OPTIMAL SURFACE TOPOGRAPHY FOR CELL ADHESION IS DRIVEN BY CELL MEMBRANE MECHANICS

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ABSTRACT

Titanium surface treated with titanium oxide nanotubes was used in many studies to quantify the effect of surface topography on cell fate. However, the predicted optimal diameter of nanotubes considerably differs among studies. We propose a model that explain cell adhesion to nanostructured surface by considering deformation energy of cell protrusions into titanium nanotubes and adhesion to surface. The optimal surface topology is defined as a geometry that gives membrane a minimum energy shape. A dimensionless parameter, the cell interaction index, was proposed to describe interplay between the cell membrane bending, intrinsic curvature and strength of cell adhesion. Model simulation show that optimal nanotube diameter ranging from 20 nm to 100 nm (cell interaction index between 0.2 and 1, respectively) is feasible within certain range of parameters describing adhesion and bending energy. The results indicates a possibility to tune the topology of nanostructural surface in order to enhance proliferation and differentiation of cells mechanically compatible with given surface geometry while suppress the growth of other mechanically incompatible cells.

Keywords titanium; nanotubes; biomechanics; adhesion; surface energy; cell membrane; bending

1 Introduction

Strong bonds between the implant and bone cells [12] is required for the long-term stability of the implant in the human body [3]. It was shown that bone cellular response is directly affected by titanium surface characteristics like roughness, chemistry, wettability or more recently studied surface topography [4]. Various methods for surface modification were employed in order to promote cell–substrate interactions [56].

The anodic oxidation is adopted to create a nanostructured titanium surface [7] by formation of TiO₂ nanotubular structures (TNTs) [38] (Fig. 1A). TNTs increase surface area that favors bone deposition and could improve therapeutic efficiency by serving as a reservoir for drug delivery [910]. The advantage of anodic oxidation is that the diameter, the wall thickness and the length of TNTs can be controlled by the process variables such as electrical current power, anodization time, temperature, applied potential, and electrolyte chemical composition [71112]. TNTs length can range from 0.1 up to 1000 μm while the inner diameter can range from 7 to 150 nm [31314].

K E Y W O R D S  t i t a n i u m ; n a n o t u b e s ; b i o m e c h a n i c s ; a d h e s i o n ; s u r f a c e e n e r g y ; c e l l m e m b r a n e ; b e n d i n g
Surface treated with TNT array present a controlled environment that allows to quantify the effect of surface topography on cell fate [15]. Nanotube diameter, rather than the other characteristics of the surface layer, exhibits critical impact on cell adhesion and proliferation [8,9,16-18]. It was further suggested that there exists an optimal diameter for TNTs that enhance osteointegration [19]. However, estimated values of the optimal diameter are contradictory. Park et al., 2017 [16] reported the optimal nanotube diameter to be 15 nm based on mesenchymal stem cell proliferation on TNT surface. They also report that the cell adhesion and spreading decreases on TNT layers with a tube diameter larger than 50 nm. Yu et al., 2010 [20] found that MC3T3-E1 preosteoblast adheres well on TNTs of diameter 20–70 nm while the cell attachment is low on TNTs of diameter 100-120 nm. Similar behavior was observed for osteoblast-like MG-63 cells that exhibit higher spreading on 30 nm TNTs whereas the larger diameter of 90 nm had the worst cell viability [9]. Both glioma and osteosarcoma cells exhibit optimal cell adhesion, migration, and proliferation on 20 nm TNTs [18]. Limited spreading on larger diameter TNTs was also reported for malignant cancer cells (T24) of urothelial origin [2].

Osteogenic differentiation of primary rat osteoblasts was observed on 35 nm (amorphous phase) and 41 nm (anatase phase) surface [21]. Das et al., 2009 [22] found 2–3 fold increase in human osteoblast attachment and spreading on 50 nm-diameter TNTs surfaces in comparison to flat Ti samples. Oh et al. reported improved adhesion of hMSC on 30 nm TiO$_2$ nanotubes and improved osteogenic differentiation on nanotubes with a diameter of 70 and 100 nm [23,24]. MC3T3-E1 osteoblast cells accelerates in the growth on 70 nm TNTs [25]. Brammer et al, 2009 [26] proposed that bone-forming ability of osteoblasts is higher if grown on TNTs of 100 nm diameter. Also Filova et al, 2015 [27] and Votrova et al, 2019 [28] concludes that optimal diameter of TNTs is around 70 nm for Saos-2 osteoblast-like cell (Fig. 1B).

Figure 1: (A) Titanium nanotubes on cpTi of average diameter 66 ± 17 nm and length 1097 ± 75 nm. (B) Immunofluorescence staining of talin in human Saos-2 osteoblast-like cells on nanostructured surface. (C) Schematic view of cell anochored into nanostructured surface. A,B adopted from Votrova et al, 2019. [28]

The divergence in results could be either caused by variations in surface topography and chemistry, due to individual fabrication protocols or by methods to assess cellular activities [29]. It is also likely, that the type of cell line affect optimal TNT’s diameter [19]. While the preference of cells to small diameter TNTs (up to 30 nm) could be explained by integrins packing [16,30], mechanism of adherence to large diameter has not been explained yet. It was suggested that migration of the cell membrane inside the crystalline nanotubes could be crucial for strong attachment [34,35]. The cell protrusions into nanotubes could strengthen the adhesive interaction of cells with the surface, and thereby potentially trigger cellular cascades that regulate cell behavior and differentiation [31]. Cell protrusion into TNTs increases contact area for attachment but requires extensive membrane deformation into tubular like structure (Fig. 1C). The aim of the present study is to quantify the overall energy cost of formation of cell protrusion into TNT. The hypothesis based on the experimental results is, that there exist an optimal diameter given by minimum of membrane protrusion energy.

2 Methods

The membrane protrusion into hollow nanotubular structure is assumed to be axisymmetric and its dimensions are determined by the shape of the nanotube. The membrane therefore forms a hollow cylinder of diameter $d$ closed by a hemispherical cup and joined to the central body along the contour shown in Fig. 2. Two energy contribution are considered in mechanics of nanotubular protrusion: the adhesion energy $F_a$ between the TNT inner surface and membrane and the deformation energy $F_b$ of the cell membrane [32].

The adhesion energy is defined as the excess energy released after the cell attaches to the surface [11]. Surface energy quantifies the formation of intermolecular bonds and depends on the contact area $A$ with a proportionality constant $\gamma$. According to our model (Fig. 2), the cell membrane is in contact with the TNT only in its central tubular segment (II). The adhesion energy could therefore be expressed as

$$F_a = -\gamma \pi dl$$

(1)
Figure 2: Parametrization of membrane protrusion into TiO$_2$ nanotube of diameter $d$. The shape is divided into three parts: (I) the spherical cup of radius $d/2$, (II) the cylindrical segment of length $l$ and radius $d/2$ and (III) the axisymmetrical collar. Principal curvature $R_1$ and $R_2$ are depicted in individual segments.

where the minus sign denotes energy release after adhesion.

Formation of protrusion requires deformation of the membrane from the mostly planar shape into the shape of thin cylinder. The bending energy of the membrane is commonly described by Helfrich energy [33]. The elastic strain energy proposed by Helfrich depends on the mean ($H$) and Gaussian curvature. As we do not expect the change in cell topology by protrusion formation, the Gauss term could be neglected because of Gauss-Bonnet theorem [34].

$$F_b = \frac{1}{2} k_b \int_A (2H - C_0) dA$$

where $k_b$ is the bending modulus of cell membrane and $C_0$ is the spontaneous curvature. Spontaneous curvature, or more precisely the spontaneous mean curvature, present a penalty for the mean curvature asymmetry [35]. The mean curvature can be expressed as an average of principal curvature values $C_1$ and $C_2$ defined as the inverse values of corresponding radii of curvatures $R_1$ and $R_2$, respectively (Appendix A).

In order to get insight into the interaction between bending and adhesion, we will analyze equilibrium of part II in Fig. 2. Contribution of part I and III could be neglected if the length of the cylinder $l$ is much greater than the diameter, i.e. $l \gg d$. The free energy is expressed from Eqs. (1) and (3).

$$F = \frac{1}{2} k_b \pi l \left( \frac{4}{d} - 4C_0 + C_0^2 d \right) - l \gamma \pi d$$

The central assumption is, that the membrane attains a shape that minimizes the overall energy. We may further assume, that there exist an optimal diameter that corresponds to energy minimum. The minimum of the energy present a stationary point and could be expressed using interior extremum theorem.

$$d_0 = \sqrt{\frac{4k_b}{k_b C_0^2 - 2 \gamma}}$$

The value of optimal diameter depends on adhesion constant and bending rigidity of the membrane. The optimal diameter exists if intrinsic curvature is higher than a threshold value. We denote this value as a critical curvature $C_{\text{crit}}$.

$$C_{\text{crit}} = \sqrt{\frac{2 \gamma}{k_b}}$$

To describe interaction between the cell protrusions and nanostructured surface, we define a dimensionless number $I_c$ denoted as cell interaction index.

$$I_c = \frac{C_{\text{crit}}}{C_0}$$

As shown above, the energy of cell membrane TNT interaction depends on mechanical properties of membrane described by the bending modulus $k_b$ and the spontaneous curvature $C_0$ and on interaction between membrane and TNT surface described by density of surface energy $\gamma$. The bending modulus of the cell range from 5 $k_B T$ for phospholipid
membrane to 200 $k_B T$ for cells, where $k_B$ is the Boltzmann constant. In the previous study of osteoblasts mechanics, the value of 100 $k_B T$ was used to describe osteoblasts bending rigidity. The cell binding energy per unit area $\gamma$ may range from 0.05 to 56 mJ m$^{-2}$ for various cell types. The spontaneous curvature of the cell membrane is determined by lipid composition and interactions between lipids and proteins. It could have either positive values (intrinsic bending inwards) or negative values (bending outwards). It was reported that lipid bilayer spontaneous curvatures ranges from -0.2 to 0.2 nm$^{-1}$.

### 3 Results

The existence of optimal diameter of cell for attachment into TNTs depends on the difference between the spontaneous $C_0$ and the critical curvature. If $C_0$ is lower than $C_{\text{crit}}$, there is no optimal diameter and cells migrate into TNTs larger than threshold. However, if $C_0$ is higher than $C_{\text{crit}}$, there exists a limited range of TNTs’ diameters in which the formation of membrane protrusion is energetically convenient (Fig. 3C,D). For higher spontaneous curvature, the TNTs’ optimal diameter range is smaller and the energy rises considerably for larger diameters (Fig. 3D).

Figure 3: Free energy of membrane protrusion ($F$) into TNT: (A,B) spontaneous curvature $C_0$ is lower than the critical curvature $C_{\text{crit}}$, (C,D) spontaneous curvature $C_0$ is higher than the critical curvature $C_{\text{crit}}$. Gray region indicate area where formation of protrusion is energetically favorable. The critical curvature $C_{\text{crit}} = 35 \mu$m$^{-1}$ and energy is calculated for $k_b = 100 k_B T$, $\gamma = 0.25$ mJ m$^{-2}$, $l = 1000$ nm for protrusion shape shown in Fig. [1].

The critical curvature is a function of binding energy per unit area $\gamma$ and bending stiffness of membrane $k_b$ (Eq. [5]). Increase in adhesion strength (Fig. 4A) and decrease in bending stiffness (Fig. 4B) enhance formation of cylindrical protrusion by lowering membrane free energy. For stiff membrane or limited adhesion between the cell membrane and the TNTs’ wall, the migration of membrane into TNTs’ is not likely to happen spontaneously (Fig. 4A,B).

Figure 5 shows the dependence of the optimal diameter of TNT ($d_0$) on the cell interaction index $I_c$, Eq. [6]. For small values of $I_c$, the contact between nanostructured surface and cell will not be formed as the energy required to bend the membrane is higher than the energy gained in forming adhesion bonds. For $I_c$ between 0.2 and 1, the optimal topology exists and it depends on the spontaneous curvature. Cells with high spontaneous curvature will prefer smaller diameter of TNTs. If $I_c$ is higher than one, the cell will prefer smooth surface against curved one.
Figure 4: The effect of (A) surface energy density $\gamma$ and (B) bending modulus of the membrane $k_b$ on free energy minimum for $C_0 = 50 \mu m^{-1}$ and $l = 1000$ nm. Solid line correspond to Fig. 3C. Optimal diameter is depicted for each curve.

Figure 5: The optimal diameter as a function of interaction index $I_c = C_{\text{crit}} / C_0$.

4 Discussion

We have hypothesized, that the cell membrane mechanics determines optimal topology of titanium nanostructured surface. The optimal surface topology is defined as a geometry that forms membrane into minimum energy shape. Cell membrane free energy accounts for the cost of the bending energy and the gain in the adhesion energy. The model explains previous experimental studies providing ambiguous values of optimal diameter of TNTs for cell growth. Model simulation show that either small diameters as observed by Park et al, 2007 [16] (Fig. 3D) or larger diameters reported by Brammer et al, 2009 [26] (Fig. 3C) are feasible within certain range of parameters describing adhesion and bending energy.

Model analysis indicate that the spontaneous curvature relative to critical curvature (Eq. 5) determines existence of optimal surface topology given by optimal diameter $d_0$. However, critical curvature value does not discriminate whether the cell is or is not attached to the surface. Therefore, we have defined a new dimensionless parameter describing the interactions between the cells and the nanostructured surface, the cell interaction index $I_c$ (Eq. (6)). The cell interaction index shows, that a certain parameters range describing cell mechanics predispose the cell to form stable protrusions into nanostructured surface of specific topology. For example, it was reported that proliferation of vascular smooth muscle cells is higher on flat TiO$_2$ surface while the endothelial cells prefer TNT surface [41, 42]. Experimental study
According to Fig. 3D, no protrusion is formed (F > I_c) in agreement with experiment. On the other hand, preference to curved nanostructured surface can be caused either by high bending modulus, low adhesion or high spontaneous curvature. The latter was reported to be high in endothelial cells [43]. The preference to small-diameter TNT surface was also observed in cancer cells [2, 18] that generally have lower adhesion strength [44] and therefore low I_c in Fig. 5.

However, if the adhesion is too low or bending rigidity too high (I_c close to zero), the cell will not adhere to the surface (Fig. 5). For example, TNT surface decrease the adherence of all bacteria [45, 46]. Gram-positive bacteria is surrounded by a bacterial wall of stiff glycan strands cross-linked into lipid bilayer [47]. This composite structure considerably increases bending rigidity [48]. High bending rigidity implies low I_c and limited protrusion into TNT wall (Fig. 5). For bacteria, TNT surface has small contact area restricted to the terminal ends of nanotubes as protrusion formation is energetically unfavourable.

Previous studies on TNT bioactivity focus mostly on material properties like surface chemistry, crystallinity, nanotube size, or water contact angles. The current study supplements previous research by study the adhesion from the perspective of the cell while the cell-substrate interactions are described by the binding energy (Eq. 1). As-synthesized TNT are an extension of the amorphous TiO_2 layer [5] and after heat treatment the crystallinity of TiO_2 is improved [45]. Titanium crystallinity (amorphous versus anatase structures) improves mechanical strength and increases hydrophilicity, which might improve cell adhesion and proliferation [21, 49]. Our results indicate, that the high cell adhesion itself (higher I_c) is required for cell attachment, but not inevitable for having an optimal TNT diameter. The same holds for the water contact angle that is another measure of surface energy [11].

The model was intentionally kept simple for clarity. However, there are many other parameters and mechanisms that could be considered in description of cell-nanosurface interactions. The adherence is described by a single adhesion energy constant γ. The cell adhesion is a complex process facilitated by charged protein-mediators [30]. The adhesion of proteins is shown to be higher for larger diameter TNT that could further facilitate adhesion [50]. The increase in surface charge could enhance protein adhesion and promotes osteoblast cell proliferation [51]. Spontaneous curvature of the membrane C_0 is one of the main investigated parameters within the current study (Figs. 3, 5). While the spontaneous curvature of lipid bilayer is mostly determined by its lipid composition, local spontaneous curvature is driven by trans-plasma-membrane or peripheral proteins [40]. Therefore, the spontaneous curvature may not be constant, but it is likely to change along protrusion. In addition, proteins not only generate curvature, but can also sense membrane curvature [52] and accumulate at curved membrane area [53]. Similarly, microgrid topography of TiO_2 stimulated hMSC adhesion and spreading area while nanotopography favoured hMSC motility, and osteogenic differentiation [54].

It is well accepted that the deformation of cell membrane, interacting with the attached cytoskeleton, affects cell proliferation and differentiation [37]. For cell adhesion, complex network of transmembrane integrins and cytoplasmic proteins is of utmost importance [55]. Extracellular components of integrins attach to extracellular matrix while their intracellular components are attached to F-actin through adapter proteins [56] and may directly affect cell nucleus shape [57]. Park et al, 2007 [16] proposed a hypothesis, that optimal diameter of nanotubes is determined by integrin size. The size of extracellular domain of integrins is about 10 to 12 nm [56] and thus close integrins packing results in optimal integrin activation. This hypothesis is supported by the measurements showing that the 15-20 nm spacing is optimal for cell adhesion, proliferation, migration, and differentiation [16, 19]. This theory was further implemented into the mathematical model of osteoblast adhesion [30]. The model well explains narrow window of optimal diameter observed by Park et al, 2009 [19] but cannot explain stability of larger diameters [8].

The integrins are also sensitive to the membrane mechanical state including the curvature [58]. It was shown, that higher concentration of integrins occurs at the neck of protrusive podosome-like structures if the substrate is porous [59]. Podosome neck correspond to part III in Fig. 2. It is reasonable to assume, that the same shape of membrane within TNT will provide similar accumulation of integrins. It was proposed, that negative membrane curvature increases separation of integrin cytoplasmic tails, which is known to promote integrin activation [60]. Therefore we complement a hypothesis of Park et al, 2007 [16] by adding the role of membrane protrusions into TNTs. The nanostructured protrusion induce negative curvature in the neck (part III in Fig. 2). Area of negative curvature results in accumulation of integrins and their activation. Actin filaments transmit the focus adhesion signal to the nucleus activating nuclear mechanotransduction pathways [57]. Park et al, 2017 observed no focal contact formation for larger TNT diameters. According to Fig. 3D, no protrusion is formed (F > 0) and therefore no region of negative curvature enhancing focal contact exists. This theory is in agreement with molecular dynamics simulation showing that nanopore-induced membrane curvature increases bioactivity locally at the neck region [61].
Figure 6: Mechanism of integrin activation at negative curvature area caused by cell membrane migration into TNT. Actin transmit the information on focal adhesion to nucleus.

5 Conclusions

The formation of membrane protrusions into TiO$_2$ nanotubes was assessed by means of cell membrane free energy. Dimensionless parameter, the cell interaction index $I_c$, was introduced to describe interplay between the cell membrane mechanics and the nanostructured surface topology. If $I_c$ is close to zero, no membrane protrusions are formed and no cell adhesion occurs. For $I_c$ greater than one, the cells prefer flat surface. For $I_c$ approximately between zero and one, there exist an optimal diameter of TNT for given cell line. This study provides a theoretical basis explaining ambiguous results of experimental studies reporting wide range of suitable TNT diameters. It was proposed, that negative curvature region at the neck of membrane protrusion may result in integrin activation and subsequent cell proliferation. The results indicates a possibility to tune the topology of nanostructural material in a way to enhance proliferation and differentiation of one cell type that is mechanically compatible with given surface geometry while suppress the growth of other mechanically incompatible cells.

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A Bending energy of membrane protrusion

Energy of membrane forming tubular structure depends on curvature of individual parts depicted in Fig. 2. According to the curvature, the membrane protrusion could be divided into three parts (Fig. 2). The first segment correspond to the hemispherical cup, where both principal curvatures equals to $2/d$ and the energy of the first segment could be expressed as

$$F_{bI} = k_b \pi \left( 4 - 2dC_0 + \left( \frac{dC_0}{2} \right)^2 \right)$$

(7)

The free energy of the central cylindrical part (Fig. 2 II) is determined by its length $l$ while the first and the second membrane curvature are $2/d$ and $0$, respectively.

$$F_{bII} = \frac{1}{2} k_b \pi l \left( \frac{4}{d} - 4C_0 + C_0^2 d \right)$$

(8)

The last part presents a neck, that connect a protrusion to the cell. The neck is modeled as axisymmetrical structure with one radius of curvature equal to $\rho$ (Fig. 2 III). The first curvature is negative as the membrane bends outwards, $C_1 = -1/\rho$. The second radius of curvature depends on the distance from axis of symmetry and could be expressed as $C_2 = \sin(\varphi)/(d/2 + \rho (1 - \sin(\varphi)))$ \cite{62} where $\varphi$ is defined in Fig. 2. For the sake of simplicity, we further assume that the radius $\rho$ equals to $d/2$. The energy could be expressed after integration of Eq. 2 over the part III as

$$F_{bIII} = \frac{1}{2} k_b \pi d \left( \frac{\pi C_0^2 d}{2} - \frac{C_0^2 d}{2} + \frac{16 \pi}{3^2 d} - \frac{8}{d} + 2 \pi C_0 - 4C_0 \right)$$

(9)

The total energy can be expressed as a sum of Eq. (1) and Eqs. (7)–(9).