Crystallised chitosan + Vitamin-E Coated Drug-Eluting Stents to Prevent Restenosis and Stent Associated Infections

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ABSTRACT

Drug-Eluting Stents (DES) was developed to reduce re-endothelialisation and thrombosis. Chitosan due to its biocompatible and biodegradable property, it is used in drug delivery and wound healing applications. Chitosan-Vitamin E (alpha-tocopherol) coated stents were prepared by seeding and crystallisation process. Drug release profile, anti-bacterial activity and biocompatibility were analysed. FESEM analysis of the coated stent evidenced the homogeneous coating of the drug-polymer mixture does not provide any surface space on the stent for the bacterial adhesion. Maximum bacterial reduction percentage was observed for biofilm-producing Staphylococcus aureus (96.6±1.04%). Escherichia coli and Pseudomonas aeruginosa showed bacterial reduction percentage of 93.3±2.51% and 93.6±2.08% respectively. Release profiles of crystalline chitosan from the coated stents indicated that the rate of chitosan release was sustained and constant with the release time. Chitosan coated stent samples showed significant biocompatibility indicated by the cell viability percentage of 96.6±1.04% when compared with the control samples (99.1±0.76%). To conclude, the drug-releasing phenomenon aided by the vitamin was correlated with its ability to prevent the formation of restenosis in atherosclerosis cases.

INTRODUCTION

Cardiovascular diseases are the primary cause of death, accounting for 20% of deaths globally (Roth et al., 1990). Atherosclerosis is the significant cardiovascular disease; deposition of fat in the coronary vessels results in a build-up of plaque, reducing the blood flow in the arteries causing symptomatic coronary artery disease. Repeated plaque formation causes total blockage of the artery leading to atherosclerosis (Rosenthal, 2014).

A stent implantation is considered as one of the successful methods for atherosclerotic cases Stefanini and Holmes (2013) by improving constant blood flow through arteries (Kastrati et al., 2007). This immediate treatment after a heart attack can be done by stent implantation, though it has some disadvantages. Hyperplasia occurs at the site of injured arterial blood vessels. These cells migrate and increase with the extracellular-matrix formation, which results in the development of neointimal tissue—this impact of the development of hyperplasia results in restenosis. About 35% of patients treated for atherosclerosis had re-occlusion within...
six months (Durgin and Straub, 2018).

Other treatments like bypass surgeries, atherec-
tomy and revascularisation process were consid-
ered to be the cost increasing factors (Jones et al.,
2014). To reduce the restenosis, the stents contain-
ing drugs are developed to reduce the formation of
neointimal tissues known as drug-eluting stents
(DES), and they were applied clinically (Mongrain
and Rodés-Cabau, 2006). Drug-Eluting Stents (DES)
was developed to reduce re-endothelialisation and
thrombosis. Sirolimus and Paclitaxel drugs are used
commonly for coating on the stent surface. These
drugs reduce the complications like the growth of
neointimal tissue, and restenosis (Honda, 2009).

Heparins are anti-coagulant agents preferentially
used for angioplasty cases for the prevention of
restenosis. He et al. (2019) synthesised differ-
ent heparin-like polymers; among them, chitin
derived chitosan having good biocompatibility and
biodegradability was analysed for its anti-coagulant
properties. In support, Wang et al. (2018) earlier
reported that chitosan has an excellent performance
in anticoagulation with a similar backbone structure
to that of heparin. Zheng et al. (2011) stated that
chitosan could also promote the growth of endothelial
cells.

Based on this biological and medical significanc,
the heparin-like chitin derived chitosan was investi-
gated for the efficacy of preventing restenosis in the
present research. This approach was carried out for
the first time by coating chitosan onto metal stents,
and the drug-releasing behaviour was studied using
vitamin E as a drug carrier. Tocopherol acetate
(Vitamin E) has been approved by FDA as a safe adju-
vant and widely used for different applications in
drug delivery like high biocompatibility, antimicro-
bial activity, and improvement of drug permeation,
antitumor activity and enhancement of drug solubil-
ity.

In the account of its anti-bacterial nature against
several Gram-Positive and Gram-Negative bacte-
rial species, Apart from the anti-coagulant prop-
erties of chitosan, it has high bio-degradability (Shi
et al., 2006) and antimicrobial property (Acharya
et al., 2005). Another significant risk associ-
ated with the coronary stents is their high infec-
tion rate due to biofilm-forming organisms like
Staphylococcus aureus and Staphylococcus epider-
midis. The patients’ proteins play a significant fac-
tor in biofilm formation leading to associated stent
infections (Mack et al., 2004).

Based on these therapeutic applications of chi-
tosan, prevention of restenosis and biofilm for-
mation was considered as the main objectives in
the present research. Chitin derived chitosan was
earlier described in our previous studies. Thus
extracted chitosan was mixed with vitamin-E and
coated onto stainless steel fabricated metal stents.
The coated stents were analysed for drug release,
anti-bacterial activity and biocompatibility.

MATERIALS AND METHODS

The present research work was carried out in the
Department of Microbiology, Annamalai University,
Chidambaram, Tamil Nadu, India. The work was
done from November 2019 to February 2020. High-
quality medical grade type 304VSS stainless steel
material (Sigma SS straight length wire #0.018)
was commercially procured and used as a coronary
stent. The stents were coated with chitosan and
Vitamin-E (alpha-tocopherol) using a modified dip-
coating method as described below.

Coating the stents with Chitosan and Vitamin-E
The stents are coated with chitosan, and a drug-
carrier called alpha-tocopherol (Vitamin-E). Two
different phases of the coating were carried out for
the effective and constant release of drugs from the
stent surface. Seeding is the initial coating process
followed by crystallisation of drugs and carriers on
the surface of the stent (Su et al., 2019).

Seeding of the stent surface with chitosan
For seeding, 1g of chitosan was prepared as
described in our previous studies (Amrutha et al.,
2018) was mixed with 5ml of n-Hexane (Sigma-
Aldrich) and sonicated (amplitude 60 for 15min
and then for 2-5min at amplitude 100) until homo-
geneous dispersion of the chitosan in hexane was
formed. After sonication, stents were mounted on
shrinkable tube placed on a needle. The needle
loaded with a stent was placed at the centre of the
vial containing the dispersed chitosan in hex-
ane (one stent per trial). All the vials were kept in
an ultrasonic bath (Shimadzu) for 10min at 30°C
to form a seeding layer. Stents were gently taken out
of the vial and allowed to dry at room temperature.
The dried and seeded stents were used for the crys-
tallisation process in the next step.

Crystallisation of chitosan and vitamin E on stent
surface
Over the seeded layer, a secondary layer using chi-
tosan with a drug carrier (alpha-tocopherol) was
coated as crystals on the surface. For crystallisa-
tion, 1g of Chitosan and 1g of Vitamin-E (alpha-
tocopherol) was weighed and dissolved in 3ml of
ethyl acetate. This solution was transferred to 20ml
screw-capped glass tube filled dropwise with 5ml n-
hexane to form a homogenous solution. Stents were
placed in this solution at 25°C/5 min for crystallisation of chitosan-vitamin E (CV_E) on the seeding layer to form crystal-carpet formation. The stents were dried at room temperature under strict sterile conditions and stored at refrigeration temperature before testing.

**FESEM analysis of chitosan-Vitamin E coated stents**

The crystallised chitosan-Vitamin E mixtures coated on the stent surface was observed using Field-Emission Scanning Electron Microscopy (FESEM). The topographic analysis was also used to identify the uniform coating of the mixtures on the stent surface. Chitosan-Vitamin E crystals on the stent were observed under the magnification of 6000X.

**Quantitative anti-bacterial activity of chitosan-Vitamin E coated stents.**

Anti-bacterial activity of chitosan-Vitamin E coated, and bare (uncoated) stents were quantitatively measured using standard bacterial adherence test against test organisms. The coated materials were placed separately in a tube with 5ml of each of the test bacteria and incubated at 37°C for 18h. During the incubation period, the bacterial cells adhere to the surface. The stent materials were separated and washed to remove the non-adherent cells.

The washed pieces were sonicated for 30 seconds to dislodge the sessile adherent. After sonication, a serial dilution of the sonicated saline was made, and the number of sessile bacteria was detected to determine the degree of adherence by viable count technique. The similar experimental set up was run in parallel for bare stent materials. Chi-square method is used to estimate the number of adhered cells on both coated and bare stents (Elayarajah et al., 2011).

The percentage reduction of adhered organisms on the coated materials was determined using a standard percentage reduction formula.

\[
\text{Bacterial reduction (\%) = } \frac{A - B}{A} \times 100
\]

Where, A = number of adhered organisms (in CFU) obtained from the bare stents,

B = number of adhered organisms (in CFU) obtained from the coated materials.

**Statistical evaluation of total viable bacteria**

The chi-square test can determine the effect of an anti-bacterial drug on bacterial adherence. The difference in the bacterial reduction percentage between the chitosan-vitamin E coated and bare stents was statistically calculated with P< 0.05 considered significant.

**Drug releasing efficacy of coated stents for the prevention of restenosis using High-performance liquid chromatography**

The efficacy of chitosan concentration released from the coated drug-eluting stents was analysed using High-performance liquid chromatography (HPLC) with a known standard. The release profile of chitosan from the biodegradable polymer matrix for a period of 120hours was studied under simulated biological conditions. Standard solutions were prepared by dissolving 10mg of crystallised CV_E drug in 10ml of the mobile phase. This mobile phase was then diluted up to 100ml. Diluted 20µl standards were injected in the HPLC column, and standard chromatogram for this standardised solution was obtained. For in-vitro kinetics study, stents were incubated in 50 ml of phosphate buffer saline (PBS – pH 7.0) solution at 37°C with constant agitation at 300 rpm in a thermal mixer. Each CV_E coated stents were removed at 30min, 1, 2, 4, 8, 12, 24, 48, 72, 96 120hours from their release vials and analysed for the amount of drug release in PBS. The experiment was carried out at room temperature. Chitosan was extracted using dichloromethane, which was later evaporated using dry nitrogen gas. The mobile phase was added to this, and the resultant supernatant was analysed for drug content by High-performance liquid chromatography (HPLC). The chitosan released at regular interval from each stent was calculated (Ankur et al., 2007).

**Biocompatibility test of chitosan-Vitamin E coated stents**

The Chitosan-Vitamin E coated stents were tested for its biocompatibility using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

MTT assay is used to evaluate the in-vitro biocompatibility of polymeric components as it is a quick, effective method for testing mitochondrial impairment and correlates quite well with cell proliferation. It is based on the use of tetrazolium salt 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide (MTT), which can be converted to an insoluble blue formazan product by mitochondrial enzymes in viable cells.

L_229 fibroblast cell line is often used to evaluate cytotoxicity/biocompatibility of any drugs and drug-coated medical devices. The fibroblast cell lines were cultivated in 12-well-microtitre plates to reach confluence growth. The samples were applied directly to the developed fibroblast monolayer. The specimens immersed in 1ml Dulbecco’s modified eagle medium (DMEM) fibroblast medium in 24-well plates for 2hours in an incubator at 37°C. The
specimens were then seeded with L929 fibroblast cell line at 10,000 cells per well according to routine cell-culture methods. The plates were incubated at 37°C and 5% CO₂ for fifteen days. Well, without drugs were included in this study. The effect of drugs in the samples on the biocompatibility of fibroblast was evaluated using the photometric MTT assay. At each time point, samples were taken from the 24-well plates and transferred into new plates for the MTT study. The MTT solution was prepared by dissolving the powder in phosphate-buffered saline at a concentration of 1 mg/ml. After 1hr of incubation, the purple crystals were dissolved by adding sodium dodecyl sulphate (SDS) in a 1:1 mixture of water and dimethylformamide (DMF) at a concentration of 20% w/v. After adding 1ml of MTT medium (0.0005mg/ml) to each well, the plates were incubated for 3hrs, rinsed and desorbed in 100ul of 70% isopropanol. After being agitated rapidly at 400rpm/min for 40min, the dyed medium was transferred to 96-well plate and read at 550nm. The biocompatibility or cell viability is expressed as a percentage of the control sample (100%) (Budman et al., 2012).

RESULTS AND DISCUSSION

FESEM analysis of chitosan-Vitamin E coated stents

The stents were coated with Chitosan-Vitamin E particles, and the topographical analysis was carried out using FESEM analysis. In our previous studies, only the surface coating of the stents with chitosan-Vitamin E particles was presented; despite the crystallised forms of chitosan on the coated stents were significantly evident during the present research work. The present analysis revealed the presence of crystallised drug particles on the stent surface; exhibiting adherence to the greatest possible extent. Chitosan coatings as large uniform and continuous layer of parallelogram shapes on the stents were observed (Figure 1). The coatings in crystal forms also increased the thickness of the stent. FESEM analysis of the coated stent also evidenced that the homogenous coating of the drug-polymer mixture does not provide any surface space on the stent for the bacterial adhesion or biofilm deposition. This was mainly by reducing the depressions on the material surfaces by the crystallised deposition of chitosan on the stent. The obtained results were found supportive when compared to the results of Basalus et al. (2009). The surface charge of anti-bacterial coatings was found to be effective in reducing bacterial adhesion and biofilm formation.

Quantitative anti-bacterial activity of Chitosan-Vitamin E coated stents

Anti-adherent activity for each coated stent materials was analysed using bacterial adherence test. The anti-adherent activity was calculated by bacterial reduction percentage. The anti-adherent activity of surface-modified stent materials against the test organisms was concentration-dependent as the reductive effect of drugs and carriers was in the range of 93.0% to 96.6% (Table 1).

Bacterial reduction percentage calculated from the CFU (colony forming units) of the Chitosan-Vitamin E coated stents against the test organisms was measured. Maximum bacterial reduction percentage was observed for the high biofilm producer, Staphylococcus aureus (96.6±1.04%). Other cultures also showed significant reduction percentage. Escherichia coli and Pseudomonas aeruginosa were reduced up to 93.3±2.51% and 93.6±2.08% respectively. Using chi-square statistical analysis, the effect of an anti-bacterial drug on bacterial adherence was determined. The difference in bacterial reduction percentage of Chitosan-Vitamin E (CV_E) coated stents and bare stents were calculated with P<0.05 considering significant. For all the test organisms, the calculated

\[
\text{Cell viability} = \frac{( \text{Treated}/\text{Control} ) \times 100}
\]
value (5.23, 5.46 and 4.95) was less than the table value (14.06). Since the calculated value was less than the table value, the assigned hypothesis was accepted.

Chitosan is a natural polysaccharide widely known for its inhibitory activity against a wide range of microorganisms. The presence of charged groups in the polymer and their ionic interactions with the bacteria cell wall constituents causes a drastic effect to microorganisms. This interaction leads to hydrolysis of the peptidoglycans in the microorganism wall, provoking the leakage of intracellular electrolytes which results in the death of the microorganism. Chitosan was found to inhibit the growth of both Gram-positive and Gram-negative microorganisms. Similarly, the effect of chitosan-coated stents on microorganisms was determined by Prasanth and Saravanakumari (2017). Anti-bacterial activity of the drug-eluting stents coated with 1X and 2X rapamycin showed significant inhibitory zones against the biofilm-producing test organisms. The inhibitory zones ranged from 18.9mm to 31.3mm during the analysis. The obtained inhibitory zones for 2X coated drug-eluting stents were found to be high when compared to that of stents coated with 1X concentration. Govindarajan et al. analysed the anti-bacterial activity of kanamycin-chitosan nanoparticles (KMCSNPs) on catheters (Kumar et al., 2016). The surface-modified stents showed significantly increased anti-bacterial activity against Escherichia coli MTCC-729 and Proteus mirabilis MTCC 425 relative to the surface of an unmodified stent.

**Drug releasing efficacy of drug-eluting coated stents**

* In vitro release study was conducted on developed Chitosan+Vitamin-E crystallised stents. Release concentration of Chitosan in PBS at a specific temperature was determined. The release study was conducted for 120h in PBS at 37°C. Release profiles of crystalline chitosan from the coated stents indicated that the rate of chitosan release was sustained and constant with the release time (Figure 2). The lag phase exhibited an initial burst effect from 0.5h to 4h (45μg, 55μg, 55μg and 55μg). Followed this lag phase, an increase in drug concentration was observed from 8 hours to 48h (85μg, 100μg, 105μg and 110μg). In PBS at pH 7.0, the hydrophilic polymer, Vitamin-E undergoes degradation during the log phase. Due to the rate of polymer degradation, the release of drugs was facilitated at a higher rate than the initial burst level concentration. During this phase, the release concentration of chitosan remained almost constant (115μg, 120μg and 120μg) from 72h to 120h, indicating the sustained rate of drugs from the coated stents (Table 2).

| S. No | Test organism                  | Bacterial reduction (%) | Bare stents | CVE coated stents* |
|-------|--------------------------------|-------------------------|-------------|-------------------|
| 1.    | *Escherichia coli*             | 0                       | 93.3±2.51  | 93.3±2.51         |
| 2.    | *Staphylococcus aureus*        | 0                       | 96.6±1.04  | 96.6±1.04         |
| 3.    | *Pseudomonas aeruginosa*       | 0                       | 93.6±2.08  | 93.6±2.08         |

* P<0.05
Table 2: Drug releasing efficacy of Chitosan-Vitamin E coated stents

| S. No. | Time (hours) | Drug release concentration (μg/ml) |
|--------|--------------|-----------------------------------|
| 1      | 0.5          | 45                                |
| 2      | 1            | 55                                |
| 3      | 2            | 55                                |
| 4      | 4            | 55                                |
| 5      | 8            | 85                                |
| 6      | 12           | 100                               |
| 7      | 24           | 105                               |
| 8      | 48           | 110                               |
| 9      | 72           | 115                               |
| 10     | 96           | 120                               |
| 11     | 120          | 120                               |

Table 3: Biocompatibility of Chitosan-Vitamin E coated stents

| S. No | Samples           | Cytotoxicity % | L929 Cell lines – MTT assay | Biocompatibility       |
|-------|------------------|----------------|-----------------------------|------------------------|
| 1     | CV_E coated stents | 0.35±0.25      | 99.6±0.57                   | No cytotoxicity (Biocompatible) |
| 2     | Control          | 0.83±0.76      | 99.1±0.25                   | No cytotoxicity (Biocompatible) |

This release of rapamycin significantly inhibited the growth of smooth muscles and platelet adhesion in the heparin-coated group than the uncoated heparin group; thus proving the prevention of restenosis and stent thrombosis. In another study, Elayarajah et al. (2011) analysed that cyclodextrin as a carrier was used for effective drug delivery mechanisms. Due to their ability to form a complex with drugs, it can act as functional carrier material and also in formulations of new drug mixtures.

These properties proved that cyclodextrin could serve as a potential carrier for effective and constant drug delivery at the targeted site. The use of tocopherol acetate (vitamin E) as a polymer in the present research offers the possibility to place hydrophilic drugs on the surfaces of hydrophobic stents, building up a slow-release drug delivery system independent of the drug charge. This slow-release drug delivery system containing crystallised chitosan-Vitamin E mixtures from stent surface could have a cytostatic effect on the neointimal growth, which is desirable in the later stages of vascular healing. Protective polymer (vitamin) in the drug-polymer mixture being hydrophilic would degrade within a short time in a vascular environment. Crystallised chitosan-Vitamin E from the coated stents slowly gets diffused into the surrounding tissues, thus preventing restenosis (Puranik et al., 2013).

Biocompatibility of Chitosan-Vitamin E coated stents

The cell viability percentage was found to be highly similar and significant (99.6±0.57%) when compared to that of control (99.1±0.25%): indicating the biocompatible properties of CV_E coated stents (Table 3). Also, no significant difference in the morphology of the L929 fibroblast cells after 24 hours of cell culturing in the cell culture media (DMEM) was evident when compared to the control samples (Figure 3).

The following literature cited observations were found to be significantly supportive of the results of our present research. To overcome the re-endothelialisation and to promote the healing, coat-
ing of chitosan-heparin in coronary stents was investigated by 

Joner et al. (2006). During the second week of observation, the growth of neointimal tissues was observed on bare-metal stents, whereas the growth of intimal tissues was integrated on the coated stent surfaces. No inflammation response was recorded in coated stents, indicating the significant biocompatibility of the coating. Figrianti et al. investigated the effect of Poly l-lactic acid with chitosan-coated on coronary stents, Figrianti et al. (2018). Cytotoxicity test was carried out by MTT Assay method. Results revealed the cell viability of the test samples in this experiment reached 90%, which is considered to be non-toxic and safe to use in the human body. And hence, as chitosan shows high cell viability, significant biocompatibility and gradual degradation it can be used for various applications like tissue engineering, wound healing, drug delivery and gene delivery systems (Mi et al., 2001).

**CONCLUSION**

As chitin derived chitosan has good biocompatibility and biodegradability, the anti-coagulant properties of chitosan were studied in the present research. To prevent restenosis due to atherosclerosis and biofilm-associated infection, chitosan-vitamin E coated drug-eluting stents were designed. The coating of the stent was carried in a two-step process: seeding and crystallisation for the effective uniform coating on the stent surface. FESEM analysis of the coated stent revealed the presence of crystallised drug particles in large uniform and continuous layer of parallelogram-shaped. Maximum bacterial reduction percentage during quantitative anti-bacterial assay showed that the homogenous crystal coating of the drug-polymer mixture does not provide any surface space on the stent for the bacterial adhesion. Release profiles of crystalline chitosan from the coated stents indicated that the rate of chitosan release was sustained and constant with the release time. Chitosan coated stent samples showed significant biocompatibility indicated by the cell viability percentage of 96.6±1.04% when compared with the control samples (99.1±0.76%). The researchers emphasise that the drug-releasing characterisations of the stents could able to prevent the biofilm formation and restenosis. However, this should be studied in detail with more optimised conditions in future.

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**Conflicts of Interest**

The authors declare that they have no conflict of interest for this study.

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