 hrs. While the Multidrug loaded CCHNS showed as lower release with %CDRof72%. Overall the drug release seemed to be slow and sustained Fig 7. Invitro Drug Release Study of Singlely Drug and Dual Drug CCHNS. It was seen from Fig.7 that the drug released directly from CCHNS with a prolonged release kinetics and showed an initial burst release kinetics. This invitro release confirmed the drug loading in polymeric CCHNS matrix. The burst release effect was observed since the drug was loaded onto blank Nanosponges. Hence, initially high amount of drug was released. It was seen that DOX showed the burst release effect the most bothinsinglely loaded and multi loaded CCHNS because, it is a hydrophilic drug which was quickly released in the phosphate buffer pH 7.4.

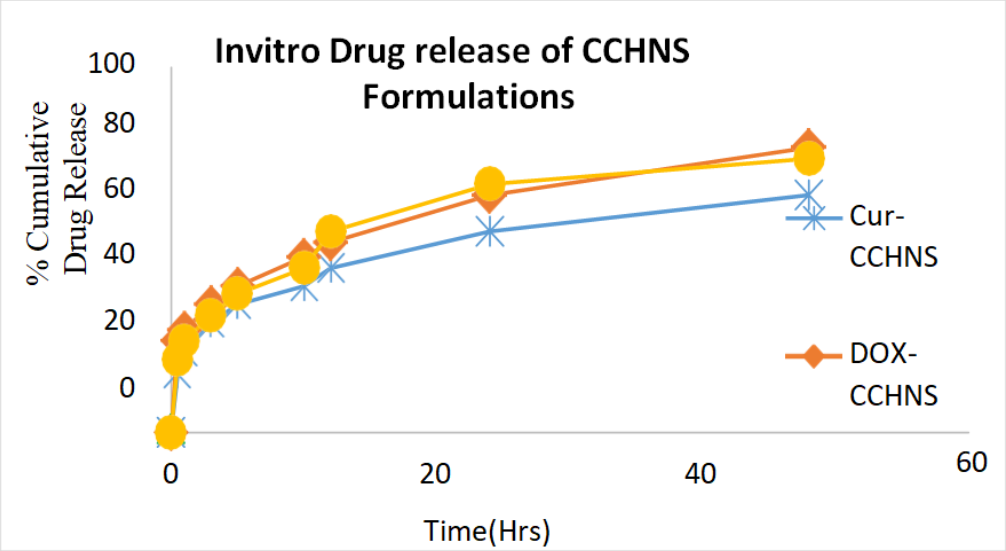

Fig 7: Intro Drug release of CCHNS Formulation

Release Kinetic Studies

Both Singlely loaded CCHNS viz Cur-CCHNS, DOX-CCHNS and Dual CCHNS were found to be best fitted to the Korsmeyer - Peppa > Higuchimodel. Korsmeyer-Peppas model explains the drug diffusion from a polymeric matrix. The value of n was 0.2930.248 and 0.367 for Cur-CCHNS, DOX-CCHNS, and Dual CCHNS which illustrates that both the formulations followed Fickian drug diffusion (n < 0.43).

Table 9: Release Kinetics Studies

Formulation	r2Values					nvalues
	zeroorder	first order	Higuchi	Hixson Crowell	Korsemeyer-Peppas	
Cur-CCHNS	0.9262	0.9753	0.9979	0.9516	0.9420	0.293
DOX-CCHNS	0.9528	0.9635	0.9814	0.9407	0.9531	0.248
Dual drug loaded CCHNS	0.9409	0.9245	0.9814	0.9726	0.9865	0.367

Invitro Permeation Studies

The diffusion cell model ensures a uniform permeation and distribution of pulmonary formulations by the continuous stirring of the receptor phase and there by maintaining temperature throughout the experiment. Drugs was estimated to permeate through the area of75 µg/cm2 over the time of 2 h from CCHNS Formulations. Fig. shows that the order of release obtained was in the following order: DOX- CCHNS> Multi drug CCHNS >Cur-CCHNS. Since DOX is a BCS Class III Drug, it shows the high solubility, low permeability followed by Curcumin which is a Class IV drug with lowest permeability. It can be seen that Nanosponge formulations are capable of overcoming the permeability issues to Class III and Class IV drugs to a great extent. These results were evidently in correlation with the results of in-vitro release studies. As the drug was entrapped in the nanocarrier system in the amorphous form, thereby facilitating the drug diffusion overtime.

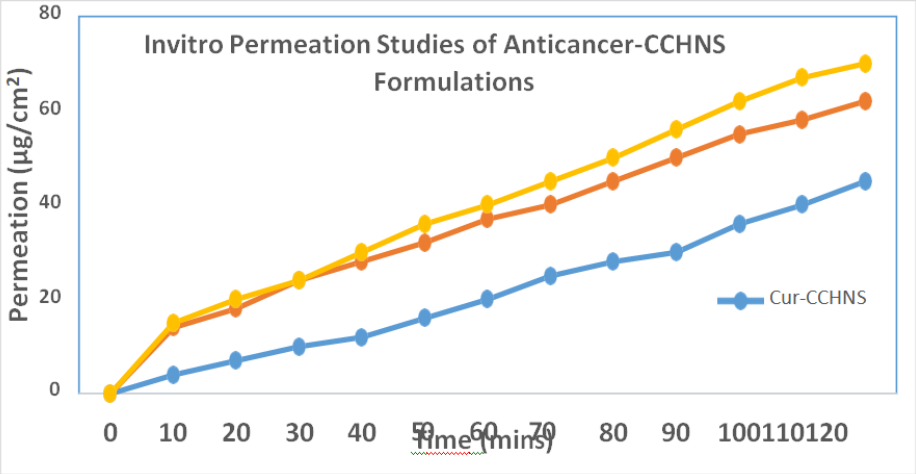

Fig 8: Invitro Permeation Studies of anticancer

Cytotoxicity

Inorder to determine the cytotoxic effects of DOX and curcumin CCHNS formulation, A549was chosen as model cell line and the MTT Assay was performed for 24 hrs. The cellviability was determined by measuring the absorbance on a micro plate reader (ELx800, Bio Tek, USA) at λ max 570 nm. A dose-response curve was constructed and the concentration of curcumin AND DOX that resulted in 50 percent inhibition of cell growth was calculated as the half maximal inhibitory concentration (IC50). Table 4.10: IC50 Values of the free and Dual Drug CCHNS on Cell line A549.

Table 10: IC50 Values of Dual drug CCHMS Formulations

Formulation	IC50	IC50 free DOX	IC50 free Curcumin	R^2
BlankCCHNS	1.8±0.4	8.64	11.2	0.9918
DualDrug CCHNS	3.2±0.5			0.9882
Cur-CCHNS	6.72±0.8			0.9726
DOX-CCHNS	5.82±0.3			0.9905

Cytotoxicity analysis of Curcumin and its formulations showed no significant difference (P>0.05) in IC50 of free curcumin as compared to Dual drug CCHNS over 24 hrs. It was observed that the findings of our study indicated that Dual drug CCHNS formulation inhibited cell viability in a concentration-dependent manner in A549 cell lines with higher efficacy than free DOX and free Curcumin (Figure 2) The lower toxicity of Dual drug CCHNS may be due to the fact that drug release from the CCHNS was slower as compared to free DOX and free Curcumin.

Cellular Uptake Studies

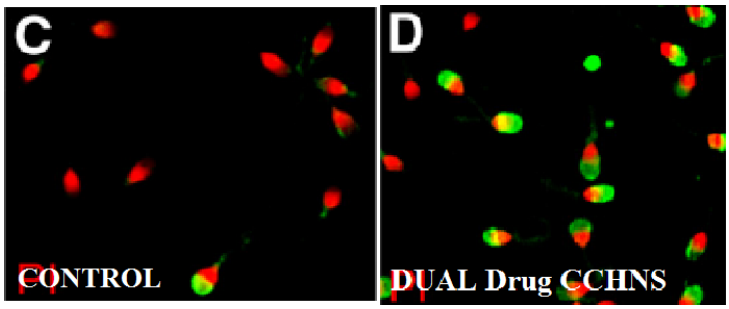

Fig 9: Cell uptake studies of Dual drug CCHNS

The cellular uptake kinetics of the various formulations encapsulating fluorescent dye FITC using fluorescence microscopy was assessed on the Human macrophage U937cellsatnumerous time points until saturation of uptake into the cells was observed.

12SEM

The surface morphology of Blank nanosponges and Drug loaded were analyzed by SEM analysis as shownin Fig.. The CCHNS nanoparticles showed flaky and clusters like morphology.

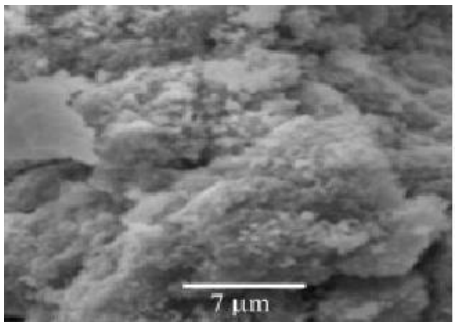

Fig 10: SEM of Dual drug CCHNS

Stability Studies

The stability of a nano formulation tested under particular conditions is very crucial for its efficacy, shelf-life and bioavailability. Hence, the formulations developed were investigated the at 2 different temperatures viz.25°C±2°C /60% ±5% RH and 4°C±2°C. The optimized formulation was found to be stable for 6 months at real time stability conditions of 4°C±2°Cand 25°C±2°C /60%±5% RH respectively.

CONCLUSION

The objective of this research work was to formulate and develop a nanosponge deliverysystem comprised of the polymers viz. β- Cyclodextrin, Chitosan and Hyaluronic acid using Ultra sound Assisted Synthesis. The prepared blank nanosponges were optimized by Box Behnken Design. The optimized batch of the Blank β-Cyclodextrin -Chitosan cross linked Hyaluronic acid Nanosponges (CCHNSs) had the particle size of 0.326 nm, PDI 0.474 and Zeta Potential of 30.8mV. The prepared Blank CCHNS were co-loaded with anti tubercular drugs viz. RIF, PYZ and IBU to obtain the Single and Multidrug loaded CCHNS. Additionally, the Blank CCHNS were also loaded for anticancer drugs viz. Curcumin and DOX. The drug loading was optimized using 2^3 Full factorial Design. The Cumulative drug release was found out to be 72, 85 and 68 for RIF, PYZ, IBU respectively and 62% from the Multi drug loaded CCHNS formulations. While the Cumulative drug release was found out to be 78 and 65 for Curcumin and DOX respectively. The Multi drug CCHNS and Dual Drug CCHNS followed Korsemeyer Peppas Korsemeyer Peppas release with anomalousnon-fickian transport. Indicating diffusion controlled release. Then-value obtained after the Korsmeyerplot was in the range of 0.57 and 0.367 respectively. The Cyotoxicity Studies were carried out using the MTT Assay using cellline A549.The IC values of 2.5 and 3.2 wereereported Multi and dual drug CCHNS respectively. This indicated that the prepared Dual Drug Nanosponge formulation is compatible without any toxicity. The cellular uptake kinetics was assessed on the Human macrophage U937 cells. The Multi and the Dual CCHNS Formulations showed the slowest up take kinetics while the hydrophobic drugs. showed the fastest uptake kinetics. The surface morphology of the Multi and Drug loaded showed flakes and clusters showing as ponge-like texture.

The optimized formulation was found to be stable for 6 months at real time stability conditions of 4°C±2°C and 25°C±2°C /60%±5% RH respectively. Based on this result as help life of 6 months can be proposed for the optimized formulation.

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