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PROLONGED AMOXICILLIN RELEASE KINETICS AND BIOCOMPATIBILITY USING CHITOSAN/PVA BLEND MEMBRANES IN-VITRO

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ABSTRACT

The present study emphases on chitosan based drug delivery system prepared by blending it with poly vinyl alcohol to modify the processability of the former and to facilitate multiple drug-loading. Blends prepared by varying the ratios of the polymers in the mixture 70:30, 50:50 and 30:70. Chitosan supports paracellular transport of drugs and as extra cellular matrices for cell culturing hence was selected for the study. Scanning electron microscope revealed that the blends show a good adherence to the monkey kidney cells, and also the distribution of the drug was uniform and well dispersed in the membranes. FTIR analysis confirmed that the blends of polymers occurred by means of hydrogen bonding. The drug release profile on a constant load of 100mg of amoxicillin which showed a prolonged release as expected and the cytotoxicity studies proved that the blends were not toxic to the cells. Hence the system could serve as a potential carrier in drug delivery and tissue engineering applications.

KEYWORDS

Chitosan, PVA, amoxicillin, cytotoxicity

1. INTRODUCTION

Fabrication of chitosan (CS) based scaffolds on drug release kinetics and for biomedical applications such as tissue engineering *in vitro* has been extensively investigated in the recent years. Because of the inherent poor processability as it is a natural biopolymer and to facilitate multiple drug-loading, a suitable polymer has to be blended to overcome the above quoted problems. Many polymers have been blended and studied for this purpose so far including collagen, PGA, n-isopropyl acrylamide and many more. These systems should also possess a longer life time when serving as a drug carrier and should support paracellular transport of the drug molecules inside the body [1]. Tissue engineering is a recently developed approach that aims to overcome the limitations of organ transplantation by providing artificial tissues grown *in vitro*. A typical method is to incorporate isolated living cells into three-dimensional polymer scaffolds and to create conditions for cells to proliferate in vitro, then transplant them back to the patient by surgical implantation or in a minimally invasive manner to develop into the desired tissues or organs. The requisites for a polymer to be successfully used in tissue engineering are that it should be bio-degradable and mimic like the tissue which needs replacement [2]. Controlled drug delivery applications include both sustained delivery over a period of days/ weeks/ months/ years and targeted (tumorous cells) delivery on a one-time or sustained basis. Controlled release formulations can be used efficiently for reducing the overdose of the drug.

The polymer scaffold controls the tissue structure by holding the cells together in a particular three-dimensional structure and by

regulating their function as a group. The polymer scaffold also allows the diffusion of nutrients, metabolites and soluble factors, acting as a surrogate for the extra-cellular matrices (ECM) of tissues in the body until cells produce an adequate ECM of their own. Such a scaffold, therefore, needs to be developed for *in vitro* tissue reconstruction as well as for cell-mediated tissue regeneration *in vivo* [3]. CS is a biosynthetic polysaccharide comprising of deacetylated chitin, a β-1,4 linked polymer of 2-amino-2-deoxy-β-D-glucopyranose produced commercially by deacytelation of chitin (**Figure1a**).

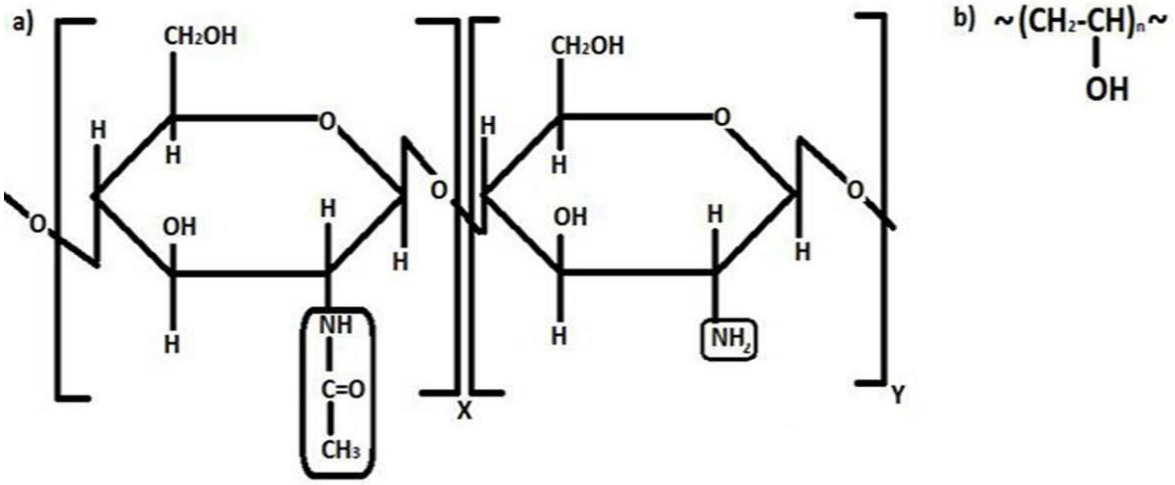

Figure.1 a) Representing the structure of Chitosan, where X>50% ➔ Chitin, Y>50% ➔ Chitosan b) Representing the Poly vinyl alcohol.

Usually these are extracted from the crustacean exoskeletons and are soluble in a slightly acidic aqueous solution. The main parameters affecting the characteristic of CS are their molecular weights (MW), degree of deacetylation (DD) representing the proportion of deacetylated units. From experiments pertaining to drug release, (employing bio-degradable polymers like N-isopropyl acrylamide) conducted earlier it was observed that with the low molecular weight polymer samples, swelling and the release of the loaded drug happened within a short period of 2h at 37^{o}C and pH of 7.4. It was observed that the amount of drug released by high MW samples for a period of 8h was equivalent to the amount of release by the intermediate MW samples for 4h. This consideration is very important in the case of CS, as the MW of the sample and the degree of deacytelation tend to influence greatly the drug release patterns of the polymer.

The reason for high interest in CS for biomedical research is due to its properties like excellent biocompatibility, biodegradability, safety and non-toxicity; it does not induce any strong immune response. CS shows promising characteristics towards improved burn wound healing and also acts as a bio-adhesive. With a positive charge and pKa value of 6.5 it readily adheres to negatively charged surfaces like the mucosal membranes and mammalian cells. CS enhances the transport of polar drugs across the epithelial surfaces and has a good regenerative effect on connective gum tissues. It also accelerates the formation of osteoblasts responsible for formation of bones. Additional positive features are its low cost due to abundance and diverse methods that enable the chemical processing, and its ability to act as hemostatic, fungistatic, anti-tumorous and anti-

cholestermic. Because CS molecules form inter-molecular hydrogen bonds the dissolution of the polymer in water is difficult and hence an acidic medium required for preparing solutions. CS currently receives a great deal of interest in medical and pharmaceutical applications. The drawbacks of CS can be overcome by blending with polymers such as PVA [4-6].

PVA is a bio-compatible and bio-degradable synthetic polymer with good adhesive properties (Figure1b). *In vivo* bio-activity studies of PVA with SBF solution were encouraging. It is an odorless, non-toxic polymer that readily and fully degrades in a quick dissolver. It dissolves in water and is often used as a protective colloid; the water molecules act as plasticizer and improve the tear strength and the elongation of PVA. Due to the ability to fabricate scaffolds containing pores, which can either limit or eliminate cell migration; as well as scaffolds with inherently high surface area, which allows for high drug loadings and the ability to overcome mass transfer limitations associated with other polymeric systems. CS scaffolds meet both the requirements of barrier function and as a drug delivery system necessary to prevent abdominal adhesion. Thus one can produce a novel bio-polymer/synthetic polymer hybrid blend. Blending of polymers for drug delivery serves the purposes of adjusting the drug release pattern as per the requirements, improves mechanical properties (important in the case of chitosan), improves the shelf-life, film formation, controlled and targeted drug delivery.

The main objective of this work was to prepare homogeneous CS/PVA blends with varying concentrations, and with constant drug loading followed by release kinetics studies. The concept of 'bio-adhesion' which is the ability of the macromolecules or colloidal particles to adhere to the cells were studied and the analyzed using the SEM [7]. This approach enables generation of CS/PVA blends which results in improvement in other physical properties of CS (e.g., hydrophilicity and mechanical properties) as well.

2. MATERIALS AND METHODS

2.1 MATERIALS

CS flakes (84% Deacetylated) were received as a gift sample from India SeaFoods, Kochi (India) and its molecular weight was found to be 1700kDa. Glacial acetic acid (99% Pure) and PVA (M. wt. 25,000) was used as received from Sigma Aldrich Inc.

2.2 Methods

2.2.1 Fabrication of CS/PVA blends

A 3% (m/m) CS solution was prepared by dissolving CS in 2% glacial acetic acid. The solution was stirred over night at room temperature to obtain a homogenous mix and was filtered to remove undissolved polymers. An 8% (m/m) PVA solution was prepared by dissolving PVA in distilled water; the solution was thoroughly stirred at 80^{o}C for four hours. The two solutions were then ultrasonicated to remove air bubbles using the ultrasonic waves. Three different blends were prepared by using measured quantities of CS and PVA, the first blend containing 25% of CS and 75% of PVA, the second with 50% CS and 50% PVA, the third, 75% CS and 25% PVA. The three blended solutions were then cast into three petridishes, washed and kept sterile prior to casting. They were later kept in an oven and maintained at 50^{o}C overnight.

2.2.2 Fourier Transform Infra-Red Spectroscopy Analysis

Fourier Transform Infrared spectroscopy of CS-PVA blend scaffolds was performed after finely grinding a portion of each sample with KBr in transmission mode using Perkin Elmer spectrophotometer RX1. The powder mixture was then crushed in a mechanical die press to

form a translucent pellet through which the beam of the spectrometer could pass.

2.2.3 Thermo-Gravimetric Analysis

The compositional analysis of the blends was done using a Mettler Toledo (TGA/SDTA 851e) thermo gravimetric analyzer (TGA). Samples were weighed and heated at 10°C/min from 40 to 500°C and weight loss for each polymer fraction was measured.

2.2.4 Scanning Electron Microscopy

The morphology of the produced scaffolds was evaluated using a Scanning Electron Microscope (HITACHI S-3400model). The morphology of the drug loaded samples was analyzed for different samples. In order to analyze the cell adherence to the prepared polymer surface the samples used for the cytotoxicity studies were dried up for a period of one day and analyzed for their attachment.

2.2.5 Water Absorption Test

Water absorption tests were performed to analyze water absorption. The samples were immersed in distilled water and were maintained at room temperature for 24 hours. The mass prior to the immersion and after the immersion were noted. The water absorption was calculated as follows,

$$\text{Water absorption } \% = \frac{W_2 - W_1}{W_1} * 100$$

W_2 & W_1 being the masses after and before immersion respectively [5, 8].

2.2.6 Drug Release

The drug release profile of all the three preparations were tested on constant loading with amoxicillin (100mg). The total amount of the drug release was studied by immersing the sample in pH 7.4 buffer solution. Using UV visible spectrum the absorbance at 217nm was evaluated for a period of time from which the percentage of drug released calculated; T90+ UV visible spectrophotometer from PG Instruments was used for the analysis [9].

2.2.7 Cytotoxicity

The cytotoxicity was evaluated based on a procedure adapted from the ISO10993-5 standard test method (indirect contact). The blends of CS-PVA were sterilized by UV radiation for 1 hour. The membranes were then immersed in a serum-free medium (SFM; containing DMEM, 1% l-glutamine, 1% lactalbumin and 1% antibiotic and antimycotic formulation) in an incubator for 24 hours to produce extraction media of varying concentrations (10, 7.5, 5, 2.5 and 1mg/mL). Monkey kidney cells were plated in 96-well plates of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, at a density of 8000 cells/well in 96-well plates. When the cultures reached confluency (typically 48 hours after plating), the tested extraction media at varying concentrations were replaced and the cells were re-incubated for 24 hours. After treatment, the tested extraction solutions were removed. Finally, the cells were incubated with 100L of a MTT-containing medium (1 mg/mL) for 4 hours. The medium was removed, the cells were rinsed with PBS (pH 7.4), and the formazan crystals formed in living cells were dissolved in 100L DMSO per well. Relative viability (%) was calculated based on the absorbance at 550nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). Viability of non-treated control cells was arbitrarily defined as 100% [10]. Cytotoxicity studies were performed at King Institute of Preventive Medicine, Guindy, India.

3. RESULTS AND DISCUSSIONS

3.1 FTIR analysis

The FTIR spectrum showed resonance peaks attributed to saccharide groups. Other peaks depicted (O–H), (CH_2), (CH_2=CH_2), (CH–O–H) and (N–H) bending of the primary amino groups, the carbonyl stretching of the amide bands, and the

N–H stretching of the primary amino groups. The purpose of the FTIR spectroscopy was to assess the polymer chemical groups involved in chitosan and PVA blend formation. Figure2 shows the FTIR spectra of different samples of chitosan and PVA individually and blends with different weight ratios, where figure 2A represents the spectrum of pure chitosan showing the characteristic peaks near 893 cm^{-1} and 1156 cm^{-1} conforming to saccharide structure.

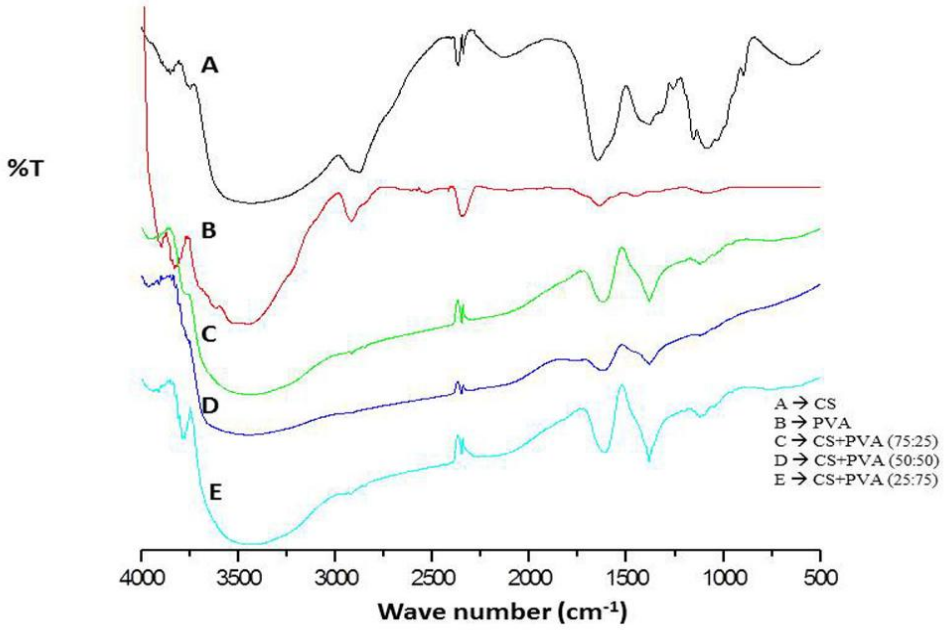

Figure.2 Illustrating the FTIR Spectrum of CS, PVA and CS+PVA

Strong absorption peaks at 1658 cm^{-1} and 1322 cm^{-1} corresponds to the amide I and III peaks which is characteristic of chitosan. The peaks between the regions ranging from 1510 cm^{-1} to 1560 cm^{-1} may be attributed to the partially deacetylated amino groups corresponding to the amide II group. The broad peak at 1030 cm^{-1} and 1080cm^{-1} corresponding to the C-O stretching vibration in chitosan and the another broad peak at 3447 cm^{-1} is attributed to the amine N-H symmetrical vibration, thereby indicating that the samples are not fully deacetylated. In case of CS/PVA blended films there was an increase in C-H intensity around 2928cm-1 which was due to the PVA and the intensity increased with the increase in PVA content [11-14]. Also all the three ratios of CS & PVA showed a characteristic stretch around 3750-3250 cm^{-1} corresponding to the OH stretch thereby it could be inferred that the polymers were blended by means of hydrogen bonding.

3.2 Thermo-Gravimetric Analysis

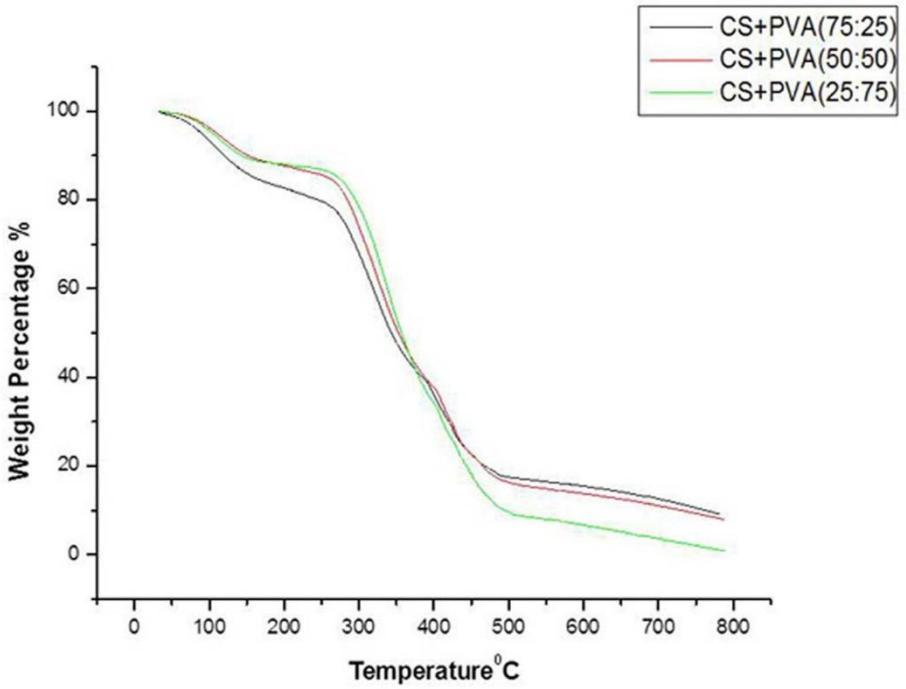

Figure.3 Shows the TGA analysis of the CS+PVA Blends Where a) 75:25 b) 50:50 c) 25:75

The thermo-gravimetric analysis of the blends confirmed the presence of both polymer fractions in the blend. Previous studies claimed that pure chitosan was found to thermally decompose at 240 °C and pure PVA at 325 °C. In figure3, the curve until ~81.75°C was due to the evaporation of water or other solvents from the surface of the blend. There was a second weight loss noted at ~154 °C or at 165 °C which could be attributed to the evaporation of any possible solvent in the pores and due to the chemically bonded water. However, in the case of the blends, the main polymer chain degradation started at 282 - 285°C. The polymer degraded completely at 440- 460°C. The curve thereafter was almost linear, indicating that the degradation of polymer had completed and the remaining was residual carbon. The degradation was gradual, meaning the polymer would degrade steadily and gradually.

3.3 Scanning Electron Microscopy

The scanning electron facility was used to study the surface-morphology of the blend prepared. SEM images were taken for blend samples loaded with amoxicillin drug (a constant load of 100mg). On studying the morphology with the help of the SEM, the image showed that superior blending occurred between CS & PVA.

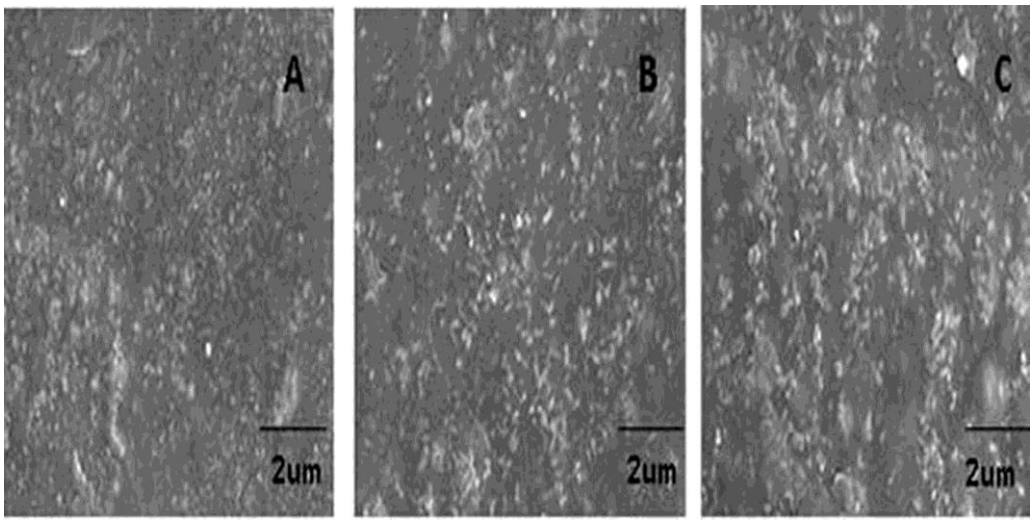

Figure.4 The SEM images of the CS+PVA Blends Where A) CS B) PVA C) CS+PVA (50:50) with Amoxicillin drugs loaded.

From figure4 it can be observed that there was an even distribution of the amoxicillin drug that was loaded. The SEM analysis showed that good blending of the polymers took place in each case; the sample which was prepared with equal amount of the two polymers (50:50) was taken for further analysis. Also the SEM analysis was carried out to determine the adherence of the cells to the pure CS, PVA and CS + PVA blend membranes (here 50:50 was selected). The SEM analysis in figure5 showed that the cell adherence was greater in the case of the blend compared to the individual CS and PVA films.

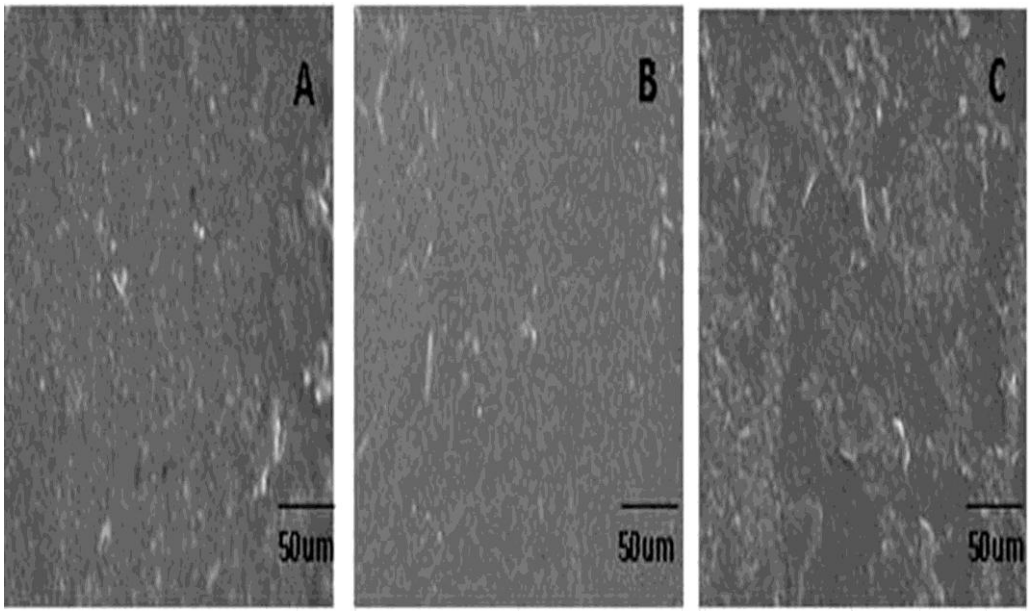

Figure.5 The SEM images of the cell adhesion of CS+PVA Blends A) CS B) PVA C) CS+PVA (50:50)

3.4 Water Absorption Tests

When all the three blends absorbed water, the samples were weighed and the water absorption was calculated. The observed pattern as shown in table 1 showed initial rapid intake. It was noted that the water absorption

percentage increased with increase in CS concentration. Visual observation also showed appreciable volume increase. The samples were tested for strength by the finger-impression test. Sample 1and 3 broke on doing so while the sample with equal proportions of the two polymers showed no impressions and was found to be stable.

Table.1 shows the results for stability test for various blends of CS+PVA

BLEND	WATER ABSORPTION %
CS+PVA(25:75)	192 %
CS+PVA(50:50)	238 %
CS+PVA(75:25)	250 %

3.5 Drug Release Kinetics

It was observed that, at the effective amount of amoxicillin loading at 100mg/g of polymer (optimized in lab), the samples showed efficient drug-release patterns. Among the three groups, the blend with equal proportions of CS and PVA showed a constant and steady release. The release rates of amoxicillin from cast film made from chitosan & PVA blend were studied in pH 7.4 at 37°C.

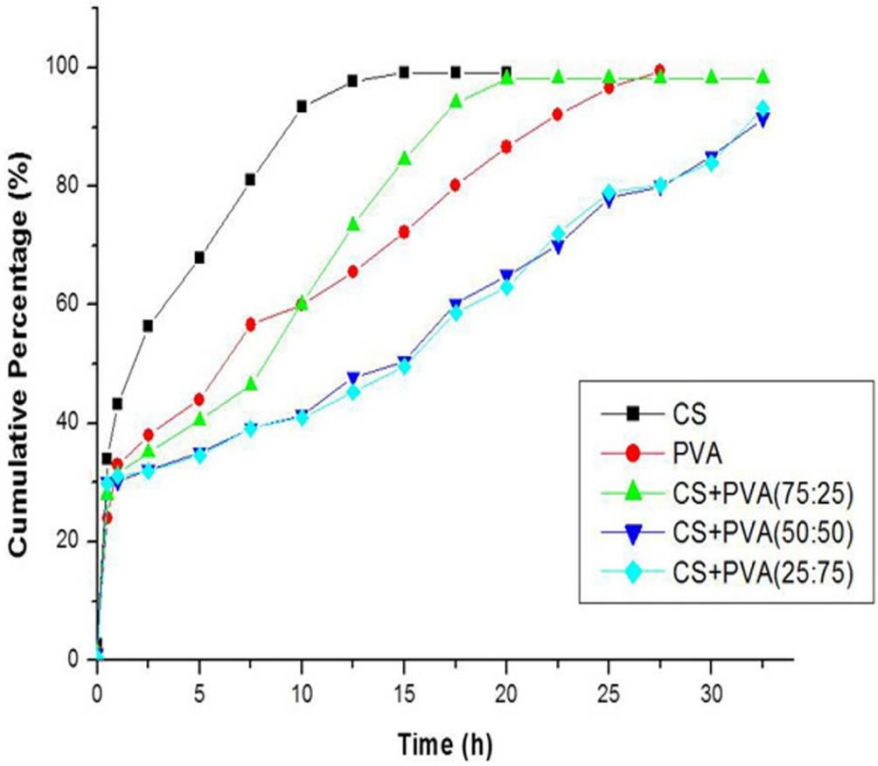

Figure.6 shows release pattern of Amoxicillin in Cs+Pva Blends at 37°C

In general, the trend of release from all three different blend films showed an initial fast release in the first few hours followed by slower release rates as shown in figure6. Investigation on all three cases of blends showed that there was an initial fast release of around 30% i.e., 30

(mg of drug) (g polymer)$^{-1}$ of the total amount of the drug. It can also be noted that in the case of the higher concentrations of CS, the release occurred in two rapid stages, initial fast release and a constant slow release thereby the released concentration reached a maximum of 98.2% in 20 hours. Even in the other two forms of blends i.e., CS+PVA (50:50, 25:75) there was an initial fast release followed by the slow release rate and a sustained release was observed for a period of 32 hours. While the amount of drug released was found to be 91.5 (mg of drug) (g polymer)$^{-1}$ in case of CS + PVA (50:50), it was found to be 93.1 (mg of drug) (g polymer)$^{-1}$ in the case of CS+PVA (75:25) further in both the cases the maximum release was found to be 97.8 and 97.1% respectively after 35 hours. Also the release study was also carried out by varying the temperature and the release was found to decrease with decrease in temperature as shown in figure 7. This result is anticipated because the release from the film is diffusion dependent and as the temperature increases the diffusion rate increases. The release study was not performed at higher temperature.

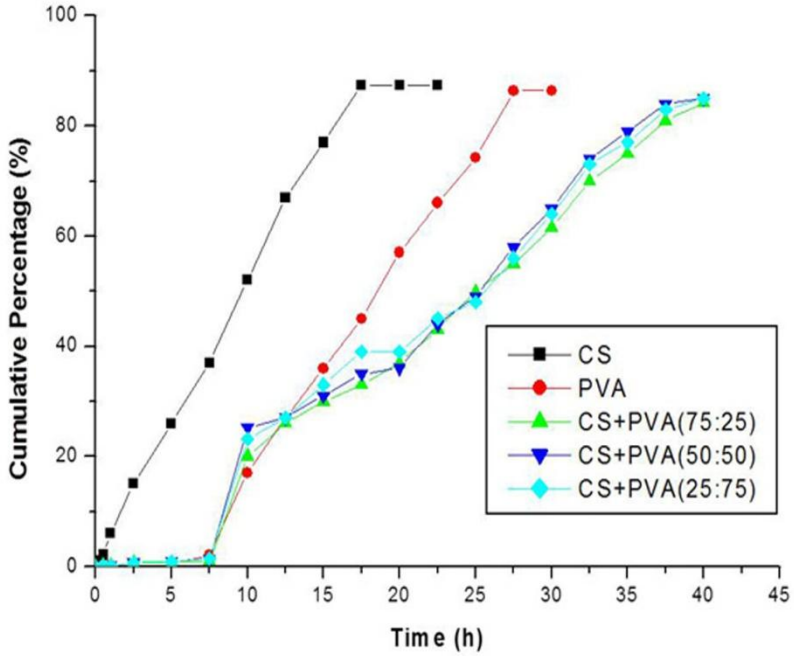

Figure.7 the release pattern of amoxicillin in CS+PVA Blends at 25°C

3.6 Cytotoxicity

Metabolically active cell mitochondria in the living cell reduce the yellow tetrazolium salt that is MTT.

Table.2 shows the Cytotoxicity results of CS, PVA and their blends at an incubation period of 72 h.

	Morphological Change	Reactivity	Total Cytotoxic Response	Score
Control	Nil	Nil	0-1	Pass
CS	Negligible amount	Very Mild	1	Pass
CS:PVA (75:25)	Negligible amount	Very Mild	1	Pass
CS:PVA (50:50)	Negligible amount	Very Mild	1	Pass
CS:PVA (25:75)	Mild Changes, some cells round/spindle shaped	Mild	2	Pass(Retest)

The reduction results in the formation of violet formazen dye from which the amount of viable cells in the sample was quantified spectrophotometrically using ELISA reader. Cell viability analyzed using the MTT assay at a period of 72h time interval (table 2) after cell seeding showed a score similar to the control group [15]. This result was found to be comparatively higher than the one observed by Souza Costa et al [5]. This reveals that the blend membrane could possibly be used in the field of tissue engineering.

4. CONCLUSION

CS based blends were successfully prepared by blending it with PVA, using acetic acid and water as the solvents for the former and the latter respectively. The drug release profiles of these blends were carefully investigated and the cell viability of the blends was also focused on as proposed. The amount of CS in the blends had a prominent effect on the above properties. The results of the various characterization tests carried out showed that the drug release profile and the cell culturing of the blends were better than that of pure forms. On comparing the results obtained, the blend with equal proportions of the polymers showed superior properties and is favorable for applications in the prolonged drug delivery and tissue engineering. The 50+50 blends withstood the stability tests after calculating the water absorption percentage. In-spite of absorbing water twice as much as the first blend, the blend bore no impressions after the test unlike the other two, one of which even broke. The water absorption property was highly desirable in case of drug delivery. The drug release profile showed a constant and steady release for an extended time. Cell growth was highly favored in the blend in comparison with the other two individually. Thus this approach could have potential application in the field of drug delivery where prolonged release is required and in tissue engineering.

5. ACKNOWLEDGEMENTS

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