Development Of A Pig Skin Model To Test Nanocomposite Materials For Antibacterial Properties

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ABSTRACT: INTRODUCTION: Polyhedral oligomeric silsesquioxane is making advancements towards medical application as an effective material for drug delivery. POSS is a unique molecule because of its structure. It is a class of organosilicon- three-dimensional compounds with cage frameworks and different degrees of symmetry. **OBJECTIVE:** Our objective was to introduce a biomedical application for POSS in area of drug delivery. For large open wounds it should have a capability to reduce bleeding and as well as provide the release of an antibiotics to reach minimal inhibitory concentrations at the site for infection coverage. If the treatment is successful, POSS can be applied in the field as a protective dressing that is capable of enhancing clot formation, prevent infection, and ultimately enhance tissue repair. **METHODS:** Two formulations of POSS were prepared and tested *in vitro* to release inhibitory concentration of Clindamycin. Clindamycin release was determined spectrophotometrically, and the MIC was determined using a bacterial pig skin wound model were determined. **RESULTS:** Minimal inhibitory concentration of Clindamycin was detected from both POSS formulation for up to 96 hours. In the pig skin wound model the POSS formulation without kaolin reduced the bacterial load more efficiently than POSS containing kaolin and was comparable to a triple antibiotic cream coverage. **CONCLUSION:** Nanocomposites of POSS can be formulated to limit bacterial growth and further reduction of bacterial load can be accomplished by attaching an antibiotic to the functional groups of the caged nanostructure of POSS.

1 Introduction

Polyhedral oligomeric silsesquioxane better known as POSS is making advancements towards medical practice. Many doctors and researchers are going beyond its common use for additives, plastics, and preceramics and are looking for ways to implement POSS into medicine. POSS is a rather unique molecule mainly because of its structure. Polyhedral oligomeric silsesquioxane (POSS) has a distinctive nanocage structure which consists of an inner inorganic core of silicon and oxygen atoms, and an outer shell of organic functional groups. The overall structure of the POSS has the potential to be functionalized for a variety of medical applications. POSS-containing materials can be engineered either as completely non-biodegradable materials or as biodegradable materials with controlled degradation rates. Polyhedral oligomeric silsesquioxanes (POSS) has a three dimensional structure which consists of an inner inorganic framework of silicon atoms ($n = 8$) linked with oxygen atoms ($n = 12$), and an outer shell of organic groups ($n = 8$). Each silicon atom is bonded to three oxygen atoms by siloxane bonds (Si-O-Si) and one carbon silicon bond (Si-C) which can be inert or reactive, allowing for the material to be functionalized. The organic side chain group (R) on the outer shell of POSS has a potentially unlimited supply of organofunctional inert or reactive groups derived from alkyl, olefin, alcohol, ester, anhydride, acid, amine, imide, epoxide, thiol, sulfonate, fluoroalkyl, silanol, and siloxide functionalities [1]. POSS also possesses one of the most important properties, bio-stability, which is the ability of a material to maintain its physical and chemical integrity after implantation in living tissue. It is considered the principal physical property when selecting polymer based composites for medical applications [2].

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Both the chemical and physical properties of POSS are ideal as a material for drug delivery. A drug delivery carrier should be thermodynamically stable, biocompatible and be able to release the drug in a sustained fashion for varying periods of time. The stability of the POSS material allows its development as a hemostatic/antimicrobial agent following trauma [3-8]. Robson et al., (1973) reported that for an open fracture with soft tissue injury that a threshold of 10^5 organism for gram of tissue can be reached within 5.17 hours [9]. Recent findings by Kamat (2011) show basic intervention such as debridement and antibiotic coverage play a significant role in limiting the risk for infection, and concluded that antibiotics should be administered as soon as possible. Application of wound cover containing antibiotics by first responders may be one of the easiest and most effective methods of treating and preventing infection [10-11]. In this study we used an *in vitro* assay to assess the ability of the material to determine the effectiveness of POSS as a drug delivery carrier. When evaluating applications for treatment of human skin, the pig skin model is more favorable because it is similar in material response to human skin. According to Godin et al., the values of the skin permeability in pigs and humans are more compatible than other animal models. Their skin contains corresponding external thickness with similar lipids on the surface.

2 Material and Methods

Clindamycin Release: In one set of experiments, clindamycin was diluted in sterile water to 30 mg/mL and mixed with 100 mg of POSS compounds. In the other set of experiments, 30 mg of POSS were added to 100 mg of POSS compound directly. Clindamycin solution in phosphate buffered saline (PBS) were scanned from 200-800 nm on a Spectrophotometer to determine the peak detection point. The detection peak was found at 210 nm. Aliquots of 100 mg of POSS containing clindamycin were added to a sterile Corning 24 well plate and 1 mL of PBS was added. Complete exchange of PBS was performed for every treatment group at 1, 2, 3, 24, and 48 hours. Each treatment group consisted of 4 wells per group and each

sample was measured in triplicate. A standard clindamycin curve was prepared (30, 3, 0.3, 0.03, 0.003, and 0 mg/mL) and performed at each sample period.

Pig Skin Wound Model: Pig skin sections (3cm x 3cm) were prepared and soaked overnight in sterile media containing 1% antibiotic/antimycotic solution. Following the overnight incubation sections were placed in five changes of sterile PBS. The prepared skin sections were placed into a Corning 6-well plate and a well (5mm wide and 3mm deep) was created in the center of the skin (Figure 1). The treatments (n=6) included: (1) naïve control, (2) POSS, (3) POSS + kaolin, (4) triple antibiotic cream, (5) POSS + antibiotic, (6) control + bacteria , (7) Clindamycin antibiotic 10uL, and (8) POSS + Kaolin + antibiotic. The aforementioned treatments along with 1 x 10^4 Staph-epi (ATCC, Rockville, MD) was applied in 50 µL aliquots to each well created in the skin. The skin was maintained in 1mLof sterile RPMI on the bottom of the plate to keep the skin hydrated and incubated in a humidified CO_2 incubator at 37°C. After incubating for 24 and 48 hours, the skin was swabbed with sterile applicators and smeared onto appropriately labeled blood-agar plates. The agar plates contained four quadrants. Quadrants1-3 were used for sampling the area within the applied material and the extra quadrant (Q4) was used for testing the media within the well. The agar plates were incubated for 24 hours and the growth was record (Figures 1a and 1b).

Figure 1a. *Example of 3cm x 3cm pig skin sections with treatment covering each well.*

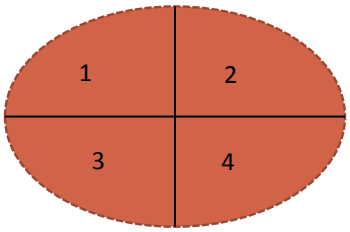


Figure 1b. Quadrant 1 = First well of treatment row, Quadrant 2= Middle well of treatment row, Quadrant 3= Third well of treatment row. and Quadrant 4 = Random skin swab outside of treatment area (media).

Statistical Analysis: Data analysis was performed utilizing Sigma Stat 12 Software Package. Descriptive statistics were performed and described as means ± standard deviation (SD) for a given number of samples (n). A test for normality (Shapiro-Wilk) was completed for each data set to determine if a parametric or non-parametric statistical test were needed. The data past the normality test, and One-Way Analysis of Variance (ANOVA) was performed [11]. An ANOVA post-hoc test was performed when the F value was significant. Tukey's test post-hoc test was used for pair wise comparisons of the data with a p value <0.05 considered statistically significant. In addition, bar graphs were prepared using Slide Write Computer Software.

3 Results

Drug Delivery of Clindamycin:

Figures 2 shows the concentration of clindamycin (µg/mL) released over times for four different formulations of POSS (POSS + liquid clindamycin 1:1, POSS +kaolin + liquid clindamycin 1:1, POSS + clindamycin salt, POSS + kaolin + clindamycin salt). The results show virtually no release of the clindamycin from either POSS formulary if it is added in liquid form. POSS without kaolin had approximately four times the detectable amount when compared with the kaolin containing POSS in the first hour. At the reaming time periods, the detectable amount of clindamycin in each group was similar (Figure 2). Plotting the data as cumulative release of clindamycin with time (Figure 3), shows 95% of the drug was released from the POSS within 48 hours while POSS plus kaolin (77.5/22.5 ratio) released approximately 45% of the drug over the same time period. The minimal inhibitory concentration of clindamycin (0.25-0.6 µg/mL) (MIC) for *Staph aureus* was obtained within the first hour from both salt containing mixtures of POSS and maintained over the 48 hour time period. Whereas, the MIC was never obtained when POSS was mixed 1:1 with liquid clindamycin.

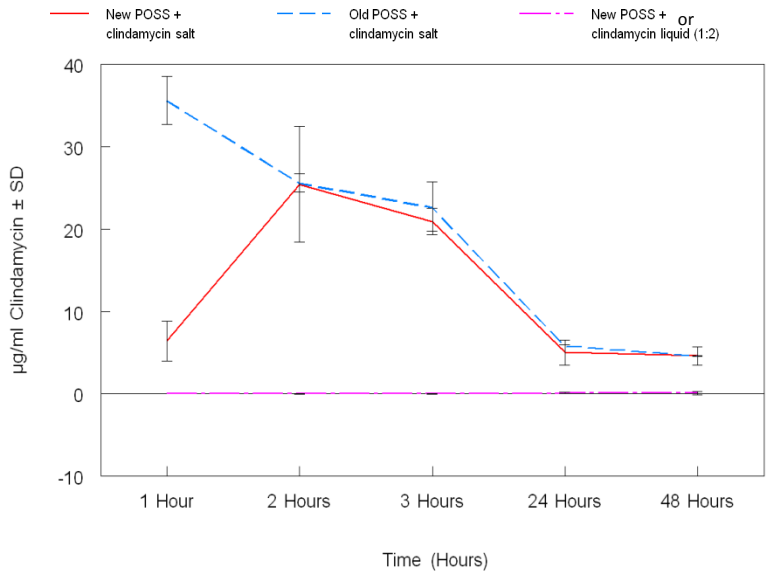


Figure 2. *Release profile of clindamycin from POSS material.*

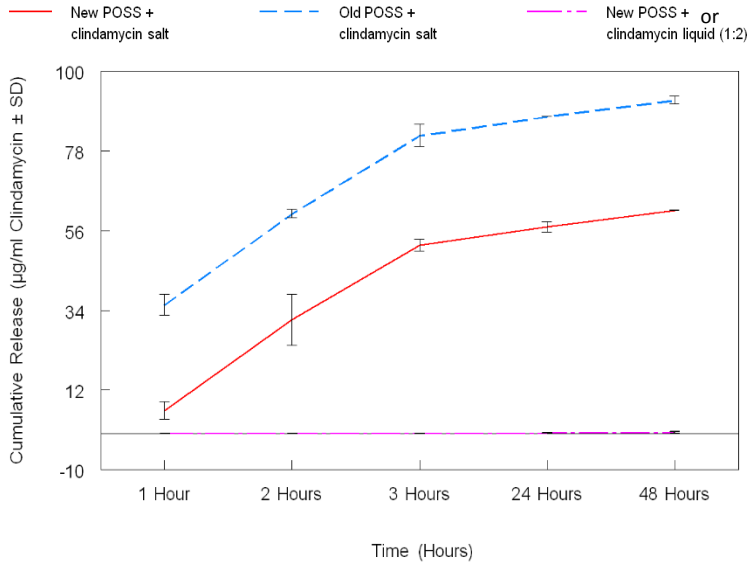
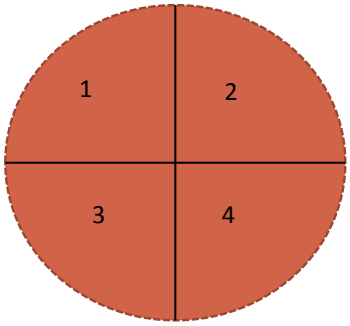


Figure 3. *Net release profile of clindamycin from POSS material.*

The in vitro Pig Wound Model:
Effectiveness of POSS and POSS containing antibiotic formulary against a bacterial challenge was investigated in the pig skin wound model over a similar 48 hour time period A pig skin model was used to determine mimic an in vivo scenario and was used to determine if POSS combined with Clindamycin salt and placed into a simulated wound for coverage would be effective in inhibiting bacteria growth comparable to triple antibiotic cream over a 48 hour time period (Refer to Figures 1A and 1B for set up of the model and collection for plating in the methods section). POSS with and without kaolin mixed with Clindamycin salt were placed into a well created in the pig skin and 1 x10^5 *Staph epi* were added. Samples were collected for plating at 24 and 48 hours and compared with saline + bacteria, triple antibiotic + bacteria, Clindamycin + bacteria, control no bacteria and control bacteria. Overall, triple antibiotic, Clindamycin and POSS formularies containing Clindamycin reduced bacterial load within the first 24 hours. By 48 hours bacteria were present in all treatment groups. It should be noted that the outer surface of the skin was also swabbed and plated to ensure that the bacteria were sequestered within the well. Soaking the skins in antibiotic media and rinsing with sterile was effective in sterilizing the skin prior to addition of the bacteria. The swabs of the outer area did not show presence of bacteria for 48 hours, which validates the usefulness of the model to simulate wound coverage (Figures 6-14).



Quadrants 1- 3= were samples taken from the center of the well at 24 and 48 hours. Quadrant 4 = Random skin swab outside of treatment area.

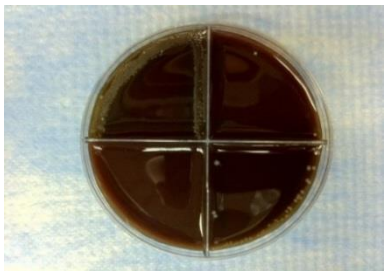


Figure 6a: *Control 24 hours*

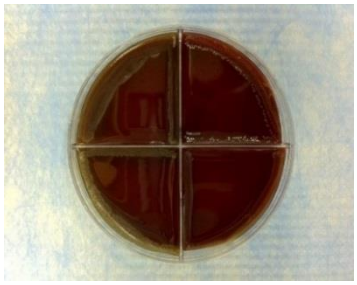


Figure 6b: *Control 48 hours*

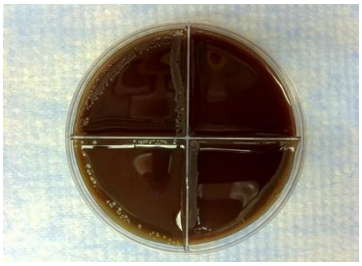


Figure 7a: *Control + bacteria 24 hours*

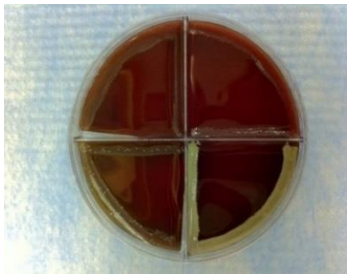


Figure 7b: *Control + bacteria 48 hours*

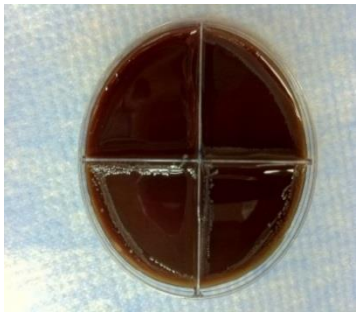


Figure 8a: *POSS 24 hours*

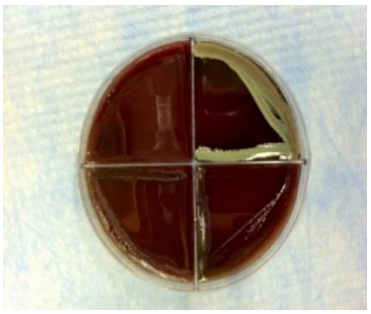


Figure 8b: *POSS 48 hours*

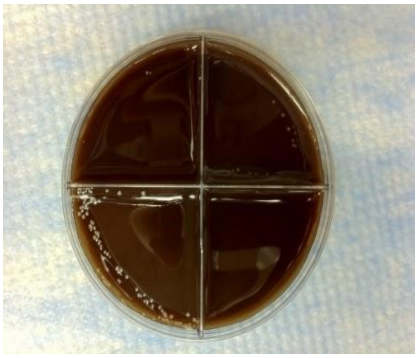


Figure 9a: *POSS with kaolin 24 hours*

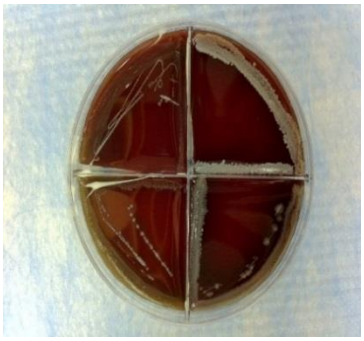


Figure 9b: *POSS with kaolin 48 hours*

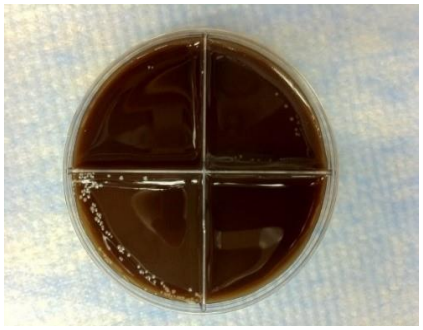


Figure 10a: *POSS SO1455 with kaolin + bacteria 24 hours*

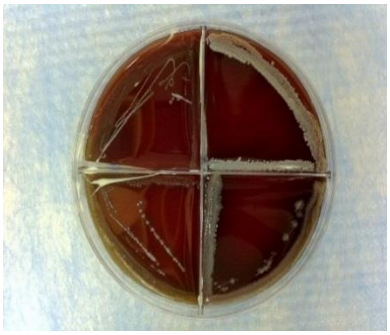


Figure 10b: *POSS SO1455 with kaolin + bacteria 48 hours*

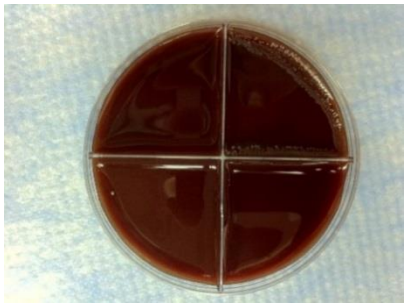


Figure 11a: *Triple Antibiotic Cream + bacteria 24 hours*

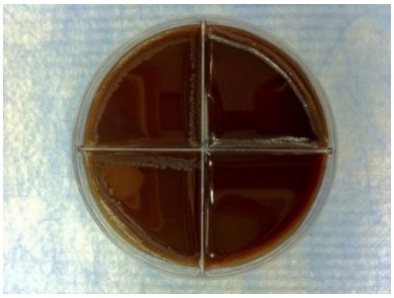


Figure 11b: *Triple Antibiotic Cream + bacteria 48 hours*

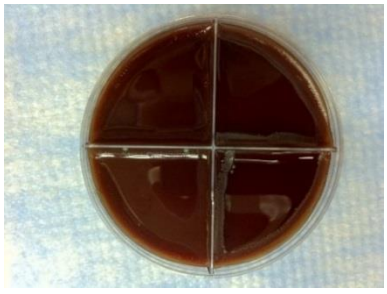


Figure 12a: *Clindamycin + bacteria 24 hours*

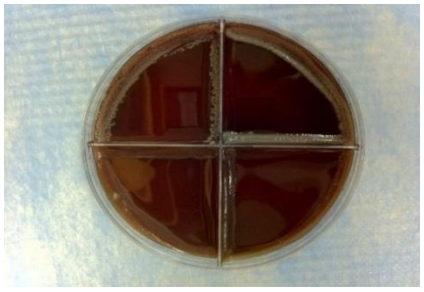


Figure 12b: *Clindamycin + bacteria 48 hours*

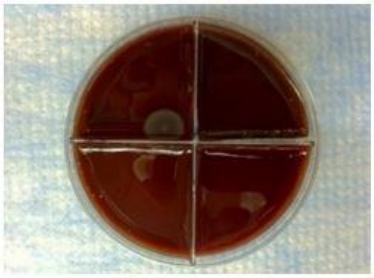


Figure 13a: *Clindamycin + POSS + bacteria 24 hours*

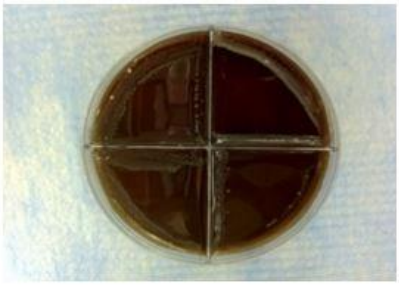


Figure 13b: *+ POSS + bacteria 48 hours*

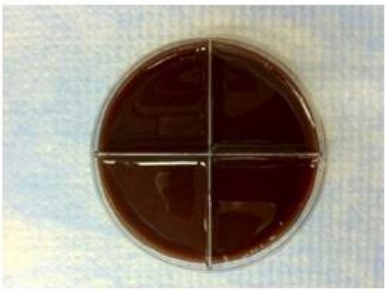


Figure 14a: *Clindamycin + POSS with kaolin + bacteria 24 hours*

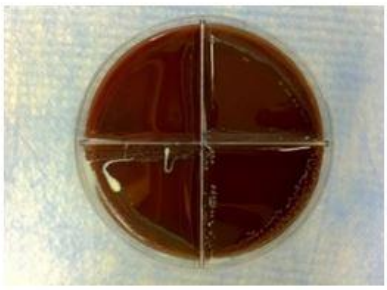


Figure 14b: *Clindamycin + POSS with kaolin + bacteria 48 hours*

4 Discussion

The literature suggests that early administration of antibiotics to a wound that has been exposed to the environment is crucial for decreasing infection, and the time to heal (Davis, 2008). The commercially available wound coverage materials do not offer this capability at present. POSS with and without kaolin nanoparticles was tested to determine if either compound had antibacterial properties. Both formulations of POSS without antibiotics where ineffective as an antibacterial agent at both 24 and 48. A dissolution study using POSS mixed with liquid clindamycin revealed that POSS was not effective in reaching the minimal inhibitory concentration (0.25- 0.6 µg/mL) (MIC) for *Staph epi* over a 48 hour period. When clindamycin salt (30 mg/mL) was mixed with 0.1 gram of either POSS Kaolin or POSS the drug was entrapped within the material. MIC was reached within 1-hour and remained at an effective MIC over a 48 hour period for both POSS formulations and was able to curtail bacterial growth over a 48 hour period using a pig skin wound model. Our results are supported by the recent work of Tanaka et al. (2008) who demonstrated an enhanced entrapment ability of dendrimers by incorporation of a hydrophobic POSS central core [12]. Their results showed that the POSS can entrap a larger amount of guest molecules without loss of affinity, and consequently, the water solubility of the entrapped guest molecules can be increased [12]. Kaolin acts as an additional barrier to prolong the retention of the drug. Overall, POSS can serve as an excellent vehicle for drug delivery. Based upon the caged structure of POSS, the side chains can be functionalized with the drugs for more controlled release over a longer period of time. Polysiloxanes can be formulated to have a quaternary ammonium salt group that can concentrate in the microorganism cell wall [13]. In fact, an array of quaternary ammonium compounds could be utilized to functionalize POSS for antibacterial applications. In one study, Majumdar et al, (2009) investigated the antimicrobial activity of functionalized POSS coatings toward Escherichia coli, Staphylococcus aureus, and the opportunistic fungal pathogen, Candida albicans using the standard Agar plating method [14]. The results showed that the composition of POSS that had been functionalized and the polysiloxane matrix affected the microbial properties. Several compositions were identified that inhibited bacterial growth, in all the microorganisms studied on the coated surfaces. Although the potential benefits warrant additional investigation as mobilized quaternary compounds, such materials are known to cause inflammatory and anaphylactic reactions [15]. Further in vivo work is necessary to determine the effectiveness of POSS for treatment and prevention of infection in open wounds.

5 Conclusions

The unique physical and chemical properties such as ultra-small size, large surface-area-to-mass ratio, and biocompatibility provide POSS with properties that overcome the limitations of other traditional therapeutic and diagnostic agents. The unique properties of POSS as well as its ability to be incorporated into a wide range of biocompatible polymers make POSS an attractive material for a versatile array of applications in medicine. POSS on its own did not result in formation of a blood clot which is consistent with the literature. Addition of kaolin to POSS enhances its hemostatic properties. When this kaolin formulary of POSS is added to a wound, it does not provide a favorable tissue response. POSS on its own is not antimicrobial against aerobic bacteria, but when combined with an antibiotic salt form of the drug was able to release the drug at concentrations that were effective to control

bacterial growth for 48 hours. Future investigation into functionalizing POSS directly to an antibiotic to be applied within the wound for greater than forty-eight hours may provide additional data as to the ability of POSS to protect a wound against bacteria.

5 Acknowledgement

6 References

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