

In-vitro Chick Chorioallantoic Membrane Study of Chitosan Capped 5-Fluorouracil Conjugated Gold Nanoparticles

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Abstract

Chitosan is a linear polysaccharide composed of randomly distributed β -(1 \rightarrow 4) -linked D-Glucosamine (DE acetylated unit) and N-acetyl-D-Glucosamine (acetylated unit). It is made by treating the chitin shells of shrimp and other crustaceans with an alkaline substance, like sodium hydroxide. Chitosan has a number of commercial and possible biomedical uses. Chitosan's properties also allow it to be used in transdermal drug delivery. Delivering therapeutic compound to the desirable site is a major problem in the treatment of many diseases. The combination of nanoparticles with Chitosan in the form of Nano composite matrices provides the high surface area required to achieve a high loading of enzymes, drugs, and a compatible micro-environment to facilitate stability. Chitosan Nanoparticles (NPs) was prepared using the ionic deletion method. 5' Fluorouracil (5'FU) loaded Chitosan nanoparticle (5FCN) was synthesized and tested for its Angiogenic activity on Cell lines by a chick chorioallantoic membrane (CAM) assay.

Keywords: 5-fluorouracil, chick chorioallantoic membrane, Gold Nano particle, Chitosan

INTRODUCTION

Recent advancements in the synthesis of various nanomaterials of different sizes, shapes and functions have established nanotechnology as an indispensable technology for agriculture [1]. Materials which show unique properties linked to their size (ranging from 1 to 1000 nm at least in one dimension) are considered nanoparticles and deemed under nanotechnology. Nano materials have some high value properties like high surface-to-volume ratio, more molecules/reactive groups on the surface; prefers Nano-encapsulation and are independent of gravity [2].

The rapid expansion of nanotechnology promises to have great benefits for society. Nanomaterial is matter at dimensions of roughly 1-100 nm, where a unique phenomenon offers novel applications. The major advantages of nanoparticles as a delivery system are in controlling particle size, surface properties, and release of pharmacologically active agents in order to achieve the site-specific action of the drug at the therapeutically optimal rate and dose regimen. Although nanoparticles offer many advantages as drug carrier systems, there are still many limitations to be solved such as poor oral bioavailability, instability in circulation, inadequate tissue distribution, and toxicity [3].

Chitin is found in the exoskeleton of some anthropoid's, insects, and some fungi. Commercial sources of chitin are the shell wastes of crab, shrimp, lobster, etc. Chitosan is usually prepared by the DE acetylation of chitin. The conditions used for DE acetylation will determine the average molecular weight (Mw) and degree of DE acetylation (DD). The DD of typical commercial chitosan is usually between 70 and 95%, and the Mw between 10 and 1,000 kDa.

The use of nanoparticles in the biological field has increased due to the superior properties that they possess when compared to their bulk counterparts such as enhanced permeability and retention and the ease with which they are taken up by the cells as demonstrated by Y. Liu et al. in the use of Nano vectors for gene delivery and K. Siegrist et al. In the case of low density carbon nanotubes [4]. Apart from

this, nanoparticles own a functional surface which gives the nanoparticles the ability to bind, adsorb and carry other compounds thus, making them suitable for drug delivery. Besides this, the nanoparticles also protect the drug from degradation, enable a prolonged release of the drug, improve the bioavailability of the drug, reduce the toxic side effects of the drug and offer an appropriate form for all routes of administration [5]. Of these nanoparticles, noble metal nanoparticles are preferred due to their optical properties, non-toxicity and biocompatibility⁸ when compared to the other metals. Majorly, gold nanoparticles can convert light or radio frequency into heat, thus enabling the thermal ablation of the targeted cancer cells [6] [7]. This type of phenomenon has been observed for gold nanoparticles [8] [9]. Hence, the ability to combine drug delivery and photo thermal therapy on gold nanoparticle based delivery platforms prove it to be a system that is capable of eliminating the cancer cells [10]. One major drawback that arises while using gold nanoparticles, is its agglomeration during reduction from its metal salts. It has reported the importance of the control of nanoparticle size [11] as the increased particle size reduces the scope for their use in drug delivery since it is well known that particles with size ranging from 10 to 50 nm are the ones that are easily taken up by the cells [11]. Thus, an effective way to prevent the aggregation of gold nanoparticles is mandatory [12]. An effective strategy for this would be the use of a stabilizing agent or surfactant. The most promising and environment friendly stabilizing agents are enzymes [15] and polymers. The role of a stabilizing agent/surfactant is played by a chitosan¹⁸. Chitosan is a biopolymer that is biocompatible, biodegradable, eco-friendly, non-toxic and has NH₂ and OH groups which act as chelating sites for drugs (5-Fluorouracil in our case) and for other molecules. Moreover, as the combination of nanoparticles with Chitosan in the form of Nano composite matrices provide the high surface area required to achieve a high loading of enzymes, drugs, and a compatible micro-environment to facilitate stability as reported by Jay Singh et al., Chitosan is suitable for use as a drug delivery carrier. There are a

number of reports on the use of gold nanoparticles and Chitosan separately for drug delivery [13]. A Nano composite system consisting of both gold and Chitosan will encompass the properties of both the moieties, which makes it a powerful choice for use in the bio-compatible, targeted delivery and sustained release of 5-Fluorouracil (5-FU).

MATERIALS AND METHODS

The chicken chorioallantoic membrane (CAM) assay is a standard assay used to evaluate the angiogenic potential of biomaterials. Angiogenesis is the formation of new blood capillaries from existing blood vessels for supplying nutrients to the cells that are distant from existing blood vessels. Angiogenesis is a complex process that is mediated by the endothelial cells that line blood vessels. Angiogenesis is a regulated process involving a number of stimulators such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), angiopoietins, activators of integrins and inhibitors such as thrombospondin, angiostatin and endostatin.

5 Days Old Fertilized Hen Eggs, Pyruvic Acid (Cat No:107360, Sigma), NaCl (Cat No.RES0926S-A, Sigma), Parafilm (#380030, Tarsons), DMSO (#PHR1309, Sigma), D-PBS (#TL1006, Himedia), 50 ml centrifuge tubes (#546043 TORSON), 1.5 ml centrifuge tubes (TORSON), 1 ml serological pipette (TORSON), 10 to 1000 ul tips (TORSON).

Equipment's used Sterilized Sharp Forcep, 18-gauge hypodermic needle (#72-567, Harvard Apparatus), Camera (Canon), Pipettes: 2-10 μ l, 10-100 μ l, and 100-1000 μ l. Assay controls Negative Control (0.9% NaCl), Positive Ccontrol (1% w/v SDS).

The chickchorio-allantoic membrane (CAM) assay for screening the effect of test samples on angiogenesis was performed according to the method given by Ribatti and co-workers (1997). Fertilized white leghorn chicken eggs were collected from a local hatchery at day '5' and checked for the damage. The eggs were cleaned with 70 % ethanol and incubated under conditions of constant humidity at 37 $^{\circ}$ c. On the 3rd day of incubation a small hole was drilled at the narrow end and 2-3 ml of albumin was withdrawn with 18-gauge hypodermic needle. The window was sealed with transparent tape and again incubated. On the 7th day of incubation a small square window was opened in the shell and sterile gel foam (3mm \times 3mm \times 1mm) piece was implanted on top of the membrane. The vehicle control group was impregnated with sterile normal 0.9% NaCl, standard group was impregnated with 1% SDS and the test group was impregnated with IC50 Concentration. The eggs returned to the incubator and they were incubated undisturbed till 24hrs. After 24hours of incubation the eggs were removed from the incubator and the CAM tissues directly beneath each sponge was resected from control and treated CAM samples. The number of vessel branch points contained in a square region equal to the area of each sponge was counted and findings from 3 CAM preparations were analyzed for each treatment group. The resulting angiogenesis index is the mean of new branch points in each set of samples.

Chitosan Coated With Gold Nanoparticles

100 ml Solution (2 ml acetic acid + 98 ml water) + 0.34 gm. Chitosan (0.34 %) + 2 ml gold solution (5nM) + 2ml sodium borohydrate solution (50 mm). Stirred for 3 hours (room temperature). PH of the solution brought to pH 5.0 using NaoH. The Solution is centrifuged at 3500 RPM for 15 minutes to separate Chitosan coated with Gold Nanoparticles. The sample is dried at 50 $^{\circ}$ C overnight. *Nivethaaa et al., (2009).*

5-Fluorouracil Loaded Chitosan - Gold Nanoparticles

100 ml Solution (2 ml acetic acid + 98 ml water) + 0.34 gm. Chitosan (0.34 %) + 3.8 mm 5-Fluorouracil + 1.4 mm sodium polyphosphate + 0.5 % v/v Tween 80+ 2 ml gold solution (5nM) + 2ml sodium borohydrate solution (50 mm). Stirred for 3 hours (room temperature). PH of the solution brought to pH 5.0 using NaoH. The Solution is centrifuged at 3500 RPM for 15 minutes to separate Chitosan coated with Gold Nanoparticles. The sample is dried at 50 $^{\circ}$ C overnight. The conjugated nanoparticle is characterized by FTIR, UV, and TG/DTA.

RESULT AND DISCUSSION

FTIR Spectrum Of 5flurouracil Linked Gold Coated Chitosan

The FTIR spectrum of Chitosan coated gold nanocomposite exhibits an NH₂ twisting peak at ~1566.50 cm⁻¹, stretching at ~ 715.07 cm⁻¹, bending at 1404.11 cm⁻¹, a peak of NH₃ + at ~1661.48 cm⁻¹. These peaks correspond well to the peaks of pure Chitosan except for minor differences that establish the formation of the nanocomposite. The splitting of the NH₃ + and NH₂ peak increases with increasing the amount of gold in the nanocomposite. This is because of the neutralization of the protonated amine group. An enhancement in the intensity of the NH₂ peak on increasing the amount of gold is an indication of the increase in the percentage of gold, incorporated into the nanocomposite. The FTIR spectrum of 5-FU encapsulated nanocomposite is shown in figure 4. All these observations show the encapsulation of 5-FU to Chitosan coated gold nanocomposite.

TG/DTA Curve Of 5flurouracil Linked Gold Coated Chitosan

Thermal Analysis (TA) techniques study the properties of materials as they change with temperature. It provides information on properties like thermal capacity, mass changes, enthalpy, coefficient of heat expansion etc. It is widely used in solid state chemistry to study solid state reactions, thermal degradation reactions, and phase diagrams. The weight loss curve indicates changes in sample structure, heat stability, and other parameters. Differential Thermal Analysis (DTA) involves exposing the material under study and an inactive reference to similar heat cycles. Any temperature distinction amongst test and reference is recorded. In this procedure, the heat flow to the specimen and reference is kept constant instead of the temperature. The peak intensities are observed at 475.9 cells, 531.5Cel, 550.6 Cel.

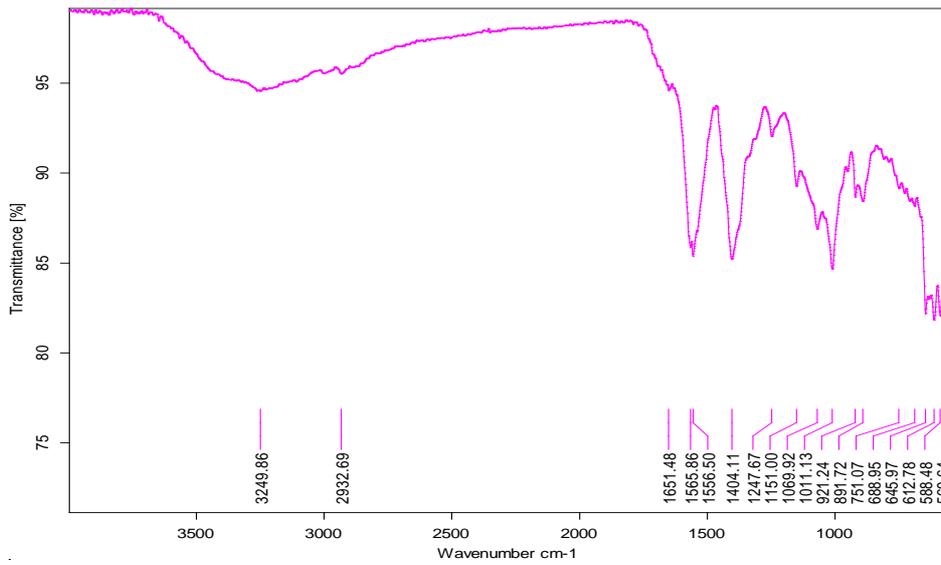


Figure 3.1. FTIR Spectrum of 5-Fluorouracil linked Gold coated Chitosan

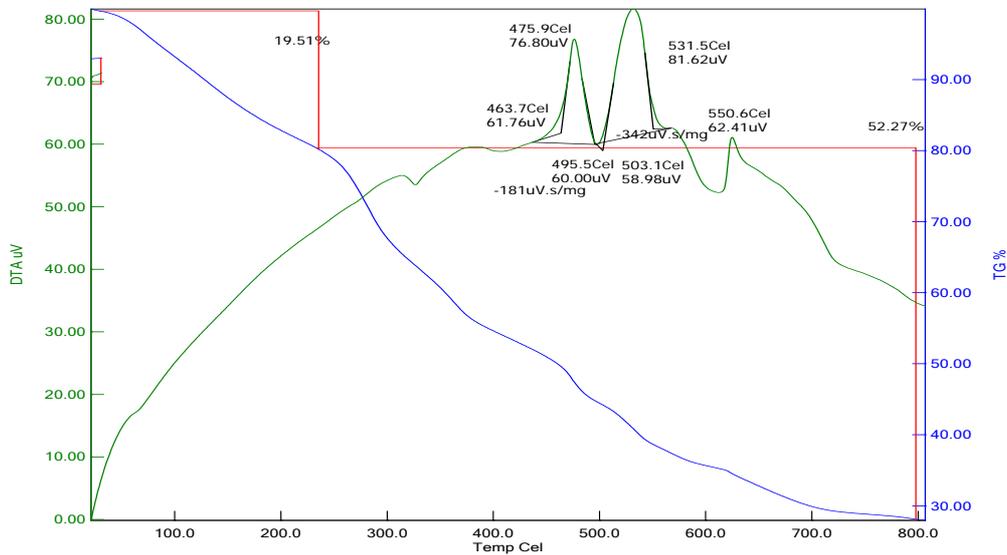


Figure 3.2. TG/DTA Curve of 5-Fluorouracil linked Gold coated Chitosan

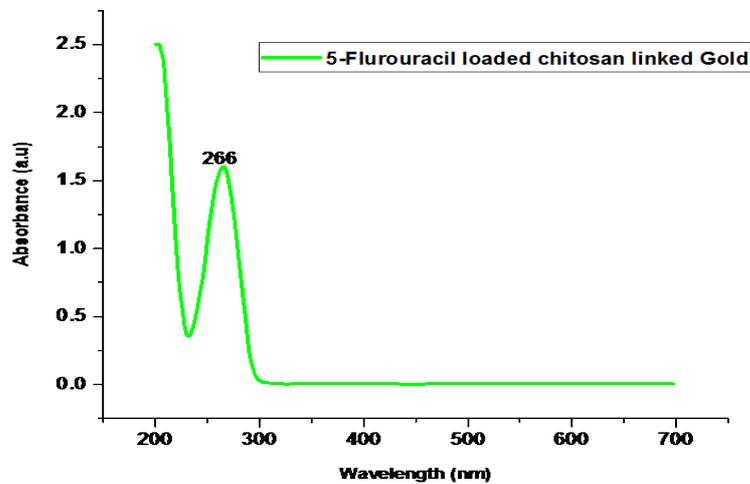


Figure 3.3. UV Spectrum of 5-Fluorouracil linked Gold coated Chitosan

UV Spectrum Of 5-Flurouracil Linked Gold Coated Chitosan

The UV-Vis spectra taken for the composite containing different weight percentages of gold is shown in figure 5. The UV-VIS spectra for the formation of gold nanoparticles was monitored and obtained. A single peak corresponding to the surface plasmon resonance of gold was observed in the wavelength range 210-300 nm. A peak in this range is generally attributed to the surface plasmon excitation of small spherical gold nanoparticles. The damped nature of the peak is indicative of the small particle size. In small particles, the mean free path of the electrons is reduced, which eventually leads to the peak dampening. The intensity of the peak increased with concentration of gold, which implies that the amount of gold binding to Chitosan increased.

Angiogenic Activity Of 5-Fluorouracil Loaded Chitosan And Gold By Chickchorio-Allantoic Membrane (CAM) Assay

Sample A (C+G) and Sample B (C+F+G) were evaluated to check the Angiogenesis Activity on the Fertilized Chick Eggs. The used Concentrations of the compounds and No of Branching Points resulted mentioned in the Table 1.

Sl.No	Test Compounds	Concentration
1	Untreated	NaCl (0.9%)
2	Standard	Pyruvic Acid
3	Sample A (C+G)	IC50 (uG)
4	Sample B (C+F+G)	IC50 (uG)

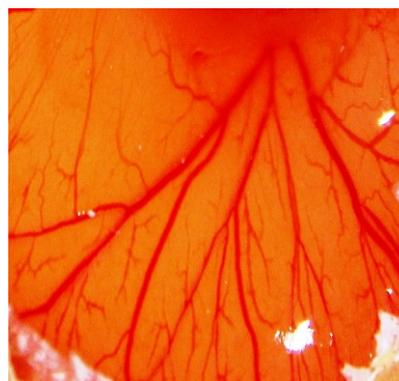
Table 1. Details of Drug Treatment to respective Fertilized Eggs.

CAM Study Of The Test Compounds Using Fertilized Hen Eggs

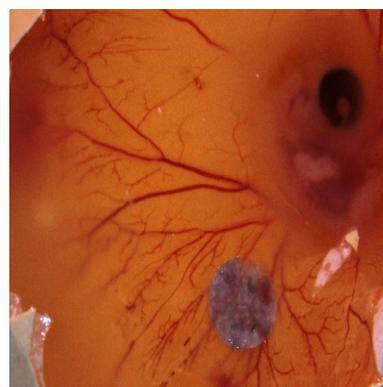
The Observations in Statistical data of CAM Study suggesting that on Fertilized Eggs, given Test Compound-A (C+G)) showing a slight increase in the growth of nerve branches with the blood vessel density 26 after the incubation of 24hours of the drug treatment. On the other hand, Test Compound-B (C+F+G)) showing a high increase in the growth of nerve branches with the blood vessel density 43 after the incubation of 24hours of the drug treatment compared to the Standard Drug showing 86% of Blood vessel Density. In this study, The angiogenesis index in the chick CAM was High in the Sample B Compared to the Sample A. The networks in CAM and the newly synthesized vessels participated actively in the circulating of blood cells. Taken together, the observations in the present study suggest that the given Test Compounds exhibit strong angiogenic actions and also may have the potential to be a useful activator of the large number of serious diseases characterized by deregulated angiogenesis.

Fertilized Hen Eggs is treated with Chitosan and gold nanocomposite under different samples, branching points count and blood vessel density is calculated for different samples. The result is tabulated in table 2. The blood vessel density is minimum at control and standard (86) and the

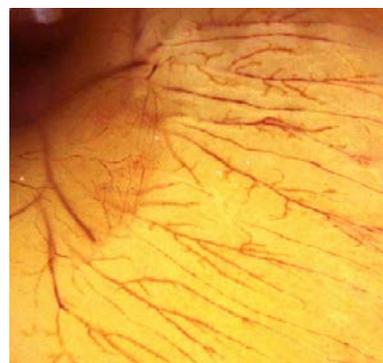
blood vessel density increase in the sample B (43.66666667).



Control



Sample A (C+G)



Sample B (C+G+F)



STD

Figure 3.4. Angiogenic activity of the drug under various samples.

Concentration Unit: uG/mL	Incubation: 24hrs			
Test	CONTROL	STD	Sample A	Sample B
Concentration	0.9% NaCl	1% SDS	IC 50	IC 50
No of Branching Points Count 1	76	168	102	121
No of Branching Points Count 2	78	161	104	125
No of Branching Points Count 3	81	164	108	120
Mean No of Branching Points	78.33333333	164.3333333	104.6666667	122
Blood Vessel Density	0	86	26.33333333	43.66666667

Table 2. Details of Drug Treatment to different samples and counting of blood vessels.

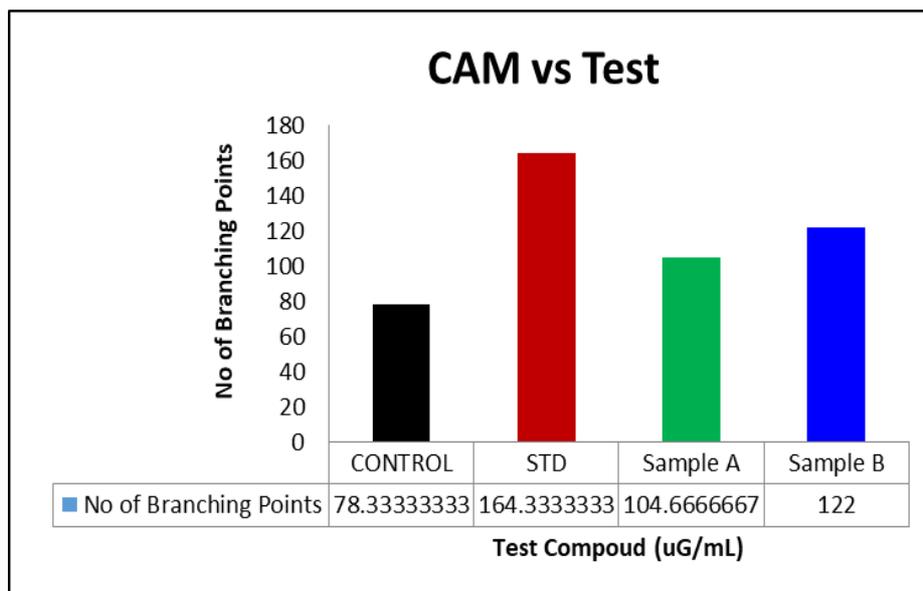


Figure 3.5. The Angiogenic activity of Chitosan loaded gold nanoparticles.

CONCLUSION

It was observed from the present investigation that, the angiogenic activity of 5-FU loaded chitosan nanoparticles provided a platform for treating cancer with biopolymer nanomaterials. Chitosan is safe to use as a carrier for the drug 5-FU, which was observed from the present investigation. This could contribute to the discovery of a new method for the treatment of various cell lines that may overcome the difficulties in the currently available procedures or therapies. The increased angiogenic activity was found even at lower concentrations of the drug. Thus synthesized nanoparticles were more efficient in the biomedical applications in cancer treatment for their high angiogenic activity.

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