Exploring doxorubicin localization in eluting TiO2 nanotube arrays through fluorescence correlation spectroscopy analysis

Tullio Monetta, University of Napoli

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Exploring doxorubicin localization in eluting TiO₂ nanotube arrays through fluorescence correlation spectroscopy analysis†

Ilaria De Santo,ab Luigi Sanguigno,ab Filippo Causa,ab Tullio Monetta and Paolo A. Netti*ab

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Drug elution properties of TiO₂ nanotube arrays have been largely investigated by means of solely macroscopic observations. Controversial elution performances have been reported so far and a clear comprehension of these phenomena is still missing as a consequence of a lack of molecular investigation methods. Here we propose a way to discern drug elution properties of nanotubes through the evaluation of drug localization by Fluorescence Correlation Spectroscopy (FCS) analysis. We verified this method upon doxorubicin elution from differently loaded TiO₂ nanotubes. Diverse elution profiles were obtained from nanotubes filled by soaking and wet vacuum impregnation methods. Impregnated nanotubes controlled drug diffusion up to thirty days, while soaked samples completed elution in seven days. FCS analysis of doxorubicin motion in loaded nanotubes clarified that more than 90% of drugs dwell preferentially in inter-nanotube spaces in soaked samples due to decorrelation in a 2D fashion, while a 97% fraction of molecules showed 1D mobility ascribable to displacements along the nanotube vertical axis of wet vacuum impregnated nanotubes. The diverse drug localizations inferred from FCS measurements, together with distinct drug–surface interaction strengths resulting from diverse drug filling techniques, could explain the variability in elution kinetics.

Introduction

Medical implants in titanium and its alloys are currently used for many applications, such as cardiovascular stents, dental implants and orthopedics, since they have good mechanical properties, biocompatibility and corrosion resistance. However, side effects associated with implantations still compromise their durability. To overcome these limitations, several strategies are under investigation. In particular, more attention is being paid to engineering the implant–body interface, which should endow not only proper mechanical properties, but also include biological requirements as the promotion of cell growth and anti-inflammatory capabilities. Nanostructured surfaces are one route to satisfy these requirements. Among diverse possibilities of nanostructured coatings, titania nanotubes, obtained from anodized titanium, can be produced in a wide range of lengths and diameters, and their morphological characteristics can be fine-tuned. Titania nanotubes have already demonstrated appropriate biocompatibility, post-implantation non-inflammatory responses, and the ability to improve attachment, function and proliferation of several cell lines, in particular bone cells.

The more relevant advancement in titania nanotubes implant coatings is the enhanced surface-to-volume ratio that magnifies drug-loading capabilities, allowing the replacement of standard drug treatments with local therapies, thus increasing drug efficacy, reducing side effects related to the systematic drug distribution throughout the body. Local therapies can address specifically the promotion of cell proliferation through the growth factor addition or reduce the post-implantation response through anti-inflammatory drug incorporation. Proteins such as bovine serum albumin and lysine, anti-inflammatory drugs such as indomethacin, gentamicin, paclitaxel, and sirolimus, as well as bone morphogenetic protein-2 growth factor were satisfactorily loaded and eluted from different titania nanotube drug-eluting platforms. Beside the advantages of local therapy treatments and enhanced loading capabilities, the main challenge in nanostructured drug-eluting coatings resides in the possibility to engineer the drug elution kinetics from nanotubes; thus, a proper comprehension of the drug release mechanism from nanotratures is required. Indeed, different works were already devoted to assess the release mechanism from titania nanotube arrays. Previous studies focused on the effect of TiO₂ nanotube length and nano-topology upon elution. These works have measured sustained release, whereas, more recently, studies have...
reported that the same platforms deliver drugs only up to a few days. There is indeed no common consent on the topic.\(^{20}\)

The main differences among the reported results might be found in the nanotopographies and surface chemistries of used platforms, together with distinct eluting molecules. The combination of diverse molecules and surface chemistries attain distinct pairs, each identified by an interaction energy value which might control the elution kinetics, giving rise to short or long release profiles. In addition, the proximity of the drug dimension to the nanostructure characteristic size (i.e. confinement ratio) could also affect the release kinetics giving rise to diverse diffusion mechanisms.\(^{21-24}\) On the other hand, filling tube efficiencies and drug localization onto the nanostructured platform may also play an important role in affecting elution.

Therefore, further investigations are needed to better characterize molecules elution from TiO\(_2\) nanotubes, thus to correlate drug-surface interaction extent and drug localization influence to the obtained elution kinetics. Here we propose a method to investigate this aspect in TiO\(_2\) nanotube arrays loaded with a model drug, doxorubicin, by two filling methods, soaking and wet vacuum impregnation, at various drug loadings and temperatures. We performed our analysis investigating doxorubicin diffusion within nanotubes by a single molecule technique, Fluorescence Correlation Spectroscopy (FCS),\(^{25,26}\) recently used to monitor diffusion in several nanosized systems.\(^{22-27,29}\) The different elution profiles obtained clearly show that the loading technique deeply influences the kinetics of elution and the elution mechanism is concentration independent and temperature activated. Our single molecule investigation elucidates drug molecular displacements within the confined geometry on a time scale not resolvable by elution kinetic measurements. The analysis can explain the relevant difference among elution kinetics obtained from titania nanotube arrays, clarifying that molecules move preferentially along the nanotube vertical axis in impregnated samples.

### Results and discussion

Titania nanotubes are produced through anodization of titanium samples in inorganic solution. Obtained platforms are characterized by an average tube diameter of 110 ± 50 nm and a tube length of 800 ± 30 nm obtained from SEM images, as shown in Fig. 1. Doxorubicin, a common anticancer drug, is used as a model eluting-drug since they feature autofluorescence, a small size of about 0.8 nm and good chemical affinity to the material. In order to compare elution kinetics of drugs loaded by different methods, both soaking and impregnation were tested as filling methods. Soaking attained less than 10% loading efficiency, whereas the adopted impregnation process obtained a better efficiency of about 49%, see Table 1.

Release from soaked and impregnated platforms was followed up to two months. The eluted drug fractions are reported in Fig. 2. Soaked structures completed drug elution already at 10 days, whereas impregnated nanotubes continued eluting up to 60 days. In addition, since loading efficiency was consistently higher for impregnated platforms, this loading method was adopted for further characterization. In order to evaluate the influence of drug concentration on the release kinetics, two different amounts of drugs were used, 10 and 20 μg, and the highest effective load accomplished was respectively 12 and 62 μg cm\(^{-2}\) into the nano tube arrays. The temperature influence on the doxorubicin release kinetics was also studied for flat samples and nanotube arrays. The release was followed at both 23 \(^\circ\)C and 37 \(^\circ\)C and a long-term drug release was observed at both temperatures. Nanotubes released higher quantities compared to control flat TiO\(_2\) surfaces, whose maximum drug eluted was indeed less than 2 μg cm\(^{-2}\) in all cases (see Fig. S1 and S2 in the ESI†). The eluted quantity increased, by increasing temperatures, by a factor of 14% for the low loading rate. Indeed, by comparing maximum eluted quantities at the highest loading rate, it results that 34 μg cm\(^{-2}\) were effectively released at 37 \(^\circ\)C, which is 55% of 62 μg cm\(^{-2}\) loaded, while 16 μg cm\(^{-2}\) were eluted at the lower temperature. 95% of the 17 μg cm\(^{-2}\) being effectively loaded, see Table 1. This increase in drug-eluted quantities is not unexpected since higher temperatures correspond to higher probability to overcome the energy barrier related to surface or intermolecular interactions. Eluted quantities from the 20 μg impregnated samples were correlated to what eluted from 10 μg platforms at the same temperature. We found that for any effective loading accomplished, a linear relationship correlates the two eluted values at each temperature, which suggests that the mechanism of elution is concentration independent in the concentration range investigated, which allows a comparison of elution results obtained at different filling concentrations (see the ESI†).

A first order kinetic model was adopted to interpret the obtained master release curves reported in Fig. 3,

\[
M(t) = M_0 + M_c(1 - \exp(-Ct))
\]

where \(M(t)\) represents the eluted quantity by the time \(t\), and \(M_0\) and \(M_c\) represent the initial fraction eluted in the burst phase and in the slow phase respectively. Thus, the main effects of the increase in temperature resulted in an increase in the mass fraction eluted in the burst phase of about 44%, and a speed up of the

![Fig. 1 Scanning electron microscopy images of TiO\(_2\) nanotubes. Left, top view, \(d = 110\) nm, scale bar represents 100 nm. Middle, top view, scale bar represents 200 nm. Right, side view, \(h = 800\) nm, scale bar represents 1 μm.](image-url)
Table 1  Comparison of loading capacity expressed in μg cm⁻² for different loading conditions and fitting parameters obtained

<table>
<thead>
<tr>
<th>Loading method</th>
<th>Load efficiency %</th>
<th>Effective load [μg cm⁻²]</th>
<th>Released fraction %</th>
<th>C [day⁻¹]</th>
<th>M₀ [μg cm⁻²]</th>
<th>Mᵢ [μg cm⁻²]</th>
<th>Mᵢ [μg cm⁻²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>23 S 2.5 μg</td>
<td>6 ± 3</td>
<td>0.9 ± 0.2</td>
<td>80</td>
<td>0.14 ± 0.06</td>
<td>—</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>VI 10 μg</td>
<td>12 ± 9</td>
<td>11 ± 4</td>
<td>94 ± 5</td>
<td>0.032 ± 0.005</td>
<td>1.8 ± 0.2</td>
<td>9.4 ± 0.6</td>
<td>11.2 ± 0.8</td>
</tr>
<tr>
<td>VI 20 μg</td>
<td>23 ± 7</td>
<td>17 ± 6</td>
<td>95 ± 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 VI 10 μg</td>
<td>22 ± 3</td>
<td>12 ± 2</td>
<td>96 ± 3</td>
<td>0.064 ± 0.009</td>
<td>2.6 ± 0.4</td>
<td>9.2 ± 0.5</td>
<td>11.8 ± 0.8</td>
</tr>
<tr>
<td>VI 20 μg</td>
<td>49 ± 2</td>
<td>62 ± 1</td>
<td>55 ± 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*S stands for soaking, VI represents vacuum impregnation. M₀, Mᵢ and Mᵢ represent the initial fraction eluted in the burst phase and in the slow phase respectively, while Mᵢ is the total quantity to be released.

Fig. 2  Comparison of doxorubicin eluted fraction with respect to the effective quantity loaded in nanotube arrays in two different ways. Empty squares correspond to eluted fraction from platforms soaked in 500 μl PBS of Dox 9.2 μM at 23 °C for 24 h, over days of release. Full right orange squares represent eluted fraction from 10 μg impregnated samples at 23 °C over days of release. Lines represent single-phase elution model fitting (C₁ = 0.03 day⁻¹, C₂ = 0.15 day⁻¹).

Fig. 3  Doxorubicin master elution profiles for nanotube arrays and for control flat platforms (full right symbols represent 10 μg loaded samples, empty right symbols 20 μg evaluated from master curves). Orange squares represent elution profiles at 23 °C. Red squares represent elution profiles at 37 °C. Data are presented as average values of two–three samples; average standard deviation at 23 °C (not reported for clarity) are 16%, while 28% at 37 °C. Lines represent single-phase elution model fitting (C₂3 = 0.032 day⁻¹, C₃7-C = 0.064 day⁻¹).

elution process. The kinetic parameter C is indeed doubling, going from 23 °C to 37 °C, and in particular, the characteristic time of the elution process attains about 30 days in the first case, while only 15 in the second one. This finding is aligned with a temperature activated elution mechanism. From the doubling of the elution velocity in a 14 K range, it is possible to compute the activation energy of the elution process which is about ~47 kJ mol⁻¹. From a straight comparison of our results with previous reports regarding soaked samples, it is clear that the activation energy of the process attained a lower value of ~27 ± 1 kJ mol⁻¹, aligned with the reported faster release process of soaked drugs.¹⁹ This result is reasonably ascribable to the different loading technique adopted, soaking in place of impregnation, coherently with our findings of a much faster release of doxorubicin in soaked platforms compared with what was found for impregnated ones.

In addition, molecular motion within the confined geometry at the single molecule level was analyzed in order to relate mobility to drug localization. We conducted FCS measurements of doxorubicin diffusion within nanotube arrays, both drug soaked and impregnated. In FCS, the fluctuating intensity is analyzed in terms of the intensity Autocorrelation Function (ACF) and then fitted to a biophysical model G(τ) (see the ESI†).²³ ²⁴ The ACF recorded in soaked nanotubes showed a higher G(0) compared to that measured in impregnated platforms. Since G(0) = 1/(N), with (N) the mean number of particles, the analysis confirms that the amount of drug loaded through soaking procedures is somewhat lower than what is obtained by impregnation (see Fig. S5 in the ESI†). In order to compare dynamics, normalized ACFs of doxorubicin diffusing in PBS bulk solution and in differently loaded TiO₂ nanotubes are shown in Fig. 4. The decorrelation time in NT is higher than in bulk, as indicated by the shifting towards longer decorrelation times of the confined ACFs. The restricted environment might exert a significant motion hindrance to doxorubicin in nanotubes, even if the molecule confinement ratio, defined as the molecule size over tube diameter, is too small to determine the single file diffusion onset.²²

Prior to the discussion of this finding, it should be stressed that in confined systems the detection volume is restricted by the unconventional geometry of the material. In particular, nanotubes restrict the detection volume to the nanotube diameter, thus resulting in a 1D diffusional system. Indeed, an inner channel diffusive mechanism would correspond to a 1D diffusive behavior, since molecule lateral displacements along the nanochannel diameter would not produce any intensity fluctuations due to the nanometric size of the diameter.²⁸ Also, movements along the nanotube outer surface would produce 1D fluctuations. In the case of inter channel diffusion, a lateral
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Fig. 4 Normalized ACF of 150 nM doxorubicin in PBS recorded in bulk (red circle), in titania nanotube arrays soaked in doxorubicin (empty gray square), and of doxorubicin vacuum impregnated (orange square). Fitting curves are shown for both ACF recorded in NTs: single component 1D fitting curve (dashed line), and two components model of both 1D and 2D contributes (red line). Doxorubicin diffuses in the inter-channel space preferentially in soaked samples where the ACF has a 2D shape, while occupying also the tube interior in VI samples attaining a 1D-like ACF shape.

displacement is permitted as well, which would induce fluorescence decorrelation in a 2D system. Since molecules that diffuse along the nanotubes vertical axis only contribute to a 1D fluctuation along the z axis, and those occupying the outer space mainly fluctuate in a 2D system filled of obstacles, the ACF due to both contributions reads

\[
G(t) = \frac{1}{\langle N \rangle} \left( 1 + \frac{\theta e^{-t/\tau_{\text{F}}} - \theta}{1 - \theta} \right) \left( 1 - F \right) \left( \frac{1}{1 + \left( t/\tau_{\text{diff,xy}} \right)} \right) + F \left( \frac{1}{1 + s^2(t/\tau_{\text{diff,z}})^2} \right)
\]

(2)

where \( \theta \) is the triplet kinetics fraction, \( \tau_F \) is the triplet time, \( F \) is the fraction of molecules diffusing along the nanotube vertical axis, and \( \tau_{\text{diff,xy}} \) and \( \tau_{\text{diff,z}} \) are the two decorrelation times in the \( xy \) lateral plane and along the \( z \) vertical axis respectively. However, the ACF shape recorded in soaked nanotubes does not report a one dimensional diffusion shape, which indicates that molecules do diffuse in the inter-channel space preferentially. The curve was indeed best fitted to a single component 2D diffusion process, resulting in a diffusion time of 105 ± 4 \( \mu s \). The ACF was also forced to fit to the two components model, having a first 1D component diffusing along the nanotube axis only, and a second 2D component diffusing among the channels. Fitting quality was indeed comparable with the single component 2D model. The fraction moving along the nanotube vertical axis was found to be less than 10%. In addition, since doxorubicin bulk diffusion ACF fitted to a 3D single component diffusion model achieved a drug diffusion time of 20 ± 3 \( \mu s \), the recorded slow down factor \( \tau_{\text{NT}}/\tau_{\text{bulk}} \) amounts to around 5. The diffusion time recorded is indeed related to the motion within a periodic nanometric structure of tubes, which hinders motion.

On the other hand, FCS could resolve a deeply different mechanism from impregnated samples, whose ACF was best fitted to eqn (2), resulting in a main fraction of 97% diffusing in a 1D fashion, having a characteristic diffusion time of 2.5 ± 0.4 ms, which is ascribable to molecules diffusing along the nanotube vertical axis, and only 3% of molecules diffusing in a 2D fashion, to be related to dynamics occurring in the interchannel space. In order to compare the vertical axis diffusion time to the bulk later mobility, \( \tau_{\text{diff,z}} \) is normalized over the \( s^2 \) dimension, giving 96 ± 15 \( \mu s \). Indeed, the computed delay is partially ascribable to the different length scales probed. In addition, a further apparent delay arises due to the presence of walls inside the confocal volume. Indeed, we demonstrated that whenever FCS measurements are carried out in proximity of barriers, several different scenarios can arise, all causing the apparent increase of molecule diffusion time. In loaded nanotubes the focus distance from the bottom wall of the tubes was around 400 nm, which is smaller than the characteristic focal volume size, thus determining a wall distance/\( f_z \) ratio of about 0.4. This affects the probed molecular mobility since the distance to be covered in order to escape the detection volume along the available side becomes longer. Indeed, the diffusion time is apparently delayed by a factor of 2.05, as computed from our modelling of FCS in wall proximity, giving a normalized value of 47 ± 8 \( \mu s \) (see the ESI†). The estimated value is only twice the doxorubicin bulk diffusion time, which can be easily justified in confined systems.

A slower second diffusion component, representative of a desorption processes, was not resolved in the fitting procedure, which could rather indicate that its characteristic diffusion time is at least on the second time scale, thus not resolvable by FCS. This large time scale would not be unexpected since the characteristic time of desorption is on the order of several hours, as shown in the release profiles.

As a result, doxorubicin molecules dwell in inter-nanochannel spaces preferentially in the case of soaked samples, indicating that a majority of loaded drugs occupy the external space, whereas impregnated drugs mostly occupy the nanotube inner volume at the elution time investigated.

**Experimental methods**

**Nanotube production**

Titanium medical grade 2 foils of 0.5 mm in thickness were rinsed in DI water and acetone and then anodized under a constant voltage of 25 V in inorganic solution (10 wt% H\(_3\)PO\(_4\), 0.5 wt% HF). After 90 min of anodization, a regular distribution of nanotubes showing an average 100 nm in diameter and 800 nm in length was obtained. Foils were rinsed in DI water cut into square platforms of 0.25 cm\(^2\) and air-dried. Control tests were conducted using Ti samples as received.

**Drug loading**

Drug loading was carried out in two different ways in order to compare loading efficiencies. Nanotubes and bare samples were soaked in 9.2 \( \mu M \) doxorubicin (Dox, Abur Oncology, UK) water solution of 500 \( \mu l \) for 24 h and then placed in 500 \( \mu l \) of fresh PBS. Another group of platforms was loaded via wet vacuum impregnation, which is a simplified lyophilization method. Platforms were first soaked in ethanol, rinsed in DI water and then air-dried. Water solutions of doxorubicin (Dox, Abur Oncology,
UK) of 1.84 mM were prepared. Volumes of 2 μl of the drug were pipetted onto the platform surfaces and spread to obtain even drug coverage. The samples were left to dry under vacuum at room temperature. The filling step was repeated until the desired amount of drug, 10 or 20 μg of doxorubicin, was loaded into the nanotube array and respectively on the flat controls. After a final 2 h drying step, platforms were quickly rinsed with 500 μl of PBS (D-PBS Euro Clone) and the rinsed solution was later analyzed. The rinsing step was achieved at 23 °C or 37 °C according to the elution temperature. Loading efficiency \( \eta \) was evaluated as \( \eta = (C_0 - C_f)/C_0 \), where \( C_0 \) represents the loading concentration and \( C_f \) the measured rinse concentration, while the effective load was computed as \( C_0 \eta/A \), where \( A \) is the platform area. Platforms were then placed in 500 μl of fresh PBS solution to monitor doxorubicin release in vitro.

**In vitro doxorubicin release**

Impregnated samples were incubated in PBS at 23 °C and 37 °C to monitor the temperature influence on the release kinetics, and followed up to two months. The external medium was changed periodically to ensure maintenance of sink conditions. Samples of 100 μl were taken after specific intervals of time to determine the doxorubicin amount. The solution was replaced with 100 μl of fresh PBS at every sample collection. The drug content was analyzed by a calibrated spectrofluorimeter at 485–535 nm for soak samples. Single molecule measurements, through FCS analysis, clarified that drug localization is profoundly different in the two loading methods adopted, and in particular, drug filled nanotube interior in impregnated samples, while mainly occupying the nanotube outer space in soaked arrays. The possibility of unraveling drug localization from the characterization of drug mobility through FCS measurements in hollow nanostructures could be generally exploited for the analysis of nanostructures employed in diagnostics and as drug release devices.

**Fluorescence correlation spectroscopy diffusion measurements**

A PBS solution of 150 nM Doxo was prepared and analyzed through FCS both in free solution and in loaded TiO\(_2\) nanotubes at 10 days of elution. Loaded nanotube arrays were placed on a coverslip facing the inverted water immersion objective lens. Focus was adjusted at around 400 nm inside the tubes. A confocal fluorescence correlation microscope, ConfoCorII (Carl Zeiss, Jena, Germany) was used to carry out FCS experiments. A 488 nm laser beam was focused by an Apochromat 40 × 1.2 water immersion objective to obtain a confocal fluorescence correlation microscope. Focal volume parameters were fit from ACF curves of 10 nM Rh6G dye diffusing in water (diffusion coefficient 4.0 × 10^{-6} cm\(^2\) s\(^{-1}\) at 23 °C)\(^{10}\) to obtain \( r_{xy} \) and \( s \), which measured 0.19 ± 0.01 μm and 5.1 ± 0.2 respectively.

**Conclusions**

Nanostructured coatings manufactured through anodization procedures on titanium foil surfaces and loaded with doxorubicin via soaking and wet vacuum impregnation methods were analyzed through both macroscopic observations and single molecule analyses in order to assess drug–surface interaction strength and drug localization influence upon the elution profile. Different elution release profiles were obtained depending on the loading method adopted. Indeed, in the case of doxorubicin loaded by soaking procedures, the characteristic time of the elution process is around seven days while attaining a length of 30 days in the case of vacuum impregnation. The activation energy of doxorubicin elution from the VI nanotube surface achieved a much higher strength compared to what was obtained for soaked samples. Single molecule measurements, through FCS analysis, clarified that drug localization is profoundly different in the two loading methods adopted, and in particular, drug filled nanotube interior in impregnated samples, while mainly occupying the nanotube outer space in soaked arrays. The possibility of unraveling drug localization from the characterization of drug mobility through FCS measurements in hollow nanostructures could be generally exploited for the analysis of nanostructures employed in diagnostics and as drug release devices.

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**Notes and references**