Therapeutic nano-device: Study of biopolymer coating on titania nanotubes array loaded with chemodrug targeted for localized cancer therapy application

Wan Nuramiera Faznie Wan Eddis Effendy¹, Rabiatul Basria S.M.N Mydin¹²*, Amirah Mohd Gazzali³, Srimala Sreekantan⁴

¹Oncological and Radiological Sciences Cluster, Advanced Medical and Dental Institute, Universiti Sains Malaysia, 13200 Bertam, Kepala Batas, Pulau Pinang, Malaysia.
²Department of Biological Sciences, 14 Science Drive 4; NUS Environmental Research Institute, National University of Singapore 117543, Singapore.
³School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800, Minden Penang, Malaysia.
⁴School of Materials and Mineral Resources Engineering, Universiti Sains Malaysia, Engineering Campus, 14300, Nibong Tebal, Pulau Pinang, Malaysia.

Email: rabiatulbasria@usm.my

Abstract. Polymer coating for drug delivery system act as command for drug release rate either for instant, sustained or extended prolonged release dosage. This coating layer also is crucial to protect the functional stability of drug that loaded into delivery platform especially the smart delivery systems. Presently, titania nanotube arrays (TNA) platform have been discussed as nanotherapeutic device for drug delivery system especially in localized cancer therapy application. The purpose of this study is to investigate the outcome of chitosan coating on cisplatin-loaded TNA. Chitosan coating on cisplatin-loaded TNA have shown extended cisplatin release activities until day 28 period compared to the uncoated cisplatin-loaded TNA. Burst release was observed within the first 6-h period for both the chitosan uncoated and coated samples. Spike stable cisplatin release from coated TNA sample was observed after day 20, which suggested polymer coating on TNA platform may provide probable an extended-release cisplatin delivery activity. Findings from this work suggest the potential of TNA as nanotherapeutic device for extended cisplatin release platform in localized cancer therapy application.

1. Introduction
Traditional drug delivery system in cancer treatment has limitation in controlling and targeting the drug release dosage which could compromise the neighboring normal healthy cells too [1, 2]. Present system required frequent administration of chemodrug into the body system which leads to high exposure potential side effect and discomfort to cancer patient [1,3] In order to overcome these limitations and increase treatment efficiency, scientists are looking on the recent innovation on of smart delivery systems for targeted cancer therapy practices [4]. In biomedical research, Titania nanotube array (TNA) have initially become contemporary interest for drug delivery platform for dental and orthopedic implant applications [4-6]. Furthermore, previous research has described the
ascendancy on TNA’s nanocylindrical structures which allows the loading and drug cargo [3,5] thus this delivery platform could become a great advantage for targeted therapeutic nano-device.

The key in developing a successful smart drug delivery system is control the drug release dosage for certain required phase. Biopolymer coating have provided a favorable advantage for drug delivery application. For instance, chitosan is reported to be suitable implant coating as this biopolymer possess antimicrobial and biocompatible properties [5,7,8]. This coating layer could also act physiological barriers to efficient drug delivery. Therefore, an extensive study on functionalized and drug release profile is required. Unagolla et al (2018), [9] reported that optimized polymer coating layer provide non-toxic environment for surrounding cells and allured cells integration. Furthermore, pivotal of coating layer is to exploit the administration and dosage of drug either for instant, sustained or extended prolonged release dosage. Therefore, it is crucial to optimized chemodrug delivery from a system with biopolymer coating. This study used an in vitro model system to evaluated the functional chitosan coating on TNA loaded with cisplatin a chemodrug used in cancer therapy.

2. Methodology

2.1 Fabrication of TNA

TNA were synthesized according to protocol as described previously Mydin et al, 2017. The anodization curve was studied by computed software named Leios Material Analysis. Field emission scanning electron microscopy (FESEM), Supra 35VP Zeiss, Germany is used to observed TNA morphology. TNA sample is initially coated with gold sputter using the SPI-module line of modular sputter coaters to create thin conductive layer for electron microscopy resolution. Prior in in vitro use the TNAs were sterilized with 70% ethanol, multiple rinsed with distilled water, and autoclaved at 121°C for 25 min.

2.2 Chemo-drug preparation and detection by spectrophotometry

Chemodrug cisplatin (Platol™) obtained from Venus Remedies, India was reconstituted using the negative pressure technique. The handling procedure is supervised under the guideline of Health and Safety Executive (HSE)/Medicines Controls Agency (MCA) [11] and Department of Pharmacy, Clinical Trial Complex, Advanced Medical and Dental Institute, Universiti Sains Malaysia. Cisplatin detection were carried out using microplate spectrophotometer reader (PowerWave™ BioTek, USA) with and without addition of Bradford color reagent (BioRad, USA) at wavelength, 595 nm and 280 nm, respectively. Absorbance reading for the cisplatin was recorded and expressed as protein concentration according to following formula:

\[ \text{Protein concentration (mg/mL)} = (\text{Absorbance of sample} - \text{Absorbance of PBS(blank)}) \times k \]

where k is coefficient factor of the Bradford reagent (0.0536).

2.3 Cytocompatibility Assay

Epithelial NPC/HK-1 cell line model obtained from Professor George Tsao, University of Hong Kong is maintained in complete RPMI-1640 (Gibco, Life Technologies) medium (10% fetal bovine serum (FBS), 2 g/L sodium bicarbonate, 12.5 mg/L HEPES, 1 X L-glutamine and 1% penicillin and streptomycin) at 37 °C in a 5% CO² humified incubator. Cell viability was assessed after 24 h treatment by CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA) according to the manufacturer’s protocol at 490 nm using microplate spectrophotometer reader (PowerWave™ BioTek, USA). The cytocompatibility assay evaluation and determination of half maximal inhibitory concentration (IC₅₀) was performed following the protocol recommendations in ISO 10993-5.

2.4 Cisplatin loading into TNA

TNA samples with measurement 1 cm x 1 cm were immersed in individual container with 0.10 µM of cisplatin for 24 h at 4 °C follow by 2 hours air-dried before polymer coating procedure.
2.5 Chitosan coating preparation
Chitosan (low molecular weight, Sigma Aldrich, St. Louis, USA) is prepared in acetic acid with continuous shaking at 60 °C for 5-h using incubator shaker (KS 4000i Control, Germany). The study samples coated with this solution by one-time dip coating technique as described by Liang et al, 2018 and air-dried under biosafety cabinet level 2 for overnight. This protocol is modified from previous studies works. Pre-optimization on 0.1 M and 0.2 M acetic acid concentration were done and subjected to biocompatibility test.

2.6 Cisplatin release profile
Cisplatin-loaded TNAs (coated and uncoated samples) were immersed in PBS at 37 °C with 100 rpm incubator shaker. Quantification of cisplatin were studied at two phases: burst release for every 30 mins and prolonged release in 28 days as described by previous studies [7].

3. Results and Discussion
3.1 The fabrication process
The fabrication of TNA was achieved by electrochemical anodization process similar to our previous work [10, 13] and monitor using Leios material analysis computer software. The current density-time curve at constant voltage were studied for 1800 seconds as shown in figure 1. During this process, bubble formation was observed which contribute to the initial oxide pore formation on titanium foil which coinciding with the peak observed in the Leios graph. This observation describes the beginning of oxide dissolution activities and nanotube formation. Study by Zhao and co-workers (2017), described the mathematical model of the nanotube formation into three phases. The initial phase known as CV curve occurred when the dissolution of oxide and the release of fluoride ion taken place. This phenomenon was caused by high electric field resulted in high content of free ion. In the Figure 1, this event can be clearly seen at the first 60 seconds (as shown by the red arrow in Figure 1). Second stage (CC curve), is the formation of nanotube in which the molding created by the bubble. In the last stage (quasi-steady stage), the grow of nanotube at steady and continuous state until the end of the anodization resulted in the consistent record of current density exponential to the time. Similar finding also described by Fereirra et al, 2019 on the nucleation of nanotubes occurs resulted by the dissolution of F presented in NH4F in CC curve hence produced vertical structure of nanotubes. As a result, TNA with average 300 nm was successfully grown on the titanium foil and applied for drug delivery study.
Figure 1. The current density-time plot using Leios material analysis computer software. The curve at constant voltage with peak at 60 seconds (red arrow) displayed starting of TNA fabrication process by electrolyte in electrochemical anodization process.

3.2 Morphology of chemotherapeutic nano-device
The fabrication of highly ordered nanotube is shown in Figure 2 (a) with diameter $76.58 \pm 10.64$ nm and length about 300 nm. Diameter obtained from the anodization procedure is very important in allowing cell interaction and drug entrapment [16]. Other study had highlighted the optimum cells integration occurs on TNA with 70 nm diameter [17] while some also mentioned the range of diameter between 30-100 nm is excellent for cell interaction [18,19]. Based on reports by others, we had chosen to fabricate TNA with diameter less than 100 nm for further study as chemotherapeutic nano-device. Closer examination on the hole like nanotubes surface in figure 2 (a) showed open-end tube and ended rounded closed surface in figure 2 (b). This topology could become an advantage to entrapment drug also supported by the finding of Somanith and co-workers. Kafshgari and co-worker (2019) also stated similar finding on open-end tube drug delivery system. From the peel-off structure observation showed hollow closed ended nanotube structure that might enables the loading of drug and also promote slower release of nanotube [21]. These parameters are important in ensuring the cisplatin loading activity and longer release can be achieved.
3.3 Cisplatin Detection and Quantification

The initial crucial detection of cisplatin release baseline at two different wavelengths, 280 nm and 595 nm were studied as suggested by previous works. Generally, detection of cisplatin was carried out by direct detection using spectrophotometric as studied by Zieske and co-workers at 280 nm, however we had unable to detect the cisplatin dilution at the same wavelength. Recently, o-phenyldiamine (OPDA) and dimethylformamide (DMF) had been used in determination of the cisplatin in research and urine as reported by Basotra et al, 2013. Alternatively, we had added Bradford color reagent into cisplatin dilution and measured at 595 nm. Figure 3 displayed the absorbance reading of cisplatin at two wavelengths. Based on the principle of Bradford assay, the Coomassie dye will bind to amino acid residue like arginine, lysine and histidine (He, 2011). Structure of cisplatin contains $\text{NH}_3$ which similar to structure of amino acid hence make detection with Bradford assay possible. As seen in Figure 3, increasing concentration of cisplatin resulted in increase intensity of blue color changes hence influence the absorbance reading.

$$y = 0.005x - 0.0102$$  
$$R^2 = 0.8889$$
Figure 3. Cisplatin detection by using spectrophotometric measurement. The quantification of Cisplatin was studied at 290 nm and 595 nm wavelengths.

3.4 Chitosan coating
Naked TNA topology is hydrophobic but addition of chitosan coating on this topology can provide hydrophilicity characteristic which will improve the cells integration [25]. Note that the solubility of chitosan is very weak in alkaline condition withal due to presence of N-acetyl glutamine (free amino group), as this compound dissolves in weak acid [9]. In this study, acetic acid used as diluent for chitosan which in line by other study [26]. Generally, acetic acid does have cytotoxic effect on cells [27]. Therefore, chitosan preparation is crucial as it need to be cytocompatible and sustained the cisplatin release activity at same time.

Present work investigates the cell viability profile of lower concentration of acetic acid between 0.1 M or 0.2 M for chitosan preparation. The selection of acetic acid concentration is based literature [12,13] which used different concentration of acetic acid ranging from 0.5 to 2.0 %. From the bar chart shown in Figure 4, the cells only and cells with TNA surface showed 100 ± 11.99% viable and 96.4 ±1.28 % viable after 24-h incubation. Treatment of cisplatin that give IC₅₀ were used as positive control for cytotoxic environment noted as cisplatin + cells with viability 40.91 ± 11.99 %. The chitosan with 0.1 M acid concentration showed 75.6 ± 11.28 % viable cells and 66.03 ± 9.82 % viable cells for chitosan with 0.2 M acid concentration. Data analysis of all the samples to control group (cells only) showed no significant difference except for 0.2 M chitosan coating on cisplatin-loaded TNA + cells with p value equal to 0.001. From the statistical analysis, we choose 0.1 M chitosan coating as our polymer layer for this work.

Figure 4. Pre-optimization of biopolymer coating with different concentration of acetic acid. The test was conducted in duplicate and expressed mean ± SD (n=3). There is no significant difference between control group and 0.1 M chitosan coating on cisplatin loaded TNA + cells while significant...
difference was noted for 0.2 M chitosan coating on cisplatin loaded TNA + cells compared to control group (cell only) with \( p \) value 0.001. Data was considered significant if \( p \) value < 0.05.

### 3.5 Cisplatin release profile

The content of cisplatin to load into TNA was determined by in-vitro cytotoxicity of the drug on the NPC cell line according to the ISO 10993-5 guideline. The cultured NPC cells was incubated directly with the TNA and also chitosan coated cisplatin-loaded TNA for 24-h. The cytotoxicity of the samples tested was evaluated with MTS reagent and the result was displayed in Figure 4. Biological evaluation for medical devices design needed to be performed to investigate the possible response occurred after the contact of cell and the device.

Figure 5 showed the prolong and sustained release activities of cisplatin from chitosan coated cisplatin-loaded TNA in two phases known as biphasic release profile. Burst release of cisplatin was observed within 6-h, with 1.287 µg/mL cisplatin detected for coated TNA and 1.653 µg/mL cisplatin for uncoated TNA. Cumulative release of the cisplatin was measured throughout the burst and prolong phase as mentioned by others [28]. Based on Figure 5b, the release of cisplatin chitosan coated cisplatin-loaded TNA was at 4.507 µg/mL while uncoated cisplatin-loaded TNA recorded at 5.433 µg/mL until 28 days’ period. Furthermore, the both samples had recorded instant release in PBS at 37 °C as labelled in red line. Highest release was observed at 210 min for both samples. Extended release phase only at day 23 showed significant release of the cisplatin for all groups (analysis of variance at \( p \) < 0.05 as determined using SPSS 24.0 software) compared between the two samples. Although there is no significant release of cisplatin between the chitosan coated cisplatin-loaded TNA and uncoated cisplatin-loaded TNA, the difference between the samples can still be seen.

TNA with dimensions stated in Figure 2 could provide prolonged and continuous cisplatin release activities. Presence of TNA capillary force might assist the entrapment of cisplatin deeper into the tube cylindrical structure hence could extend the cisplatin release performance as described by Zhang et al, 2017 which supported by this study where longer cisplatin release period is required for uncoated cisplatin-loaded TNA. Further statistical analysis using the post hoc Bonferroni test showed that cisplatin release activity increased significantly on chitosan coated cisplatin-loaded TNA when compared to the uncoated cisplatin-loaded TNA. Findings from this study observed extended steady-rate cisplatin release from chitosan coated cisplatin-loaded TNA. Further prolong experimental period is required to profile the cisplatin release from chitosan coated cisplatin loaded TNA as in 28-d study did not showed maximum release profile. Additionally, modification of pH and temperature of the soaking buffer should be studied to better control the release pattern according to the specific application. Nevertheless, this system could become a promising smart delivery system that might benefit traditional chemodrug treatment.
Figure 5. Biphasic release of chemodrug loaded TNA (a) Release profiles of cisplatin from chitosan coated cisplatin-loaded TNA (coated) and uncoated cisplatin-loaded TNA (uncoated), inset showing the details within the first 6 h. (b) Prolong release profile for 28-d was studied and the data was recorded daily. All the data are expressed as mean (n = 4) and analysed statically by post hoc Bonferroni with significant data expressed with p value < 0.05.

4. Conclusion
Chitosan coating cisplatin loaded TNA has been successfully fabricated thus acts as nano reservoir for drug delivery activity in targeted therapy. Prolong and sustained release activity of cisplatin had been achieved throughout 28-d of study as result from the addition of chitosan coating. This finding may provide potential nano therapeutic device for targeted cancer therapy in biomedical field.
5. Conflict of interest
All authors declare no conflicts of interest.

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