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Biosynthesis of tamoxifen coated chitosan biopolymer, its characterization, DNA damage study and anticancer study against breast cancer cell line

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Abstract

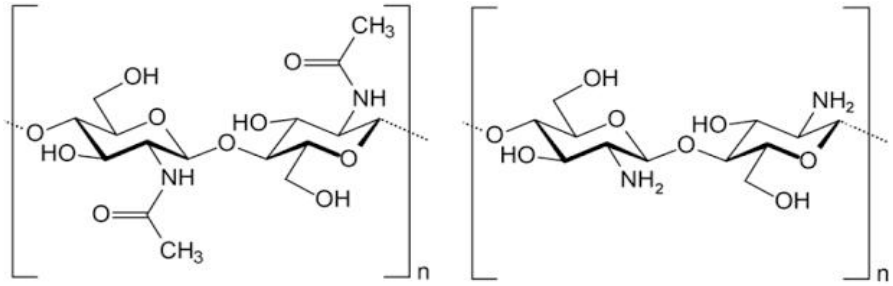
Tamoxifen has a broad spectrum of anticancer activity. The main aim of the study is to determine the biosynthesis of tamoxifen coated chitosan biopolymer which can be characterized by SEM and FTIR. The loading efficiency of oyster shell chitosan shows higher value than that of the shrimp shell chitosan. The ampicillin and E-coli culture were introduced into tamoxifen drug coated with biopolymer results in the isolation of DNA and plasmid. It determines the DNA damage activity of tamoxifen drug coated biopolymer (chitosan). The *in vitro* drug release study plays an important role in expressing the penetration activity of tamoxifen drug from the dialysis bag into the water and the drug releasing capacity is 48%. DMEM provides enough nutrients for the breast cancer cell line (MCF - 7) to grow properly. The mixed proportion (10µl and 20µl) of Tamoxifen drug coated biopolymer introduced into 96 microtitre well plates besides breast cancer cell lines (MCF 7) in CO_2 incubator for 72 hours. Readings taken from ELISA reader shows that Cell death in 10µl sample of oyster shell chitosan coated tamoxifen drug was 59.41%. 10µl sample of oyster shell chitosan coated tamoxifen has shown high anticancer activity in MCF - 7 cell lines.

Keywords: Chitosan, tamoxifen, ampicillin, breast cancer, ELISA

1. Introduction

Chitin, a homopolymer of N-acetyl-D-glucosamine (Glc- NAc) residues linked by β-1-4 bonds, is the most widespread renewable natural resource following cellulose. The main source of chitin is crustacean waste, which is also the main cell wall material in most of the fungi. Chitin and its derivatives have high economic value owing to their versatile biological activities and agrochemical applications. The natural antibacterial and/or antifungal characteristics of chitosan and its derivatives have resulted in their use in commercial disinfectants (Zouhour Limam *et al*., 2011)[(12)].

Chitosan is a linear polysaccharide obtained by the process of deacetylation of chitin. The linear polysaccharide (chitosan) is composed of randomly distributed β – linked glucosamine and N- acetyl D – glucosamine. It is made by treating the chitin shells of shrimp and other crustaceous species with an alkaline substance like sodium hydroxide. Chitosan has number of commercial and possible biomedical uses. Since they are biodegradable non-toxic and bio-compatible, these derivatives have a potential for use in a variety of medical applications (R. Vivek *et al*., 2013)[8].



Chitin Chitosan

Chitin has major applications in many fields like agriculture, medicine, etc. (Linden J *et al*., 2000). Chitin is used in wine making as a fining agent; it also helps to prevent spoilage. It is used as a self healing polyurethane paint coating in industries (Shahidi F *et al*., 1999; Hosokawa J *et al*., 2000; Gaellstedt M *et al*., 2005)[6, 2, 1].

The chitin is a hard substance with crystalline structure and it is white and the base material is variety of animals and lobster shells. Chitin is also found in the outer covers of snails and fungi. They are found in diatoms, nematodes, anthropoids, shrimps, crabs, krill and squid, yeasts and invertebrates. Degraded molecules of chitin/chitosan exist in soil and water. Chitin – chitosan can be characterized by XRD (X –ray Powder Diffraction), FTIR (Fourier Transform Infrared Spectroscopy) and SEM (Scanning Electron Microcopy). A scanning electron microscope is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons. The term Fourier transform infrared spectroscopy orginates from the fact that a Fourier transform is required to convert the raw data into the actual spectrum. In the present study, the action of biopolymer (chitosan) against breast cancer cell lines (MCF – 7) and the DNA damage study of oyster chitin have been undertaken.

Cancer means a range of diseases along with uncontrolled proliferation and invasion of other organs. Cancer cells are passes through the blood or lymphatic system to other parts of the body (Zahra Saeidi *et al*., 2016)[9]. Breast cancer starts when cells in the breast begin to grow out of control. These cells usually form a tumor that can often be seen on an x-ray or felt as a lump. The tumor is malignant (cancerous) if the cells can grow into (invade) surrounding tissues or spread (metastasize) to distant areas of the body. MCF-7 cells are useful for *in vitro* breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium. These include the ability for MCF-7 cells to process estrogen, in the form of estradiol, via estrogen receptors in the cell cytoplasm. This makes the MCF-7 cell line an estrogen receptor positive control cell line. In addition to retaining their estrogen sensitivity, MCF-7 cells are also sensitive to cytokeratin (Soule *et al*., 1973; Levenson AS *et al*., 1997)[7, 3]. Tamoxifen is a commonly used anticancer drug. It is most often used against breast cancer, carcinomas, osteosarcoma and soft-tissue sarcomasa. Tamoxifen citrate (tamoxc) is the drug of choice for estrogen-mediated treatment among breast cancer patients (Ranjit Singh *et al*., 2012)[5].

2. Materials and Methods

Collection and extraction of chitosan

Shrimp and oyster shells were collected from the Ukkadam market, Coimbatore, Tamilnadu, India. Chitosan was extracted from the shells of shrimp and oyster shell chitin. 10 ml of 2 % acetic acid was added and kept in water bath at 40° C for 1 hr. 1 ml of water was added and incubated at water bath 50° C for 2 – 3 hours. Neutralized the chitosan and kept in overnight incubation, then chitosan was extracted from chitin.

Synthesis of biopolymer with tamoxifen drug

1 ml of phosphate buffer was added into the extracted chitosan and centrifuged at 8000 rpm for 10 minutes. Readings were recorded at 254 nm in the UV Vis Spectrophotometer. Tamoxifen was added into the pellet, centrifuged and then readings were recorded.

Drug release study

Tamoxifen was added into the pellet removed from the oyster shell chitosan because it had a high value compared to shrimp shell chitosan. The pellet was dissolved with 1 ml of phosphate buffer and poured into the dialysis bag. It was incubated at room temperature for 1 hr and readings were recorded at 254 nm.

Drug loading efficiency = $(W_0/W) \times 100$

W_0 - Total concentration of drug, W - Concentration of chitosan

Isolation of DNA and plasmid from bacteria

E coli culture was incubated for 24 hours, centrifuged and taken as ampicillin pellet was plasmid and without ampicillin pellet was DNA. It was again centrifuged with 700 µl of TE buffer, 20 µl of lysozyme was added into the centrifuged pellet. 150 µl of SDS and 300 µl of sodium acetate were centrifuged at 12,000 rpm for 10 minutes. 700 µl of ethanol was added into the pellet for DNA purification. 40 µl of TE buffer was added, centrifuged and 20 µl of lysozyme was added for the lysis of cellwall. Solution B containing 0.2 g SDS and 0.16 g NaOH in 20 ml distilled H_2O and Solution C containing 4.9218 g sodium acetate in 20 ml distilled H_2O were added and incubated in a freezer. Then centrifuged at 6000 rpm for 6 minutes. To purify the plasmid, 700 µl of ethanol was added and later stored for further use.

DNA nicking assay

1 µl of TE buffer was added in both isolated DNA and plasmid; 10 µl of Fenton's reagent was added and kept for incubation for 3 hours at room temperature. Then, the DNA was viewed under UV Transilluminator.

MTT assay

100 µl of MCF 7 cell line, BSA and DMEM medium was mixed and poured into T –Flask the medium appeared pink colour. It was incubated in a CO_2 incubator for 72 hours. 10 µl MTT dye was added again and incubated for 72 hours. Readings were noted at 570 nm in an ELISA reader.

3. Results and Discussion

The mechanism of action of Tamoxifen which itself is prodrug, having relatively little affinity for its target protein. Tamoxifen drug was coated with both the chitosan compounds which resulted in high absorbance in oyster shell chitosan. Plasmid and DNA isolation is used to study the DNA damage activity. The drug in the dialysis bag was released through porous membrane in which the drug releasing capacity into water was 48 %. MCF – 7 cell lines were grown properly in DMEM in a CO_2 incubator. Finally, readings from the ELISA reader showed high values in 10 µl concentration of the sample. Cell death occurred in 10µl sample of oyster shell chitosan coated with tamoxifen drug was 59.41 %

Characterization of oyster shell chitosan

Scanning Electron Microscopy (SEM)

a) Scanning electron microscope showed the cell structure of drug coated chitosan

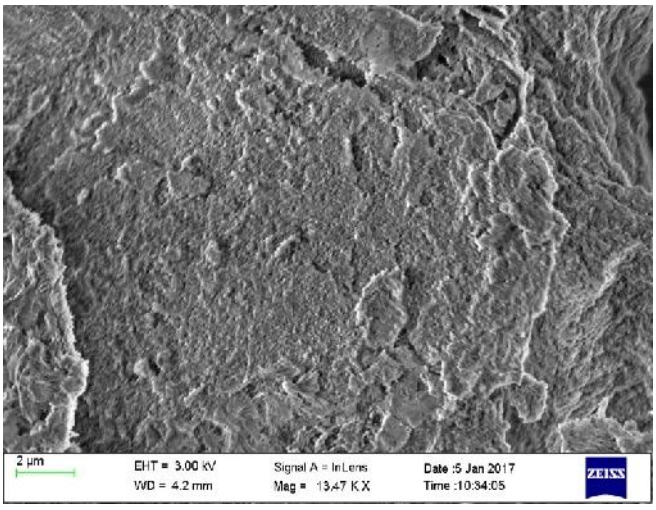


Figure 1. Drug coated biopolymer

b) Scanning electron microscope showed the image of oyster chitosan

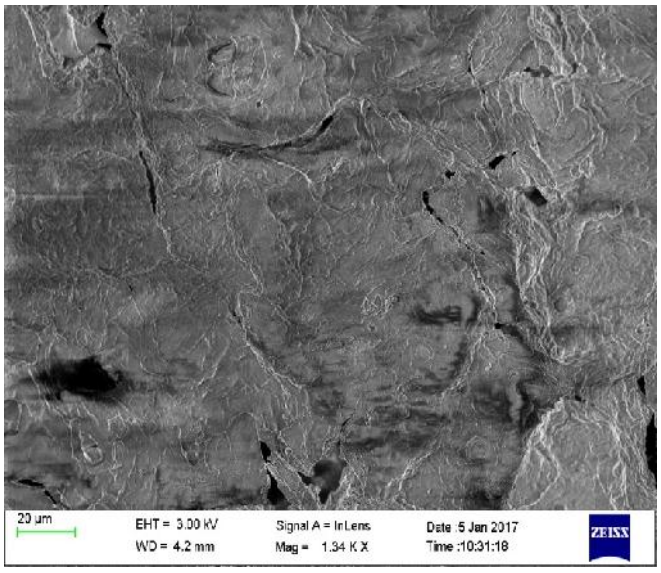


Figure 2. Oyster shell chitosan

Fourier Transform Infrared Spectroscopy (FTIR)

a) The highest peak of drug coated with biopolymer was 2250 nm

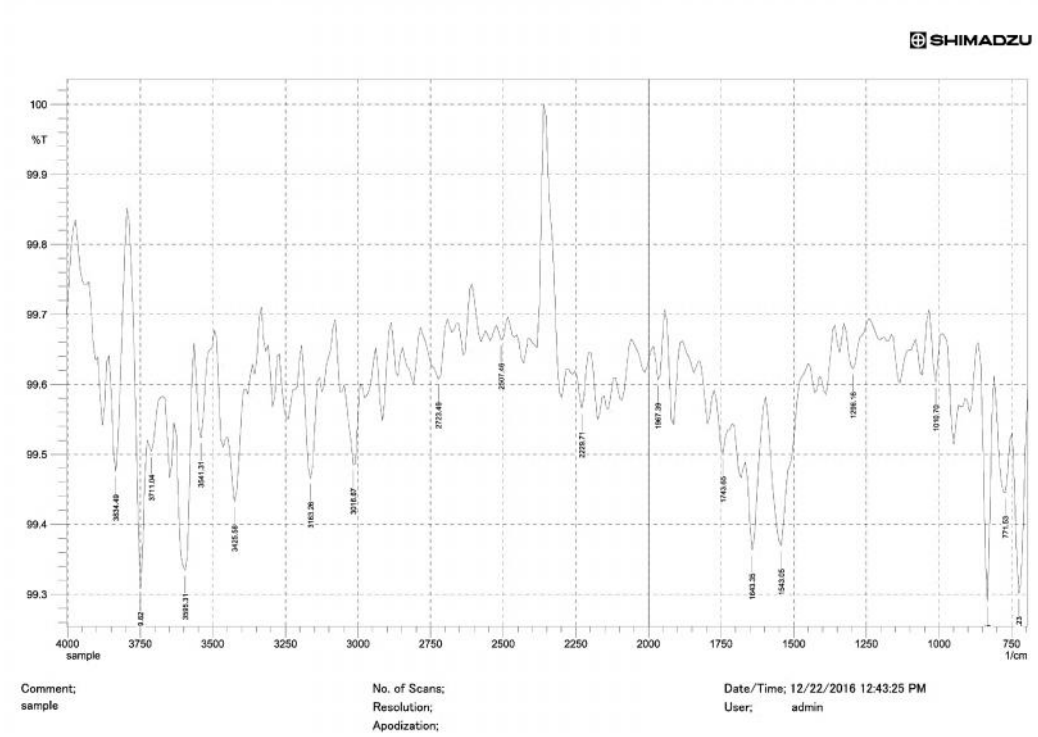


Figure 3. Drug coated with biopolymer

b) The highest peak of drug coated with biopolymer was 3350 nm

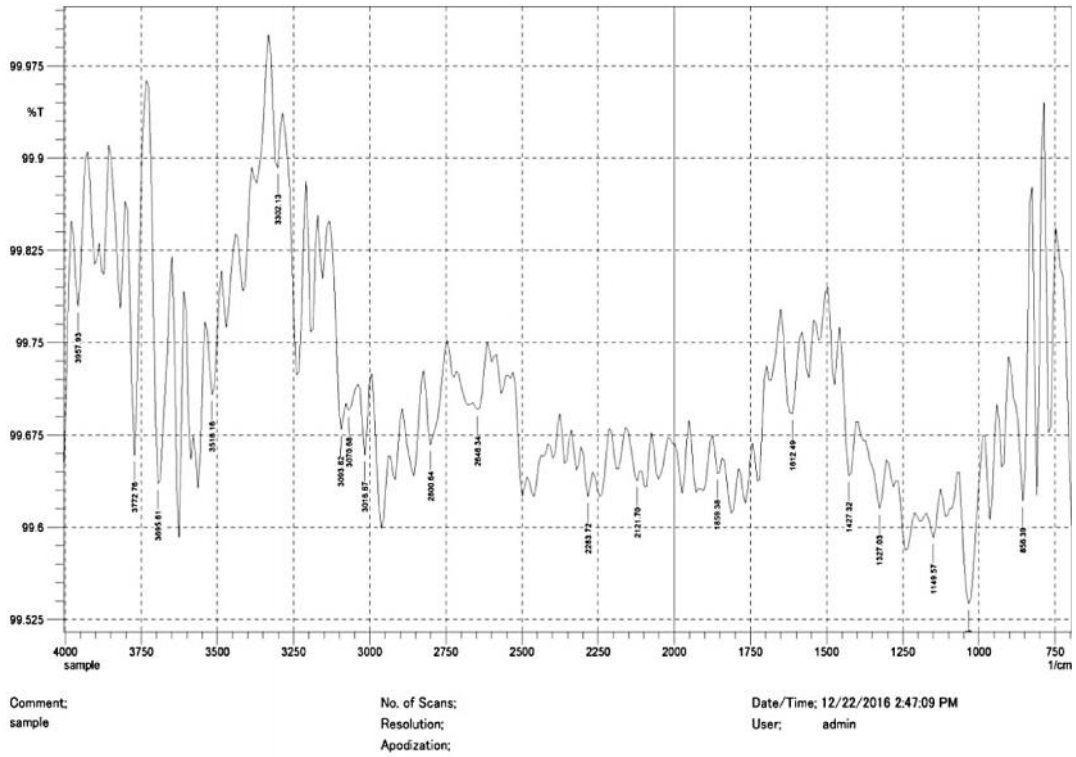


Figure 4. Oyster shell chitosan

Comparison of oyster and shrimp chitosan

1 ml of phosphate buffer was added into both oyster and shrimp shell chitosan and centrifuged at 10,000 rpm for 10 minutes. Tamoxifen drug coated into the centrifuged pellet and readings were observed at 254 nm.

Table 1. Oyster shell chitosan

CHITOSAN POWDER	DRUG WITH CHIOTOSAN	DRUG FREE CHITOSAN (CONTROL)
0.06	1.5	1.057
0.125	1.5	0.862

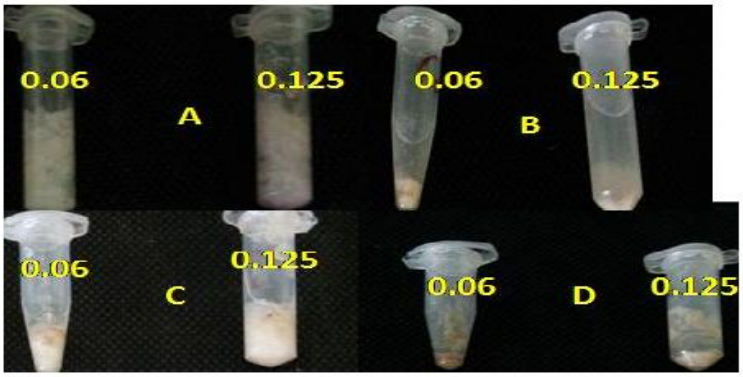


Figure 5. Shrimp and oyster shell chitosan

A) Control of shrimp shell chitosan
B) Control of oyster shell chitosan
C) Shrimp shell chitosan
D) Oyster shell chitosan

Tamoxifen coated oyster shell chitosan has shown higher value of concentration when compared with shrimp shell chitosan.

Drug loading efficiency of chitosan from oyster shell

DRUG LOADING EFFICIENCY= W_0 / W X 100
((W_0) = 0.025)
DRUG LOADING EFFICINCY = 0.025 / 0.06 X 100 = 41.66 %
DRUG LOADING EFFICINCY = 0.025 / 0.125 X 100 = 20 %

The concentration 0.06 g for oyster shell chitosan with drug loading efficiency was 42 % and 0.125 g for oyster shell chitosan with drug loading efficiency was 20 %. Drug loading efficiency of oyster shell chitosan was 42 % higher.

DNA damage activity

DNA and plasmid were isolated from bacteria and it determines the DNA damage activity of tamoxifen drug coated biopolymer (chitosan).

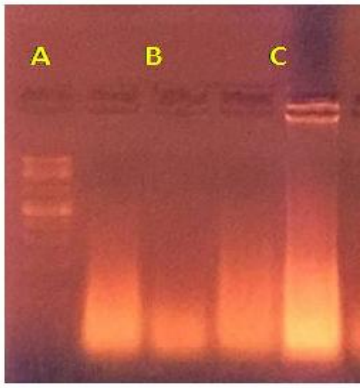


Figure 6. Activity of DNA damage
A) Control
B) DNA
C) Plasmid

In vitro drug release study

Chitosan powder and phosphate were added. Then centrifugation was carried out at 8000 rpm. Tamoxifen drug was added and kept overnight in an incubator. The reading was noted at 254 nm.

TABLE 2. Values of chitosan

SHRIMP SHELL CHITOSAN	OYSTER SHELL CHITOSAN
0.673	1.5

Oyster shell chitosan has shown the highest concentration.

Drug coated oyster shell chitosan has to be immersed into the dialysis bag and then the drug released through porous membrane. The drug releasing capacity after 24 hours was observed to be 47.99 %.

Anticancer study

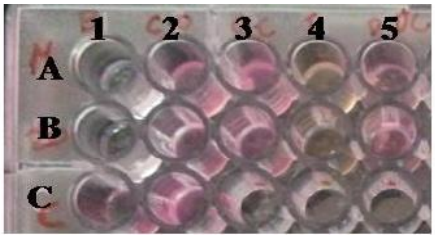


Figure 7a. Samples were added into the 96 well plates

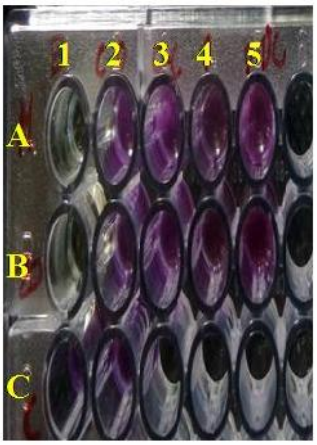


Figure 7b. After cell maturation colour changes were observed

1) Blank
2) Shrimp shell chitosan
3) Oyster shell chitosan
4) Tamoxifen drug
5) Drug coated oyster shell chitosan

A) 10µl
B) 20µl
C) Control

The activity of tamoxifen coated chitosan has shown the ability to act against breast cancer cell lines at a higher value of 10 µl concentration.

Anticancer activity was measured by using this formula**,**

Cell death = Control – Sample / Control X 100

Table 3. Values of anticancer activity obtained by ELISA

CONCENTRATION	10 µl CONC.	% OF 10 µl CONC.	20 µl CONC.	% OF 20 µl CONC.
Control	2.962	-	2.962	-
Blank	0	0	0	0
Shrimp shell chitosan	1.661	43.92 %	1.797	39.33 %
Oyster shell chitosan	1.582	46.59 %	1.656	44.09 %
Tamoxifen drug	1.357	54.18 %	1.709	42.30 %
Oyster shell chitosan coated with drug	1.202	59.41 %	1.586	46.45 %

Human serum albumin nanoparticles were modified by adding an outer coating of the chitosan to improve the therapeutic index of tamoxifen against MCF-7 breast cancer cells (Ranjit Singh *et al.*, 2012)[(5)]. Chitosan is characterized by high antibacterial and fungicidal activities. This study also indicated the possibility of exploiting the chitosan as an effective inhibitor of bacteria and fungi (Zouhour Limam *et al.*, 2010)[(10)].But,the present study was evaluated the coating of drug with biopolymer (chitosan) has ability to act against breast cancer cell lines (MCF – 7). In this study, tamoxifen coated chitosan does not act as an effective inbihitor of bacteria and fungi.

Conclusion

Chitosan is a multifunctional polymer with many valuable applications. In the present study, it was found that chitosan has excellent biological properties such as biodegradability and immunological, antibacterial and antimicrobial activity. Also, drug coated chitosan has wide applications in anticancer activity.

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