



CHITOSAN AS A COATING AGENT ON THE DRUG ELUTING STENTS TO PREVENT BIOFILM FORMATION DURING STENT IMPLANTATION

Amrutha M¹, Uma^{2*} and P. Sivagurunathan²

¹Research scholar, Department of Microbiology, Annamalai University, Chidambaram, India

²Associate Professor, Department of Microbiology, Annamalai University, Chidambaram, India

*Corresponding Author Email: m.amrutha1980@gmail.com

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ABSTRACT

Arteriosclerosis is the build-up of fatty cells and tissues within blood vessels. Coronary stents implantation is considered as a common method for atherosclerosis cases. Formation of neointimal tissue and high infection rate are the major complications found in stent implantation. The delivery of drugs directly to the site of vascular injury via polymer-drug coated stents can retard the organisms responsible for biofilm formation. Chitosan is widely-used as an antimicrobial agent due to its high biodegradability, nontoxicity and antimicrobial properties. Vitamin E has been approved by FDA as a safe adjuvant and widely used in drug delivery systems. Thus the main objective of this research is to prevent biofilm formation by drug-carrier (Chitosan-vitamin) mixtures. Biofilm formation assay was done using Microtitre Plate method for the determination of biofilm producing organisms. Chitosan was extracted from exoskeletons of crab shells. The stents were prepared and seeded with lyophilized chitosan and a carrier. FESEM analysis and Anti-biofilm assay were done for image of uniform coating in stents and anti-bacterial activity. The inhibitory zones ranged from 32mm to 30mm for drug-carrier coated stents were observed during the analysis. The maximum inhibitory zone of 32mm against high biofilm producing *Escherichia coli* were observed on coated stents. The Chitosan coated stents slowly gets diffused into the tissues preventing Biofilm formation. Thus the chitosan-vitamin coated stents are considered as a novel biomedical product which prevents biofilm formation.

Keywords: Drug-carrier mixture, Chitosan, Vitamin-E, Stent implantation, Anti-biofilm assay

INTRODUCTION

The leading cause of death world-wide; accounting for 30% of all death is due to cardiovascular diseases¹. Arteriosclerosis is the major causes of cardiovascular disease. The build-up of fatty cells and tissues within blood vessels results in arteriosclerosis. The build-up of fatty cells and tissues is not detected until complete blockage occurs which prevents the flow of blood leads to heart attack, stroke and sometimes death. Thrombosis, Restenosis or Re-occlusion and Biofilm formation are the common complications associated with arteriosclerosis. All these complications were reported during or after coronary stent implantations (Angioplasty) to remove fatty cells and tissues (plaque) within blood vessels².

Coronary stents implantation is considered as a common method for atherosclerosis cases³. Stent implantation results in arterial injury and vascular smooth muscle cells are further activated. These cells migrate and proliferate with extracellular-matrix formation resulting in the development of neointimal tissue, which leads to restenosis⁴. Certain growth factors with cellular or acellular elements and the interaction of cytokines was considered as the pathophysiology of restenosis⁵. About 35% of even successfully treated atherosclerotic lesion was reported to re-occlude within 6 months. Systemic approaches, including anticoagulation, antiplatelet therapy, antispasm therapy, lipid alterations, and anti-inflammatory therapy, have failed to reduce the restenosis rate⁷. Revascularization process, bypass surgeries and atherectomy were considered to be the cost increasing factors⁶.

Prevention of restenosis in arteriosclerosis cases were reported to be failed due to lack of essential drug concentrations at the

target site⁸. Drugs should be delivered at the stent implanted site. This can be done by delivering medication directly to the site of vascular injury via polymer-drug coated stents⁹. These stents are also known as drug-eluting stents. Inhibition of vascular smooth muscle cell proliferation can be done by drug eluting stents coated with different drugs like sirolimus, chitosan and paclitaxel under *in vitro* and *in vivo* conditions¹⁰. Another major risk associated with these stents is their high infection rate. *Staphylococcus aureus* and *Staphylococcus epidermidis* are the biofilm producing pathogens that colonize the implants in patients by adhering on their own proteins causing infection¹¹.

Chitosan, an aminopolysaccharide biopolymer, has a unique chemical structure as a linear polycation with a high charge density, reactive hydroxyl and amino groups as well as extensive hydrogen bonding. It displays excellent biocompatibility, physical stability and processability. Owing to its high biodegradability, and nontoxicity and antimicrobial properties, chitosan is widely-used as an antimicrobial agent either alone or blended with other natural polymers¹². D- α -tocopheryl polyethylene glycol succinate (Vitamin E TPGS or TPGS) has been approved by FDA as a safe adjuvant and widely used in drug delivery systems. The biological and physicochemical properties of TPGS provide multiple advantages for its applications in drug delivery like high biocompatibility, enhancement of drug solubility, improvement of drug permeation and selective antitumor activity. Notably, TPGS can inhibit the activity of ATP dependent P-glycoprotein and act as a potent excipient for overcoming multi-drug resistance (MDR) in tumor¹³.

The main objective of this research is to prevent bacterial infection during stent implantation in arteriosclerosis, A

preliminary attempt was made whether the drug-carrier (Chitosan-vitamin) mixtures could retard the biofilm formation. Coating of drug-carrier (Chitosan-vitamin) mixtures was done. Efficacy of chitosan release concentration from the coated drug-eluting stents for the prevention of biofilm formation, anti-biofilm assays was conducted.

MATERIALS AND METHODS

Procurement of biofilm producing bacterial cultures

Biofilm producing bacteria were purchased from Microlab, Coimbatore, India. Aerobic test organisms like (*Staphylococcus epidermidis*, *Proteus mirabilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*) have ability to produce biofilm. The strains selected for the study was mainly based on their ability to surface colonize on the stent materials. Each culture was subcultured and maintained in their selective media and it was reconfirmed by staining technique.

Biofilm determination of the challenge organisms: MTP method¹⁴

A microtiter plate procedure is an indirect method for estimation of bacteria in situ and can be modified for various biofilm formation assays¹⁴. A Biofilm forming ability of each test organism is evaluated using the microtitre plate method described by Christensen et al. In brief, all the test organisms (*Staphylococcus epidermidis*, *Proteus mirabilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*) were allowed to grow in the 96 well titre plates for 48 hours. All the wells are washed with double distilled water and added crystal violet dye to evaluate the remaining adhered organisms. These adhered organisms were considered to be as the biofilm growth on the inner surface of plate. Added About 100µl of 90% ethanol was added to each stained well. After incubating the plates, the optical density of the stains in ethanol layer are measured. Test organisms having optical density of 600nm wavelength were considered as biofilm producers which were read using ELISA reader (Braun, Germany). Based on the OD value, the adherence of organism in the silicate tubes and titre plates were classified as mentioned in Table: 1.

Extraction of Chitin and Chitosan¹⁵ (using a standard acetylation)

Extraction of chitin and chitosan: The chitin and chitosan sequence involved the crushing and washing of the discarded exoskeletons as described by Kim *et al.*, 1999¹⁶ and by the Sonat Corporation¹⁷. The crabs exoskeletons were placed in 250 ml beakers and treated in boiling sodium hydroxide (2% and 4% v/v) for one hour in order to dissolve the proteins and sugars thus isolating the crude chitin. Since there was little knowledge about what to expect from the 2% and 4% sodium hydroxide (NaOH) concentrations, the criteria established to assess the best results between the two concentrations were simply looking for any visible physical change such as colour and/or texture. Based on the fact that both sodium hydroxide concentrations yielded no visual physical change in the crab exoskeleton, the 4% NaOH was selected for use in the chitin preparation, which is the concentration used by the scientists at the Sonat Corporation¹⁷. After the samples were boiled in the sodium hydroxide, the beakers containing the crab shell samples were removed from the hot plate, placed in the hood and allowed to cool for 30 minutes at room temperature. The exoskeletons were then further crushed to pieces of 0.5-5.0 mm using a Hamilton Beach, 7-speed blender.

Deminerlization: The grounded exoskeletons were divided into 4 sub-crab samples weighing approximately 25 g each. Each sub-sample was deminerlized with 100 ml of HCl using concentrations 0.5% or 1.0% and the remaining two samples with 5% or 10% acetic acid (CH₃COOH) concentrations. The samples were allowed to soak for 24 h to remove the minerals (mainly calcium carbonate). The deminerlized crab shell samples were then treated for one hour with 50 ml of a 2% NaOH solution to decompose the albumen into water soluble amino-acids. The remaining chitin was washed with deionized water, which was then drained off. The chitin was further converted into chitosan by the process of deacetylation.

Deacetylation: The deacetylation process was carried out by adding 100 ml of 50% NaOH to each sample and then boiled at 100°C for 2 h on a hot plate. The samples were then placed under the hood and cooled for 30 min at room temperature. Afterwards the samples were washed continuously with the 50% NaOH and filtered in order to retain the solid matter, which is the chitosan. The prepared chitosan was then placed in 250 ml beakers and labelled according to the treatment used. The samples were then left uncovered and oven dried at 120°C for 24 h. The chitosan was then in a creamy-white form. The moisture percentage of the crab shell samples was then evaluated.

Procurement of commercial Metal stents (Implant)

The metal stent like implant was fabricated using SS mesh in the present study. The mesh were fabricated and designed like angioplast stent with the standard size. The developed stents were further subjected for coating with chitosan and carrier (Vitamin E).

Seeding catheter surface with lyophilized chitosan and a carrier

Process is composed of two steps (seeding and crystallization). For seeding, 50 mg of chitosan was weighed, grinded for 3 min and 1.6 mg of grinded chitosan was transferred into 5ml glass vial. 4ml of n-Hexane (Sigma-Aldrich) was added to this vial and sonicated (amplitude 60 for 15min and then for 2-5min at amplitude 100) until homogeneous dispersion of Chitosan in hexane was formed. After sonication, stents were mounted on shrinkable tube placed on needle and needle loaded with stent was placed at the centre of the vial containing the dispersed Chitosan in hexane (one stent per trial). These vials were then placed in the ultrasonic bath (Shimadzu) for 10 min at 30°C to form seeding layer. Stents were gently taken out of the vial and allowed to dry at room temperature. These dried and seeded stents were preceded to the next crystallization step.

Crystallization of lyophilized chitosan on seeded implant surface

For crystallization, 50mg of Chitosan and 1% of Vitamin-E (alpha tocopherol) as drug carrier was weighed and dissolved in 3 ml of ethyl acetate. This solution was transferred to 100 ml glass tube and this tube was filled drop wise with 65ml n-hexane to form homogenous solution. Stents were placed in this solution at 25°C/5 min for crystallization of Chitosan on seeding layer to form crystals carpet formation, and then dried overnight.

FESEM analysis Topographical analysis of coated and uncoated Implants

The surface coatings of the drugs and carrier on stent materials were observed using Scanning electron microscopy. SEM evaluation was also used to know the uniformity of coating of chitosan-vitamin mixtures over the stents. The topographic analysis of coated and uncoated stents was prepared for SEM using a suitable accelerating voltage (10 KV), vacuum (below 5 Pa) and magnification (X 3500). Metal coating was used as the conducting material to analyze the sample.

Qualitative Anti-biofilm assay¹⁸ (antibacterial activity)

Qualitative antibacterial activity of drug-eluting stents was performed as per the method described by El Reheby et al., 2009¹⁸. This method was also called as slurry dip-coating technique. The stents are coated with chitosan under controlled sterile conditions. The pre-measured size of 5mm in length was selected for coated and uncoated preparations (drug and carrier coated and uncoated materials). All the materials were washed in phosphate buffered saline (PBS) to remove any surface accumulation of drug. All test materials were placed on the surface of Nutrient agar (Hi Media) plate which was seeded with overnight broth culture of each test organisms (*Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*). All the plates were incubated at 37°C for 24h to observe the zone of inhibition around the coated stents. The antibacterial activity of each coated stents was expressed as the diameter of the zone of inhibition.

RESULTS

Biofilm determination of the challenge organisms: MTP method

In Table: 1, the optical density (OD) values and biofilm index of the test organisms were presented based on the biofilm classification described by Christensen *et al.*, (1985)¹⁹. Out of five test organisms, three showed significant biofilm index value indicating as strong biofilm producers. The test organisms considered strong biofilm formers in MTP assay were *Staphylococcus aureus* (0.292), *Escherichia coli* (0.250) and *Pseudomonas aeruginosa* (0.194). The differences in OD values observed during the analysis were due to the amount of crystal-violet (dye) absorbed by the test organisms in the microtitre well. Appropriate control was maintained throughout the test.

Table: 1 Screening test bacteria for biofilm formation by MTP method

Test Bacteria	Biofilm formation (OD 570nm)*	Biofilm index
<i>S. epidermidis</i>	0.288	High
<i>S. aureus</i>	0.291	High
<i>E. coli</i>	0.260	High
<i>P. mirabilis</i>	0.265	Moderate
<i>P. aeruginosa</i>	0.194	Moderate

Table: 2 Anti-bacterial activity of Carrier coated and Drug carrier coated stents against test organisms

S. No	Test organism	Inhibitory zones (mm)	
		Carrier coated	Drug carrier coated
1.	<i>Escherichia coli</i>	24	32
2.	<i>Staphylococcus aureus</i>	21	31
3.	<i>Pseudomonas aeruginosa</i>	22	30

FESEM analysis Topographical analysis of coated and uncoated Implants

This study describes a novel coating methodology for therapeutic agents using chitosan-vitamin mixtures. The drug-carrier coatings were evenly applied on the material surface through treatment. Stents were coated and topographical analysis was carried out using SEM. Representative SEM images of chitosan-vitamin coatings on the stents exhibits extremely large uniform and continuous layer in stents. The difference in the morphology of uncoated and coated stents was clearly evident in Figure 1a and Figure 1b respectively.

Qualitative Anti-biofilm assay (antibacterial activity)

The diffusing ability of the chitosan from the drug-eluting stents to retard the growth of test bacteria seeded on Nutrient agar (Hi Media) plate was calculated based on the zone of inhibition. In Table: 2, revealed the antibacterial activity of drug-eluting stents for all the test organisms. About 21mm and 31mm were observed on carrier coated and drug carrier coated stents against *Staphylococcus aureus*. Carrier coated and drug carrier coated stents showed 22mm and 30mm against *Pseudomonas aeruginosa*. Maximum inhibitory zone of 24mm and 32mm was observed against *Escherichia coli* on carrier coated and drug carrier coated stents. No inhibitory zones were observed for uncoated materials (Figure 2).

DISCUSSION

Biofilm forming ability of each test organism is evaluated using the microtitre plate method described by Christensen *et al.*¹⁹. The test organisms considered strong biofilm formers in MTP assay were *S. aureus*, *E. coli* and *P. aeruginosa*. These organisms showed OD values ranging from 0.194 to 0.288. The differences in OD values were due to the amount of crystal-violet (dye) absorbed by the test organisms in the microtitre well. The high and moderate biofilm producers were identified by the colour intensities formed in the microtitre plates. This assay will allow researchers to rapidly screen for differences between strains or growth conditions prior to performing labour - intensive microscopic quantification.

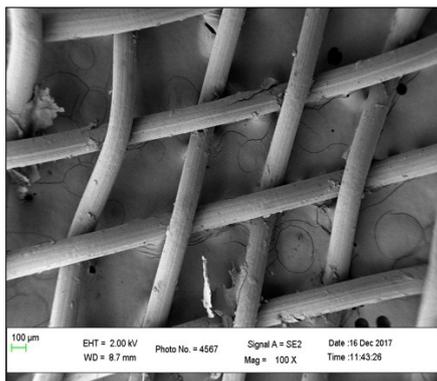


Figure 1a: FESEM analysis of uncoated stent

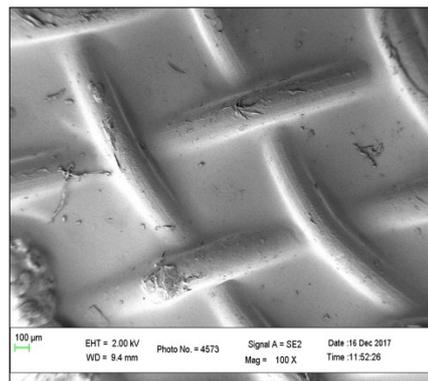


Figure 1b: FESEM analysis of coated stent



Escherichia coli



Staphylococcus aureus



Pseudomonas aeruginosa

Figure 2: Antibacterial activity Carrier Coated and Drug Carrier Coated stents

Scanning electron microscope determines the difference between the surface of coated and uncoated stent materials. The drug-carrier coatings were evenly applied on the material surface through treatment. The topographical analysis of coated stents thus revealed the presence of crystallized chitosan-carrier particles on the stent surface; exhibiting adherence to the greatest possible extent. SEM analysis of the coated stent also evidenced that the homogenous coating of chitosan-carrier 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 crystallized deposition of chitosan on the stent. The obtained results were found supportive when compared to the results of Olena Rzhapishevska et al²⁰. The researchers found that the surface charge of antibacterial coatings were more efficient in reducing bacterial adhesion and biofilm formation.

Antibacterial activity of developed chitosan-vitamin coated stents showed significant inhibitory zones against the biofilm producing test organisms. The inhibitory zones ranged from 32mm to 30mm during the analysis. Interestingly, maximum inhibitory zone of 32mm against high biofilm producing *Escherichia coli* were observed for chitosan-vitamin coated stents. Other biofilm producers like *Staphylococcus aureus* and *Pseudomonas aeruginosa* also showed significant inhibitory zones measuring 31mm and 30mm respectively (Figure 2). The carrier vitamin-E on the stent surface assisted the elution of drug from the stents after placing on the agar surface. Degradation of carrier occurs at a sustained rate when the stent was exposed on the moistened agar surface. This in turn the carrier makes the drug to release at constant rate from the agar surface to inhibit

the biofilm producing organisms. In Figure 2, the clear inhibitory zones around the drug-eluting stents against the biofilm producing test cultures were presented. The rate of degradation of the carrier was considered to be directly proportional to the rate of release of drugs²¹. When the rate of degradation was high, then the release concentration was also found out to be high. Similar condition was experienced by Matl et al., 1963²² during their research when they analysed the release of gentamicin and teicoplanin from PTFE vascular stents grafted with carriers like DL-lactic acid, tocopherol acetate and dynasan. Zarida et al 2015²³ reported that the rate of release of tobramycin and gentamicin was due to the rate of degradation of the drug containing calcium phosphate beads. Comparative analysis was found supportive during this analysis. Two main mechanisms have been suggested as the cause of the inhibition of microbial cells by chitosan. One means is that the polycationic nature of chitosan interferes with bacterial metabolism by electrostatic stacking at the cell surface of bacteria. The other is blocking of transcription of RNA from DNA by adsorption of penetrated chitosan to DNA molecules. In this mechanism the molecular weight of chitosan must be less than some critical value (~5000 Da) in order to be able to permeate into cell. The antimicrobial activities of chitosan are greatly dependent on its physical characteristics, most notably molecular weight (Mv) and degree of deacetylation (DD). Chitosan with a higher degree of deacetylation tends to have a higher antimicrobial activity¹². Thus Chitosan from the coated stents slowly gets diffused into the surrounding tissues preventing the microbial infection.

Similar works were done using Chitosan as a coating for local delivery of antimicrobials for prevention of acute implant infection. The objectives of the study were to measure the release of antimicrobials from chitosan coatings, determine efficacy of eluted antimicrobials against bacteria, in vitro, and evaluate toxicity of eluted drugs to host cells/tissues²⁴.

Implantable sensor devices require coatings that efficiently interface with the tissue environment to mediate biochemical analysis. Polymer Brush-Functionalized Chitosan Hydrogels as Antifouling Implant Coatings by Irene Buzzacchera *et al.*, 2017²⁵ offers a facile route to functionalizing implantable sensor systems with antifouling coatings that improve hemocompatibility and pave the way for enhanced device integration in tissue²⁶.

Chitosan were chemically bonded to titanium coupons via silane-glutaraldehyde molecules and that chitosan has the potential to be used as a biocompatible, bioactive coating for orthopaedic and craniofacial implant devices²⁷.

The conjugation of an aminoglycoside antibiotic (streptomycin) to chitosan could efficiently damage established biofilms and inhibit biofilm formation. This was suitable to eradicate biofilms formed by Gram-positive organisms, and it appeared that antibiotic contents, molecular size and positive charges of the conjugate were the key to retain this anti-biofilm activity. The antibiotic covalently linked to carbohydrate carriers can overcome antibiotic resistance of microbial biofilms and might provide a comprehensive solution to combat biofilms in industrial and medical settings²⁸.

Chitosan has been investigated as a coating for implant materials to promote osseous integration, and as a potential vehicle to deliver therapeutic agents to the local implant-tissue interface. The coating of chitosan onto implant alloy surfaces has been achieved via chemical reactions and electro-deposition mechanisms as well as by other methods such as dip coating and layer-by-layer assembly. This work examines the different mechanisms and bond strengths of chitosan coatings for implant alloys, coating composition and physicochemical properties, degradation, delivery of therapeutic agents, such as growth factors and antibiotics, and in vitro and in vivo compatibilities²⁹.

Chitosan coating is beneficial to maintaining the storage quality and prolonging the shelf life of postharvest fruits and vegetables, which is always used as the carrier film for the antimicrobial agents. The effects of chitosan-based coating on the storage quality, microbial safety, and shelf life of fruits and vegetables were introduced. Their results indicated that chitosan-based coating with different antimicrobial agents would probably have wide prospect in the preservation of fruits and vegetables in the future³⁰.

Chitosan coating eliminated faecal coliforms and lowered counts of *Staphylococcus* spp. Chitosan coating retarded lipid oxidation in all the meat products during storage. The efficacy of chitosan coating in inhibiting *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas fluorescens* was validated. Thus chitosan can be coated on ready NP that contains a core of magnetic material usually a mixture of magnetite (Fe₃O₄) and maghemite (γ-Fe₂O₃). Their chitosan covering provides them with free amino and hydroxyl groups that enable the possibility to bind to a diversity of chemical groups and ions, leading to a number of applications such as protein and metal adsorption, guide drug and gene delivery, magnetic resonance imaging,

tissue engineering and enzyme immobilization. Furthermore, this type of nanoparticle could be used in hyperthermia treatment for destroying malignant cells³².

CONCLUSION

In this research, Chitosan – vitamin E mixtures was crystallized and coated onto the stent materials to retard biofilm formation. During the study, it was found significant that the drug-carrier coated stents could able to retard the growth of biofilm forming organisms when tested using standard assay protocols. The developed drug-carrier coated stents in the present study revealed the significance of the coronary implantation for patients in preventing arteriosclerosis due to biofilm formation. The sustained release of drugs from drug eluting stents fulfilled the primary aim of developing an anti-infective method for post-operative infection caused by biofilm organisms. The research work is appropriate for the field of Pharmacology because, the developed stents is considered as a novel biomedical product with a combination of drug and carrier mixtures.

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