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WESLLEY FELIX DE OLIVEIRA

AVALIAÇÃO DA ADESÃO E PROLIFERAÇÃO CELULAR EM NANOTUBOS DE DIÓXIDO DE TITÂNIO FUNCIONALIZADOS COM A LECTINA CRAMOLL 1,4

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração Biotecnologia, da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Mestre em Ciências Biológicas.

Orientadora: Profª. Drª. Maria Tereza dos Santos Correia

Co-orientadoras: Drª. Giovanna Machado e Drª. Germana Michelle Medeiros e Silva

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BANCA EXAMINADORA

_____

Profª. Drª. Maria Tereza dos Santos Correia
Departamento de Bioquímica – UFPE (Presidente)

_____

Profª. Drª. Adriana Fontes
Departamento de Biofísica e Radiobiologia – UFPE (Examinador 1)

_____

Prof. Dr. Paulo Euzébio Cabral Filho
Departamento de Biofísica e Radiobiologia – UFPE (Examinador 2)

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“O potencial para novas ideias é o mesmo para todas as pessoas. Resta saber como e onde aplicá-lo; além de disciplina, determinação e empenho para transformar o potencial em ações concretas.”

Robson Feitosa

RESUMO

Implantes ortopédicos e dentários são usualmente fabricados com o titânio (Ti) por causa de sua biocompatibilidade. Os implantes estão susceptíveis a uma ineficaz e demorada integração com o tecido ósseo circundante, osseointegração, podendo prolongar o processo de cicatrização e propiciar infecções no local. Os nanotubos de dióxido de titânio (TNTs) têm sido uma estratégia de modificação de superfícies de implantes de Ti. Ademais, as propriedades osteogênicas dos TNTs podem ser melhoradas pela sua funcionalização com moléculas bioativas. Portanto, o presente trabalho objetivou imobilizar a lectina purificada das sementes de *Cratyllia mollis* (Cramoll 1,4) em TNTs revestidos com filmes automontados dos polieletrólitos hidrocloreto de polialilamina (PAH) e ácido poliacrílico (PAA). Por conseguinte, verificar a ação das diferentes superfícies sobre a adesão e proliferação de células semelhantes a osteoblastos. Foi objetivado também realizar uma revisão detalhada na literatura sobre como as biomoléculas podem ser usadas para funcionalizar os TNTs e melhorar a aplicabilidade dessas nanoestruturas na biomedicina. Os TNTs foram obtidos pelo processo de anodização e a técnica *Layer-by-Layer* (LbL) possibilitou o revestimento dos TNTs com cinco camadas alternadas de PAH e PAA. A morfologia dos TNTs foi avaliada pela microscopia eletrônica de varredura. As superfícies dos nanossistemas foram caracterizadas pelas técnicas de espectroscopia no infravermelho por transformada de Fourier (FTIR) e microscopia de força atômica (AFM); a caracterização eletroquímica também foi feita pela espectroscopia de impedância eletroquímica (EIS). A atividade de Cramoll 1,4 após funcionalizar os TNTs foi avaliada através do teste de ligação à ovoalbumina. A adesão das células nas diferentes superfícies foi analisada pela microscopia de fluorescência e a proliferação celular pelo ensaio com o corante resazurina. Os TNTs apresentaram diâmetro e espessura média da parede de 70,9 e 10,1 nm, respectivamente, e o processo de LbL não modificou a morfologia dos TNTs. Tendo a última camada do LbL sobre os TNTs (TNTs-LbL) formada com o PAH, permitiu a disposição de grupos amina para a interação eletrostática com os grupos carboxila de Cramoll, e, assim, promoveu a imobilização de Cramoll 1,4 (TNTs-LbL-Cramoll). A análise de FTIR possibilitou a confirmação da lectina no nanossistema TNTs-LbL-Cramoll pois evidenciou o aparecimento das bandas Amida I e Amida II. Através da AFM foi verificado que após as etapas de modificação da superfície dos TNTs houve aumento da rugosidade. A EIS mostrou maior resistência à transferência de elétrons na presença de Cramoll 1,4 nos substratos. Essa lectina continuou com sua bioatividade quando imobilizada nas matrizes nanotubulares pois conseguiu se ligar à ovoalbumina. A microscopia de fluorescência demonstrou uma maior adesão celular nos TNTs-LbL e nos TNTs-LbL-Cramoll quando comparadas às superfícies com apenas TNTs. A lectina, principalmente nas concentrações de 80, 160 e 320 µg/mL, no nanossistema TNTs-LbL-Cramoll estimulou maior proliferação celular em comparação com as outras matrizes. Biomoléculas podem ser usadas para carregar e/ou revestir os TNTs e, assim, aprimorar as aplicações biomédicas dessas superfícies nanoestruturadas. Portanto, os resultados sugerem que a funcionalização de implantes de Ti baseados em TNTs com a lectina Cramoll 1,4 pode promover uma maior rapidez no processo de osseointegração.

Palavras-chave: Nanotubos de dióxido de titânio. Implante. Cramoll. Osseointegração.

ABSTRACT

Orthopedic and dental implants are usually manufactured with titanium (Ti) because of their biocompatibility. Implants are susceptible to an ineffective and delayed integration with the surrounding bone tissue, osseointegration, which can prolong the healing process and propitiate infections in the local. Titanium dioxide nanotubes (TNTs) have been a strategy for modifying Ti implant surfaces. In addition, osteogenic properties of TNTs can be improved by their functionalization with bioactive molecules. Therefore, the present work aimed to immobilize purified lectin from *Cratyllia mollis* seeds (Cramoll 1,4) on TNTs coated with self-assembled films of poly(allylamine hydrochloride) (PAH) and poly(acrylic acid) (PAA). Thereafter, to check action of the different surfaces on adhesion and proliferation of osteoblast-like cells. It was aimed also to perform a review in literature about how biomolecules can be used to functionalize TNTs and improve the applicability of these nanostructures in biomedicine. TNTs were obtained by anodization process and the Layer-by-Layer (LbL) technique allowed the coating of TNTs with five alternating layers of PAH and PAA. TNTs morphology was evaluated by scanning electron microscopy. Nanosystem surfaces were characterized by Fourier-transform infrared spectroscopy (FTIR) and atomic force microscopy (AFM) techniques; electrochemical characterization was also performed by electrochemical impedance spectroscopy (EIS). Activity of Cramoll 1,4 after to functionalize TNTs was evaluated by ovalbumin-binding assay. Adhesion of cells on different surfaces was analyzed by fluorescence microscopy and cell proliferation by resazurin dye assay. TNTs present an average diameter and wall thickness of 70.9 and 10.1 nm, respectively, and LbL process did not modify TNTs morphology. Having the last layer of LbL on TNTs (TNTs-LbL) formed with the PAH, allowed the arrangement of amine groups for the electrostatic interaction with carboxyl groups of Cramoll, and thus promoted the immobilization of Cramoll 1,4 (TNTs-LbL-Cramoll). FTIR analysis enabled the confirmation of lectin on TNTs-LbL-Cramoll nanosystem because evidenced the onset of Amida I and Amida II bands. Through AFM it was verified that after the modification steps of TNTs surface there was increase of roughness. EIS showed greater resistance to electron transfer in the presence of Cramoll 1,4 on substrates. This lectin continued with its bioactivity when immobilized in the nanotubular arrays because it was able to bind to the ovalbumin. Fluorescence microscopy demonstrated an increase cell adhesion on TNTs-LbL and TNb-LbL-Cramoll when compared to bare TNTs surfaces. The lectin, mainly at concentrations of 80, 160 and 320 µg/mL, on TNTs-LbL-Cramoll nanosystem stimulated greater cell proliferation compared to the other arrays. Biomolecules can be used to charge and/or coat TNTs and, thus, enhance biomedical applications of these nanostructured surfaces. Therefore, the results suggest that functionalization of Ti implants based on TNTs with the Cramoll 1,4 lectin can to promote a faster osseointegration process.

Key-words: Titanium dioxide nanotubes. Implant. Cramoll. Osseointegration.

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LISTA DE ABREVIATURAS E SIGLAS

AFM – *Atomic force microscopy*
APTES – (3-aminopropil)-trietoxisilano
ATR – *Attenuated total reflectance*
BMP-2 – Proteína morfogenética óssea 2
CBD – Domínio de ligação a carboidratos
ConA – Concanavalina A, lectina de *Canavalia ensiformes*
cpTi – Titânio comercialmente puro
CramoII – Lectina de *Cratylia mollis*
CRD – Domínio de reconhecimento a carboidratos
DAPI – *4',6-diamidino-2-phenylindole*
DMEM – *Dulbecco's modified Eagle's medium*
EIS – *Electrochemical impedance spectroscopy*
FBS – *Fetal bovine serum*
FTIR – *Fourier-transform infrared spectroscopy*
GRGDS – *Glycine-arginine-glycine-aspartic acid-serine peptide*
HOS – *Human osteosarcoma cell line*
LbL – *Layer-by-Layer*
MeαMan – Metil-α-D-manopiranosídeo
MSCs – Células-tronco mesenquimais não diferenciadas
MTT – *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*
NH_4F – *Ammonium fluoride*
OGP – Peptídeo de crescimento osteogênico
PAA – Ácido poliacrílico
PAH – Hidrocloreto de polialilamina
PBS – *Phosphate buffered saline*
PEMs – *Polyelectrolyte multilayers*
Q – *Constant phase elemento*
R_{CT} – *Charge transfer resistance*
RGD – Peptídeo Arginina-Glicina-Ácido Aspártico
RGDC – Peptídeo Arginina-Glicina-Ácido Aspártico-Cisteína
RT – *Room temperature*
R_Ω – *Electrolyte resistance*

SAFs – *Self-assembled films*

SEM – *Scanning electron microscopy*

Ti – Titânio

Ti-6Al-4V – Titânio-6alumínio-4vanádio

TiO_2 – Dióxido de titânio

TNTs – Nanotubos de dióxido de titânio

XRD – *X-ray diffraction*

Zw – *Warburg element*

SUMÁRIO

1 INTRODUÇÃO

Implantes metálicos têm sido cada vez mais utilizados pela população em envelhecimento ofertando-lhe uma maior qualidade de vida. Os principais implantes ortopédicos compreendem as próteses para a substituição de articulações e dispositivos para a fixação de fraturas e estabilização da coluna (SANSONE; PAGANI; MELATO, 2013). Já os implantes dentários são comumente usados em casos de perda dentária resultante de doenças e traumas (THOMAS, 2014). No entanto, uma das principais falhas na reabilitação terapêutica promovida por implantes dentários e ortopédicos é devido à uma insuficiente osseointegração desses implantes levando a um afrouxamento asséptico do tecido ósseo circundante ao biomaterial (RAPHEL et al., 2016).

A citocompatibilidade e a preservação do fenótipo diferenciado das células circundantes ao implante são características fundamentais para os biomateriais cuja osseointegração é o principal objetivo (CAMPOCCIA; MONTANARO; ARCIOLA, 2013). O titânio (Ti) é um metal muito utilizado na fabricação de implantes osseointegrados. A excelente biocompatibilidade do Ti é ofertada principalmente pela formação de uma camada de dióxido de titânio (TiO_2) estável em sua superfície que garante a este metal, por exemplo, uma elevada resistência à corrosão (PIRES; BIERHALZ; MORAES, 2015; SAINI et al., 2015).

Modificações nas superfícies dos implantes de Ti são requisitadas para facilitar a osseointegração desses implantes. Uma estratégia de modificação promissora é através da fabricação de nanotubos de TiO_2 (TNTs) na superfície do Ti. Os TNTs, cujas dimensões são comparáveis às estruturas em nanoescala do osso, melhoram a bioatividade das células na superfície do Ti e ainda esses TNTs podem ser funcionalizados com biomoléculas ativas capazes de melhorar a osteogênese na interface osso-implante (GULATI et al., 2016).

Lectinas correspondem a um grupo de proteínas que têm a propriedade de reconhecer e se ligar a carboidratos livres ou presentes em glicoconjugados. Essas proteínas são onipresentes na natureza e podem ser aplicadas para fins diagnósticos e/ou terapêuticos (COELHO et al., 2017). Uma lectina glicose/manose específica extraída de sementes da leguminosa *Cratylia mollis*, chamada Cramoll 1,4, alberga uma grande versatilidade de aplicações biomédicas, tais como ação mitogênica (MACIEL et al., 2004; SILVA et al., 2014).

Nesse sentido, o presente trabalho promoveu a funcionalização de TNTs com Cramoll utilizando os polieletrólitos hidrocloreto de polialilamina (PAH) e ácido poliacrílico (PAA). Por conseguinte, as diferentes superfícies das matrizes nanotubulares foram analisadas quanto a capacidade de adesão e estímulo da proliferação de células semelhantes a osteoblastos. Ademais, foi feito um levantamento bibliográfico para explicar as principais técnicas de imobilização de biomoléculas nos TNTs e as recentes abordagens de como tais superfícies funcionalizadas podem ser aplicadas para fins biomédicos.

2 OBJETIVOS

2.1 GERAL

Funcionalizar os TNTs com a lectina Cramoll para avaliar a ação *in vitro* desses nanossistemas sobre a adesão e proliferação celular. Fazer revisão literária sobre as principais aplicações biomédicas dos TNTs funcionalizados com biomoléculas.

2.2 ESPECÍFICOS

- Obter as sementes de *Cratylia mollis*, purificar Cramoll 1,4 e testar sua atividade hemaglutinante;
- Sintetizar os TNTs;
- Imobilizar Cramoll 1,4 sobre os TNTs previamente revestidos com os polieletrólitos hidrocloreto de polialilamina e ácido poliacrílico;
- Caracterizar os parâmetros morfológicos e físico-químicos dos nanossitemas;
- Analisar os efeitos dos diferentes nanossistemas sobre a adesão e proliferação de células semelhantes a osteoblastos;
- Realizar um levantamento bibliográfico sobre o processo de imobilização e ação das biomoléculas funcionalizadas nos TNTs para aplicação em implantes e biossensores.

3 REVISÃO DE LITERATURA

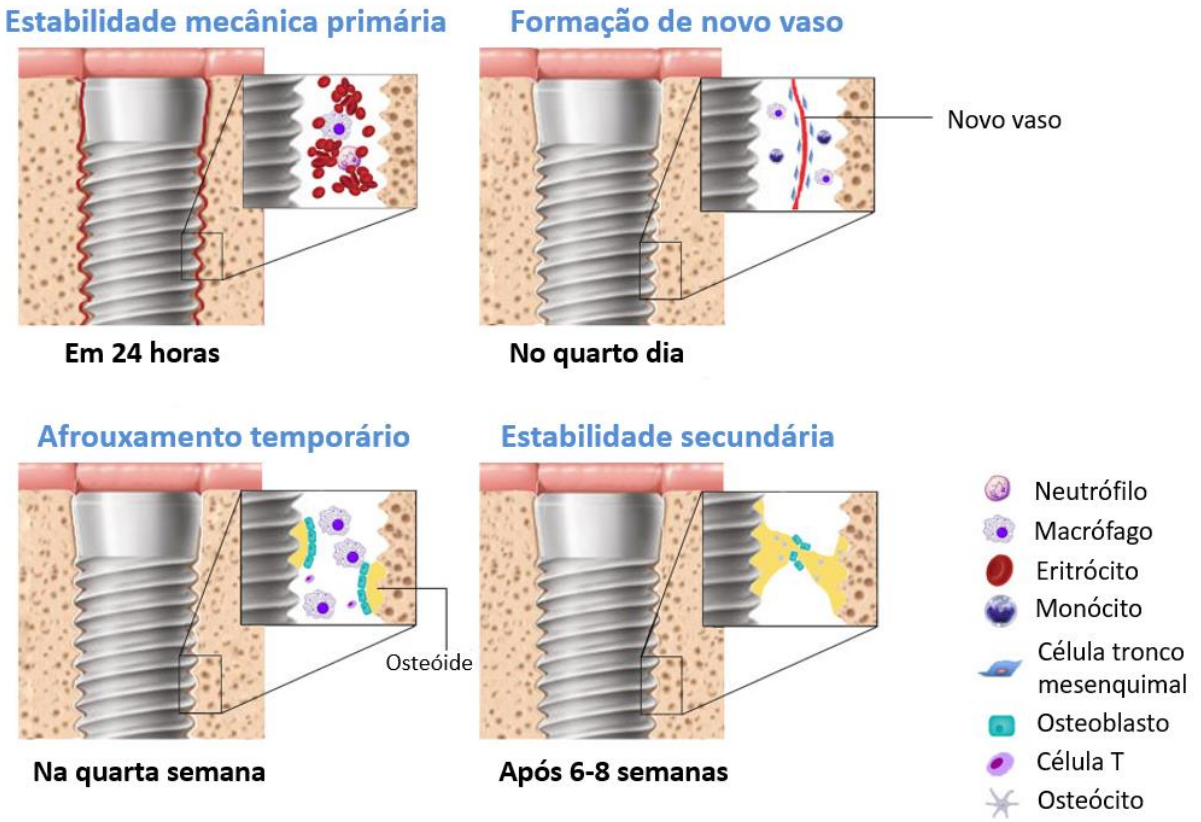
3.1 PROCESSO DE OSSEOINTEGRAÇÃO

A osseointegração pode ser definida como uma ancoragem direta estrutural e funcional de um implante ao osso vivo remodelado sem qualquer componente intermediário, tal como tecido fibroso, na interface osso-implante (BRANEMARK, 1983; REDDY, 2015). Albrektsson et al. (2017) propuseram uma nova definição para osseointegração como sendo uma reação ao corpo estranho onde o osso interfacial é formado como uma reação de defesa para proteger o implante dos tecidos (ALBREKTSSON et al., 2017).

O osso reage à colocação de um implante através de um processo de cicatrização similar à ossificação produzida após uma fratura óssea, porém o osso neoformado fica em contato com a superfície do material aloplástico, o implante (ALBERTINI et al., 2015). A remodelação óssea é resultante da função acoplada de duas células ósseas que são interdependentes, os osteoblastos que são as células formadoras do osso e os osteoclastos que promovem a reabsorção óssea (TRINDADE; ALBREKTSSON; WENNERBERG, 2015). Os osteócitos são as novas células oriundas de osteoblastos que ficam presas na matriz óssea neoformada e podem atuar, por exemplo, como mecanossensores percebendo sinais de tensão pelo desenvolvimento de microfissuras através de seus corpos celulares (CHUG et al., 2013; DU et al., 2016).

Os eventos biológicos que acontecem na osseointegração estão ilustrados na Figura 1. O primeiro componente biológico a entrar em contato com um implante endósseo após a sua inserção é o sangue, posteriormente algumas proteínas são adsorvidas na superfície do biomaterial. Essas interações iniciais levam à formação de um coágulo de fibrina em poucos minutos que por sua vez atua como um reservatório de citocinas (MAVROGENIS et al., 2009; ALBERTINI et al., 2015). As células conseguem interagir com as proteínas presentes nos implantes por meio de receptores celulares chamados de integrinas, capazes de reconhecer a sequência de aminoácidos conhecida como RGD (Arginina-Glicina-Ácido Aspártico) presente em proteínas adesivas, tais como fibronectina (ALBERTINI et al., 2015).

Figura 1 – Cronograma da osseointegração de implantes dentários em relação às mudanças ao longo do tempo.



Fonte: Adaptado de WANG; ZHANG; MIRON, 2016.

A população celular que ocupa inicialmente a superfície do implante é composta principalmente por células inflamatórias, dentro das 24 h após a inserção do implante os neutrófilos dominam o local, mas nos 2 a 4 dias seguintes há uma maior infiltração de monócitos e macrófagos no espaço peri-implantar (WANG; ZHANG; MIRON, 2016). Essas células são responsáveis pela remoção de detritos através da fagocitose e também secretam grandes quantidades de citocinas e fatores de crescimento que são necessários, por exemplo, para o processo de angiogênese e, inclusive, a fibrinólise leva à formação de um espaço para o crescimento de novo vaso sanguíneo (ALBERTINI et al., 2015; WANG; ZHANG; MIRON, 2016). Portanto, 4 dias após a implantação o crescimento de novos vasos sanguíneos produz um tecido de granulação que ocupa o espaço entre o implante e o osso, este tecido é caracterizado pela presença de células-tronco mesenquimais não diferenciadas (MSCs), mas que podem receber uma osteoindução, ou seja, diferenciar em pré-osteoblastos e posteriormente em osteoblastos maduros devido a influência de citocinas específicas e à topografia do implante (MAVROGENIS et al., 2009;

ALBERTINI et al., 2015; WANG; ZHANG; MIRON, 2016).

A formação óssea na superfície do implante é iniciada em torno do sétimo dia em que as células osteogênicas formam um tecido osteóide (matriz orgânica não mineralizada) e após 14 dias a interface osso-implante é ocupada pelo osso reticular, rico em fibras colágenas, estruturas vasculares e osteoblastos (MAVROGENIS et al., 2009; ALBERTINI et al., 2015). Essa formação de tecido ósseo imaturo na região peri-implantar possibilita a estabilização secundária em torno do implante, enquanto a estabilização primária é resultante de um ajuste de fricção do implante com o osso hospedeiro no momento da implantação. A estabilização primária diminui ao longo do tempo pois o osso que fica em contato direto com o implante morre e é reabsorvido pelos osteoclastos. Já a estabilidade secundária pode resultar na ligação óssea, caso a superfície do implante permita a osteogênese de contato (KUZYK; SCHEMITSCH, 2011).

A osteogênese pode ocorrer em duas direções, a partir da superfície do implante em direção ao osso, conhecido como osteogênese de contato; e do osso em direção à superfície do implante, nomeado como osteogênese à distância (CHUG et al., 2013). A osteogênese à distância apresenta lenta cicatrização, já na osteogênese de contato, o osso trabecular (imaturo) formado assegura a fixação biológica do implante, pois uma fina camada de tecido ósseo calcificado e osteóide é depositado por osteoblastos diretamente na superfície do implante (MAVROGENIS et al., 2009).

O osso reticular é frágil e pobre em cristais de fosfato de cálcio, e transforma-se primeiro em um osso rico em fibras paralelas e depois no osso lamelar, que é o tecido mineralizado capaz de suportar cargas mecânicas. Este processo de aposição óssea, ou seja, de síntese e mineralização da matriz óssea, dura cerca de 4 semanas (ALBERTINI et al., 2015). Após 8 a 12 semanas, a interface peri-implantar é completamente substituída por osso lamelar maduro em contato direto com a superfície do implante (WANG; ZHANG; MIRON, 2016).

Embora a remodelação do osso possa ser considerada como fase tardia da cicatrização peri-implantar, a remodelação ocorre ao longo do processo de cicatrização e continuamente em todos os ossos do corpo (KUZYK; SCHEMITSCH, 2011). A renovação do osso maduro peri-implantar nos implantes osseointegrados é confirmada pela presença de espaços medulares com osteoclastos, osteoblastos, vasos sanguíneos e linfáticos próximos à superfície do implante (MAVROGENIS et

al., 2009). A remodelação do osso em contato com a superfície do implante continua ao longo da vida útil do biomaterial (KUZYK; SCHEMITSCH, 2011).

3.2 BIOMATERIAIS

O conceito de biomaterial elaborado pelo *National Institutes of Health Consensus* de 1982 ainda é utilizado, sendo biomaterial definido como qualquer substância ou combinação de substâncias, de origem natural ou sintética, que pode ser usada por qualquer período de tempo, como um todo ou uma parte de um sistema, no intuito de tratar, aumentar ou substituir quaisquer tecido, órgão ou função do corpo (SMITH et al., 2015). Há uma crescente demanda em relação ao uso de biomateriais à medida que a população envelhece, sendo capazes de aumentar a qualidade de vida das pessoas devido à sua vasta aplicação que varia desde reposição de articulações e membros, artérias e peles artificiais até lentes de contato e substituição de dentes (BHULLAR; LALA; RAMKRISHNA, 2015).

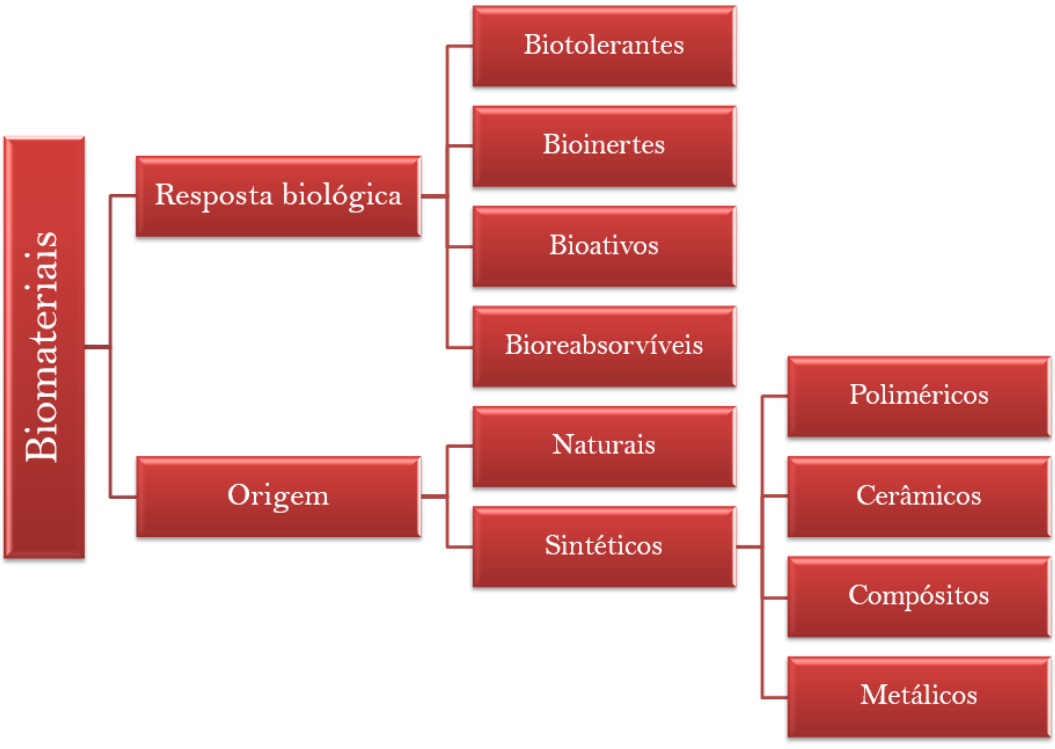
A biocompatibilidade pode ser entendida como a capacidade de um material ser usado numa íntima conexão com os tecidos vivos sem causar efeitos adversos aos mesmos (ASRI et al., 2017). Portanto, a biocompatibilidade é umas das principais características dos biomateriais, permitindo que o material implantado tenha uso prolongado no corpo humano sem causar qualquer influência negativa no ambiente local ou sistêmico com o qual ele está em contato, como tecidos moles, osso, composição iônica do plasma ou fluidos extra e intracelulares (MANAM et al., 2017).

No entanto, outro conceito denominado de bioadaptabilidade tem sido atribuído aos biomateriais levando em consideração as características do material e os aspectos biológicos dentro do microambiente de inserção e mecanismos moleculares envolvidos. A bioadaptabilidade destaca três fatores necessários para serem levados em consideração para a concepção de novos biomateriais: o microambiente criado pelos biomateriais deve ser harmonizado com o microambiente do tecido a ser reparado; deve haver adaptabilidade das propriedades mecânicas dos biomateriais ao tecido nativo; e os biomateriais devem possuir biodegradabilidade adaptável que combine com a nova formação tecidual (WANG, 2016).

Os biomateriais podem ser classificados quanto a resposta biológica no local onde o mesmo é inserido e quanto a sua origem conforme está esquematizado na Figura 2. Baseando-se na reação tecidual quando implantados, os biomateriais podem

ser classificados em biotolerantes, bioinertes, bioativos e bioreabsorvíveis. Os biotolerantes, tais como aço inoxidável e polimetilmetacrilato, não são necessariamente rejeitados pelo organismo e ficam rodeados por uma cápsula fibrosa. Os bioinertes, como a zircônia e o titânio, quando implantados têm mínimas interações com os tecidos adjacentes podendo ser envolto por uma cápsula fibrosa. Os bioativos, como a hidroxiapatita e o biovidro, estimulam uma resposta biológica, por exemplo, por meio da troca de íons promovendo a formação de ligações químicas com um tecido macio (cartilagem) ou duro (osso) (AHUJA; AHUJA; SINGH, 2015; BARFEIE; WILSON; REES, 2015; FERNANDES; ELIAS; VALIEV, 2015). Já os biomateriais bioreabsorvíveis são dissolvidos após sua inserção no organismo e lentamente são substituídos pelo tecido como o osso. O fosfato tricálcio e o ácido polilático-poliglicólico são exemplos de materiais reabsorvíveis (AHUJA; AHUJA; SINGH, 2015).

Figura 2 – Classificação dos biomateriais de acordo com a resposta biológica e origem.



Fonte: O Autor (2017).

Os biomateriais também podem ser classificados de acordo com sua origem. Os que são derivados naturalmente incluem os biomateriais à base de proteínas, tais como colágeno e gelatina; podem ser também compostos por polissacarídeos, por

exemplo, celulose e quitina/quitosana; e ainda derivados de tecidos descelularizados, oriundos de vasos sanguíneos e fígado, por exemplo (HA et al., 2013). Enquanto os de origem natural são conhecidos como biomateriais biológicos, os de origem artificial são chamados de biomateriais biomédicos, sendo estes últimos subdivididos em poliméricos, cerâmicos, compósitos e metálicos (VALLET-REGÍ, 2001).

Polímeros têm sido utilizados em diversas aplicações, por exemplo, na engenharia de tecidos para regeneração tecidual, implantação de dispositivos médicos e órgãos artificiais entre outras áreas médicas. Essa versatilidade no seu uso pode ser atribuída a suas propriedades únicas, tais como flexibilidade, peso leve, podem ser facilmente fabricados em produtos com forma desejada e acessíveis em várias composições com adequadas propriedades físicas e mecânicas (BANORIYA; PUROHIT; DWIVEDI, 2017). Vale salientar que além dos polímeros sintéticos, por exemplo o polivinilpirrolidona e o polietilenoglicol, existem também os de origem natural, ou biopolímeros, tais como polissacarídeos, proteínas e proteoglicanos (MOGOŞANU; GRUMEZESCU, 2014).

Os materiais cerâmicos possuem fortes ligações interatômicas que formam estruturas com elevado grau de compactação, tornando-os duros, mas muito suscetíveis a fraturas. Podem ser utilizados para a fabriação de instrumentos de diagnóstico, preenchimentos ósseos e próteses ortopédicas; exemplos desses materiais incluem a alumina, hidroxiapatita e o gesso (PIRES; BIERHALZ; MORAES, 2015).

Os compósitos são os materiais formados pela combinação de dois ou mais materiais cujas fases distintas são separadas numa escala maior do que a atômica (PARIDA; BEHERA; MISHRA, 2012; AFFATATO; RUGGIERO; MEROLA, 2015). No material compósito, feito de colágeno e hidroxiapatita, por exemplo, cada componente retém suas propriedas físicas, químicas e mecânicas separadas (AFFATATO; RUGGIERO; MEROLA, 2015; AHUJA; AHUJA; SINGH, 2015).

A seleção de um biomaterial depende da sua aplicação médica específica, mas entre as classes anteriormente citadas, os biomateriais metálicos são os dispositivos médicos mais utilizados, sendo aplicados comumente na confecção de implantes dentários e ortopédicos (próteses e dispositivos de fixação) (MAHAPATRO, 2015; CHEN; THOUAS, 2015). Para o uso seguro e adequado por um longo tempo sem haver rejeição é necessário que os implantes metálicos possuam algumas características, tais como possuir excelente biocompatibilidade, propriedades

mecânicas adequadas, alta resistência ao desgaste, possibilitar uma apropriada osseointegração nos casos dos implantes osseointegrados e, não menos importante, tenham elevada resistência à corrosão (CHEN; THOUAS, 2015).

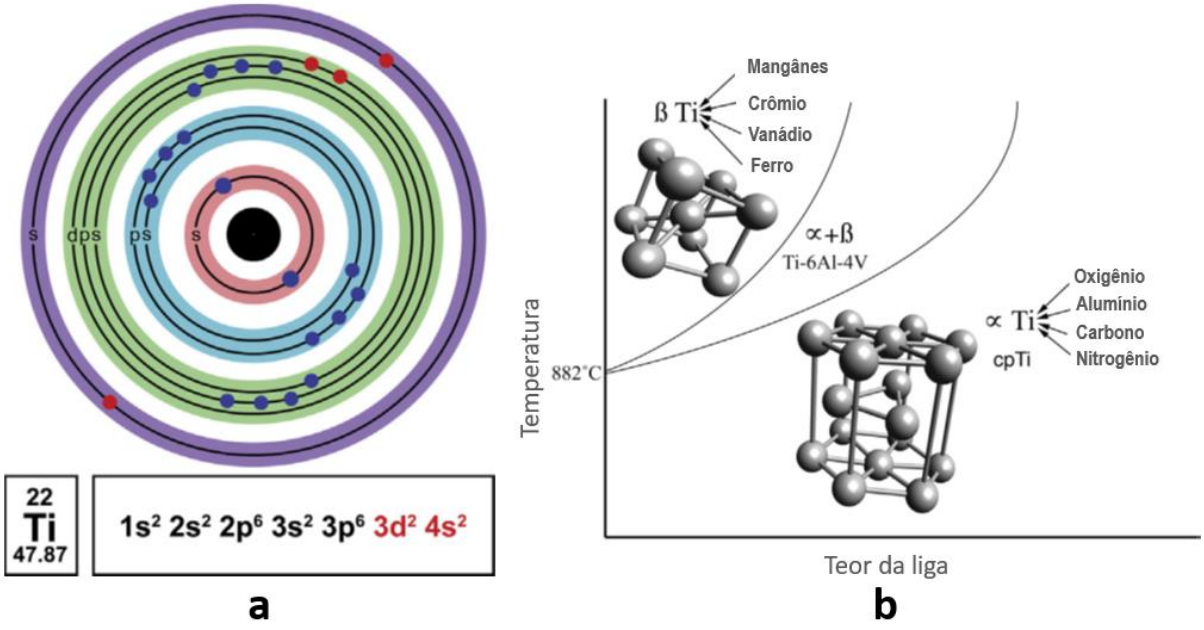
Os biomateriais logo após serem implantados no ambiente *in vivo* entram em contato com fluidos corporais extracelulares, tais como sangue e fluido intersticial. Os materiais metálicos podem sofrer corrosão após a implantação por causa de elementos corrosivos presentes nos fluidos corporais humanos, como os íos hidrogênio, cloreto e o oxigênio dissolvido, além de aminoácidos e proteínas solúveis que atuam como eletrólitos e aceleram a corrosão. Os fluidos corporais atuam como uma solução tampão e apresentam um pH em torno de 7,35-7,45, contudo após o processo de implantação esse pH diminui para cerca de 5,2 ou 5,6 e retorna aos valores normais de pH em torno de duas semanas. Portanto, o nível de corrosão devido a alterações nos valores de pH é insignificante. As consequências mais comuns da corrosão de materiais metálicos são toxicidade e alergia, pois os íons metálicos liberados pela corrosão podem combinar-se com biomoléculas como proteínas e células (HANAWA, 1999; DEVINE et al., 2009; ASRI et al., 2017). Por isso a resistência à corrosão é de suma importância para a escolha do metal que comporá o biomaterial metálico.

Alguns metais comumente utilizados para fabricação de biomaterias metálicos incluem vanádio, ferro, crômio, cobalto, níquel, tântalo, nióbio, molibdênio e tungstênio. Ademais, o titânio (Ti) possui excelente biocompatibilidade devido principalmente à camada de óxido estável formada em sua superfície e, inclusive, tem sido o metal de escolha para a fabricação de implantes dentários (SAINI et al., 2015; FERRARO, 2016).

3.2.1 Implantes de titânio

O Ti é o nono elemento mais abundante encontrado na Terra e é pertencente ao grupo de elementos de transição, possuindo número atômico de 22 e massa atômica de 47,88. Assim como pode ser observado na Figura 3a, os elétrons no Ti são arranjados em torno de seu núcleo em quatro níveis energéticos, possuindo 2, 8, 10 e 2 elétrons, respectivamente. A configuração eletrônica do Ti é também mostrada na Figura 3a e os subníveis de energia $3d^2$ $4s^2$ têm elétrons de valência levemente mantidos (PRASAD et al., 2015).

Figura 3 – Estruturas dos elétrons de valência (a) e cristalinas (b) do Ti.



Fonte: Adaptado de PRASAD et al., 2015.

Sendo caracterizado como um elemento alotrópico, o Ti pode existir em duas formas cristalinas como pode-se observar na Figura 3b; em temperaturas abaixo de 882 °C o Ti puro apresenta uma estrutura hexagonal compacta, conhecida como fase α; e numa temperatura superior a 882 °C se transforma na estrutura cúbica de corpo centrado, chamada de fase β (LI et al., 2014; PRASAD et al., 2015). O Ti comercialmente puro (cpTi) pode ser classificado em quatro graus de acordo com seu conteúdo de oxigênio, por exemplo, o grau 1 possuindo o mínimo (0,1%) e o grau 4 o máximo (0,4%) do teor de oxigênio. Todavia, as diferenças mecânicas entre esses graus do cpTi é principalmente a presença de contaminantes presentes em pequenas quantidades (SAINI et al., 2015). Portanto, as ligas de Ti podem ser criadas pela reação desse metal com elementos específicos para criar fases α, β ou α+β estáveis em temperatura ambiente. Alumínio, carbono, nitrogênio e oxigênio, por exemplo, estabilizam a fase α do Ti; enquanto elementos como vanádio e ferro estabilizam a fase β. Titânio-6alumínio-4vanádio (Ti-6Al-4V) é uma liga de fase α+β em temperatura corporal, enquanto o cpTi é um cristal de única fase α; tanto o cpTi quanto a liga Ti-6Al-4V são os mais comumente utilizados na confecção de implantes (PRASAD et al., 2015).

Algumas características que tornam o Ti ser muito utilizado na fabricação de implantes dentários e ortopédicos compreendem sua alta resistência à corrosão,

módulo de elasticidade comparado ao do osso, elevada tendência de osseointegração, mas sua baixa ou inexistente reação com o tecido circundante ao implante é devido ao fenômeno conhecido como passivação (PIRES; BIERHALZ; MORAES, 2015; SAINI et al., 2015). Este fenômeno acontece pela reação espontânea do Ti com o ar e a água, visto que o oxigênio é relativamente eletronegativo devido à sua vacância de apenas 2 elétrons se liga facilmente com elétrons de valência do Ti formando um filme de dióxido de titânio (TiO_2), geralmente de espessura nanométrica (PIRES; BIERHALZ; MORAES, 2015; PRASAD et al., 2015).

Modificações na superfície do Ti estão sendo requisitadas para melhorar seu desempenho em biomateriais. Esses tratamentos de superfície objetivam, entre outros aspectos, fornecer uma melhor molhabilidade, maior adesão e proliferação celular nos implantes e, consequentemente, aperfeiçoar a osseointegração para promover rápida cicatrização e menor duração do tratamento (JEMAT et al., 2015). Os tratamentos na superfície do Ti para uso em implantes podem ser categorizados de acordo com o mecanismo de modificação em mecânicos, químicos e físicos.

Os tratamentos mecânicos para modificação tais como polimento, jateamento e usinagem, modificam a rugosidade do material resultando em superfícies ásperas ou lisas e assim podem melhorar a adesão, proliferação e diferenciação celular. Os tratamentos físicos depositam materiais na superfície de implantes, por exemplo, pulverização térmica, catódica e por plasma. Já os tratamentos químicos com ácidos ou bases, sol-gel, tratamento eletroquímico (oxidação anódica), entre outros, alteram a composição da superfície do implante (LIU; CHU; DING, 2004; BARFEIE; WILSON; REES, 2015). Adicionalmente a essas técnicas que modificam a rugosidade superficial ou induzem a formação de uma camada de óxido de Ti, existe também a possibilidade de recobrimento dessas superfícies. Esse revestimento pode ocorrer com compostos inorgânicos, por exemplo as cerâmicas, ou com moléculas orgânicas, tais como polímeros sintéticos e as de origem natural, biomoléculas, para que possam promover uma resposta celular na interface tecido-implante (CIVANTOS et al., 2017).

A obtenção de uma rugosidade superficial em nanoescala tem sido cada vez mais requisitada para a elaboração de implantes de Ti avançados, pois essa nanotopografia atinge dimensões nanométricas de elementos fundamentais da matriz óssea, conseguindo, por exemplo, induzir a osteogênese (CIVANTOS et al., 2017; HUANG et al., 2017). Assim, os nanotubos de dióxido de titânio (TNTs) têm fornecido uma nanotopografia de superfície favorável para melhorar o desempenho clínico de

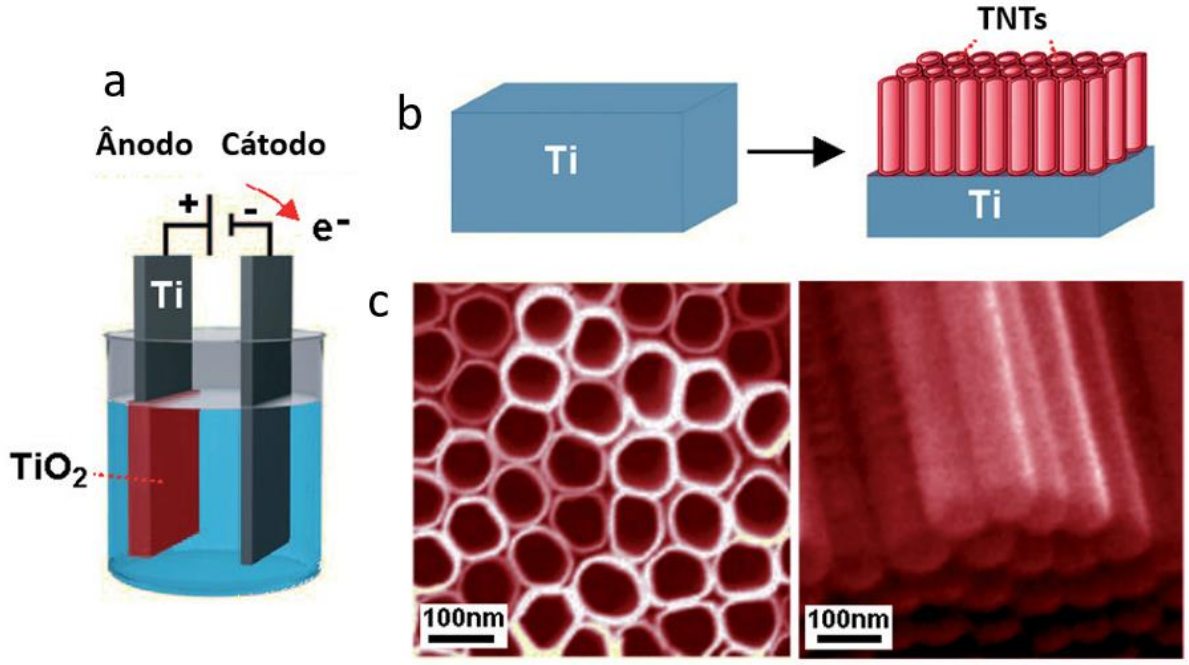
implantes osseointegrados, sendo considerados uma estratégia promissora e confiável de modificação de superfícies dos implantes de Ti (HUANG et al., 2017; MANJAIAH; LAUBSCHER, 2017).

3.2.1.1 Nanotubos de dióxido de titânio

TNTs fabricados no Ti são promissores para uso em implantes principalmente por causa de sua melhor bioatividade comparada às superfícies lisas e com micro-rugosidade, capacidade de aumentar as funções das células ósseas, estudos *in vivo* que reconhecem seu potencial terapêutico e também pela facilidade de funcionalização química e/ou biológica (GULATI; IVANOVSKI, 2016). Várias técnicas podem ser usadas para a síntese de TNTs em grandes quantidades e com morfologia controlada, por exemplo, tratamento hidrotermal de nanopartículas de TiO_2 e deposição sol-gel, todavia o processo de anodização eletroquímica, ou oxidação anódica, tem sido uma das técnicas que tem atraído mais atenção devido, por exemplo, por ser um método fácil de obtenção dos TNTs e pelo baixo custo (FILHO; ROCCO, 2013; RAHMAN; HOSSAIN; DAS, 2016).

A anodização é uma técnica de passivação eletroquímica usada para aumentar a espessura da camada natural de óxido formada na superfície do metal (ESCADA; NAKAZATO; CLARO, 2017). A oxidação anódica é uma reação de óxido-redução que se processa num eletrólito contendo íons fluoreto no qual dois eletrodos são imersos, um ânodo que é o Ti a ser anodizado conforme esquematizado na Figura 4a e um cátodo, como por exemplo a platina (SMITH et al., 2013; CIPRIANO; MILLER; LIU, 2014). Esse método resulta na formação de TNTs auto-organizados, bem definidos, alinhados perpendicularmente e dispostos hexagonalmente na superfície metálica como mostrado na Figura 4b; além disso, como pode-se observar na Figura 4c, esses TNTs são abertos na parte superior e fechados na parte inferior (ROY; BERGER; SCHMUKI, 2011; GULATI et al., 2016).

Figura 4 – Célula eletroquímica e processo de anodização para a formação da camada de TiO_2 sobre o Ti (a) com matriz de TNTs auto-organizados e alinhados verticalmente (b). Imagens de microscopia eletrônica de varredura da superfície superior e inferior da morfologia típica das estruturas dos TNTs (c).

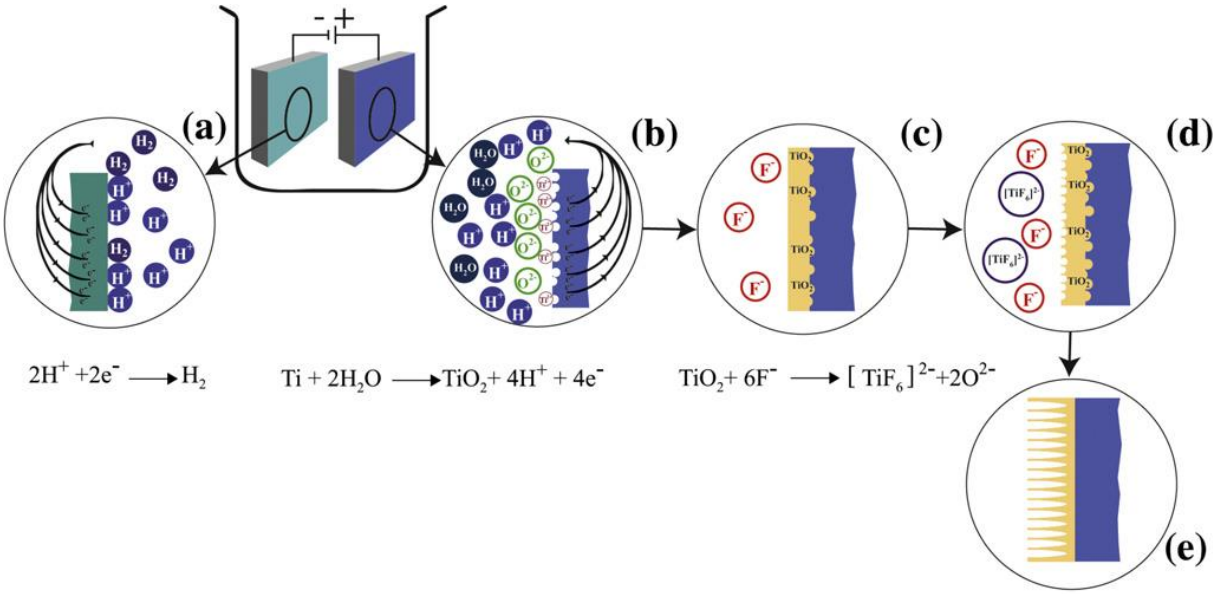


Fonte: Adaptado de GULATI et al., 2012a.

A corrente elétrica gerada por uma fonte de energia impulsiona o processo de anodização, a corrente flui do ânodo metálico para o cátodo e o crescimento do óxido do metal é iniciado (CIPRIANO; MILLER; LIU, 2014). As principais reações que ocorrem na anodização são mostradas na Figura 5. A reação que ocorre no ânodo (5b) descreve o crescimento do óxido na superfície do Ti, pois durante os primeiros segundos de reação o Ti começa a ser oxidado formando os íons Ti^{4+} ao liberar elétrons na solução eletrolítica, esses íons reagem com o ânion O^{2-} proveniente da dissociação da água ocasionado pelo campo elétrico, e assim, forma-se o TiO_2 (MACAK et al., 2007; KATEŘINA et al., 2015). Os íons fluoreto (5c) têm a capacidade de formar complexos $[TiF_6]^{2-}$ que são solúveis em água e estes complexos promovem um ataque químico (dissolução) do TiO_2 formado. Logo em seguida há um aumento da corrente devido a formação de uma estrutura porosa (5d). Quando a taxa de crescimento dos nanoporos na interface óxido-metal é a mesma da taxa de dissolução do óxido na interface óxido-eletrólito a espessura do óxido torna-se constante. Posteriormente ocorre a separação dos poros permitindo o crescimento contínuo dos

nanotubos (5e) no substrato de Ti (MACAK et al., 2007; CIPRIANO; MILLER; LIU, 2014).

Figura 5 – Crescimento de TNTs regulares: reação catódica (a), reação anódica (b), estado de transição da camada de TiO_2 (c), início da formação dos nanotubos (d) e TNTs (e).



Fonte: MINAGAR et al., 2012.

Geralmente após o processo de anodização os TNTs são amorfos que por sua vez podem ser convertidos nas estruturas cristalinas anatase e rutilo através de tratamentos térmicos. A fase anatase aparece numa temperatura de 400 °C; a 600 °C há a presença de anatase e rutilo, enquanto numa temperatura de 800 °C tem apenas a fase rutilo nos TNTs quando tratados por essas diferentes temperaturas durante 3 h (YANG et al., 2011). Essas mudanças na cristalinidade do TiO_2 têm sido executadas após a anodização como forma de aperfeiçoar a aplicação dos TNTs em implantes. A cristalização dos TNTs aumenta a resistência à corrosão do material, sobretudo a fase anatase induz melhor crescimento ósseo e até maior adesão e ativação plaquetária quando comparados aos TNTs amorfos (YU et al., 2010; BAI et al., 2011; ZHANG et al., 2018).

Adicionalmente às estratégias de melhoria no desempenho dos TNTs para aplicação em implantes osseointegrados, essas matrizes nanotubulares têm sido funcionalizadas com diferentes biomoléculas. As lectinas constituem uma classe dessas moléculas bioativas com futuro promissor para a funcionalização dos TNTs (OLIVEIRA et al., 2017).

3.3 LECTINAS

Lectinas são proteínas de origem não imunológica que possuem pelo menos um domínio não catalítico capaz de se ligar de forma reversível e especificamente a monossacarídeos, oligossacarídeos e glicoconjugados (DIAS et al., 2015). Essa ligação é possibilitada por causa de seu domínio de reconhecimento a carboidratos (CRD), também conhecido como domínio de ligação a carboidratos (CBD), localizado dentro de sua estrutura polipeptídica; e uma característica representativa dessas proteínas é a capacidade de aglutinar eritrócitos (JUAN et al., 2017).

As lectinas são amplamente distribuídas na natureza, sendo encontrada desde vírus até animais e plantas (DIAS et al., 2015). Nas plantas, as lectinas podem ser encontradas em vários órgãos, tais como folhas, raízes, rizomas, bulbos, tubérculos, cascas, mas são particularmente abundantes nas sementes de leguminosas; por exemplo, a Concanavalina A (ConA) obtida de sementes da leguminosa *Canavalia ensiformes* que foi inclusive a primeira lectina purificada em larga escala e comercializada (INGALE; HIVRALE, 2013; JUAN et al., 2017).

Com o advento das técnicas de genômica e transcriptômica que possibilitaram analisar as sequências das lectinas de plantas, essas proteínas foram classificadas em 12 famílias distintas de acordo com os domínios das lectinas relacionadas estrutural e evolutivamente. Essas famílias são: homólogas da aglutinina de *Agaricus bisporus*, amarantinas, homólogos de quitinase classe V, família cianovirina, família da aglutinina de *Euonymus europaeus*, família da aglutinina de *Galanthus nivalis*, proteínas com domínio de heveína, jacalinas, proteínas com um domínio de lectinas de leguminosas, domínios de lisina, família da aglutinina *Nicotiana tabacum* e família ricina-B (VANDENBORRE; SMAGGHE; VAN DAMME, 2011).

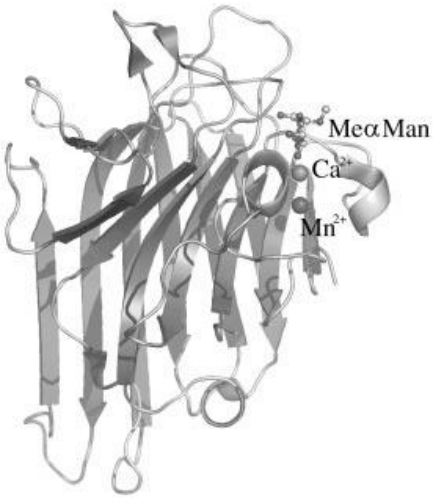
Essas proteínas são consideradas como valiosas ferramentas biotecnológicas devido a sua capacidade em se ligar aos açúcares e glicoconjugados presentes nas células, tecidos e fluidos biológicos provendo possíveis efeitos diagnósticos e terapêuticos. A interação das lectinas com carboidratos de superfície celular podem induzir diversos eventos intracelulares culminando, por exemplo, com a proliferação celular (COELHO et al., 2017).

3.3.1 Lectinas de *Cratylia mollis*

A leguminosa *Cratylia mollis* é nativa do semiárido pernambucano, popularmente conhecida como feijão camaratu, pertencente à tribo *Phaseoleae*, subgrupo *Dioclinae*. Das sementes de *C. mollis* foram purificadas quatro formas moleculares de lectinas ou isoformas, as quais foram denominadas de Cramoll 1, Cramoll 2, Cramoll 3 e Cramoll 4. As isoformas 1, 2 e 4 são glicose-manose específicas, já a isoforma 3 é uma glicoproteína ligadora de galactose. No processo de purificação dessas isolectinas os extratos de sementes de *C. mollis* são inicialmente submetidos ao fracionamento salino com sulfato de amônio (F0-40%) seguido de uma centrifugação. O precipitado obtido pode ser utilizado para a purificação de Cramoll 3 enquanto o sobrenadante passa por um novo fracionamento salino (F40-60%) e outra centrifugação. O sobrenadante adquirido contém a Cramoll 2 e o precipitado pode ser submetido à cromatografia de afinidade em Sephadex G-75 para a obtenção de uma preparação contendo as isoformas 1 e 4 (Cramoll 1,4), as quais podem ser separadas por cromatografia de troca iônica numa coluna de CM-celulose (PAIVA; COELHO; 1992; CORREIA; COELHO, 1995).

Cramoll 1 possui 236 aminoácidos e tem 82% de homologia com a ConA. A estrutura terciária de Cramoll 1 como mostrada na Figura 6 apresenta três folhas β conectadas por *loops* e o sítio de ligação ao monossacarídeo metil-α-D-manopiranosídeo (MeαMan) fica situado próximo aos locais de ligação ao cálcio e mangânes (SOUZA et al., 2003).

Figura 6 – Estrutura terciária de Cramoll 1.



Fonte: SOUZA et al., 2003.

Estudos utilizando tanto a Cramoll 1 quanto a preparação Cramoll 1,4 (também denominada pCramoll) evidenciaram diversas aplicações biotecnológicas para essas lectinas (SILVA et al., 2014). A versatilidade de Cramoll 1,4 caracterizando-a como valiosa ferramenta biotecnológica é atribuída a diferentes estudos, tais como: imobilização em Sepharose para atuar como matriz de afinidade para purificação de glicoproteínas (SILVA et al., 2011); ação cicatrizante na sua forma livre (MELO et al., 2011a) ou quando imobilizada em filme de galactomanana (ALBUQUERQUE et al., 2017); ação anti-helmíntica contra *Schistosoma mansoni* (MELO et al., 2011c); foi utilizada em biossensores como biomoléculas para fazer o reconhecimento de glicomarcadores em diferentes sorotipos de dengue (AVELINO et al., 2014) e realizar a diferenciação do câncer de próstata e hiperplasia prostática benigna (SILVA et al., 2016); caracterização histoquímica de tecido cancerígeno (LIMA et al., 2010); ação imunomoduladora e anti-inflamatória (MELO et al., 2010). Cramoll 1,4 também possui ação antitumoral estando livre ou encapsulada em lipossoma, tanto como pCramoll (ANDRADE et al., 2004) quanto na forma recombinante (CUNHA et al., 2016) e atividade mitogênica em linfócitos (MACIEL et al., 2004) e esplenócitos (MELO et al., 2011b). Essas ações sobre a proliferação celular também foram determinadas para outras lectinas, tais como a lectina ASL (purificada dos tubérculos de *Arisaema speciosum*) e a GANL (obtida das brânquias de *Aristichthys nobilis*). Essas atividades foram atribuídas à capacidade das lectinas se ligarem à membrana celular via sítios contendo glicoconjugados, podendo levar à uma sinalização celular até culminar em distintas respostas celulares (VIKRAM et al., 2010; YAO; PAN; ZHOU, 2012).

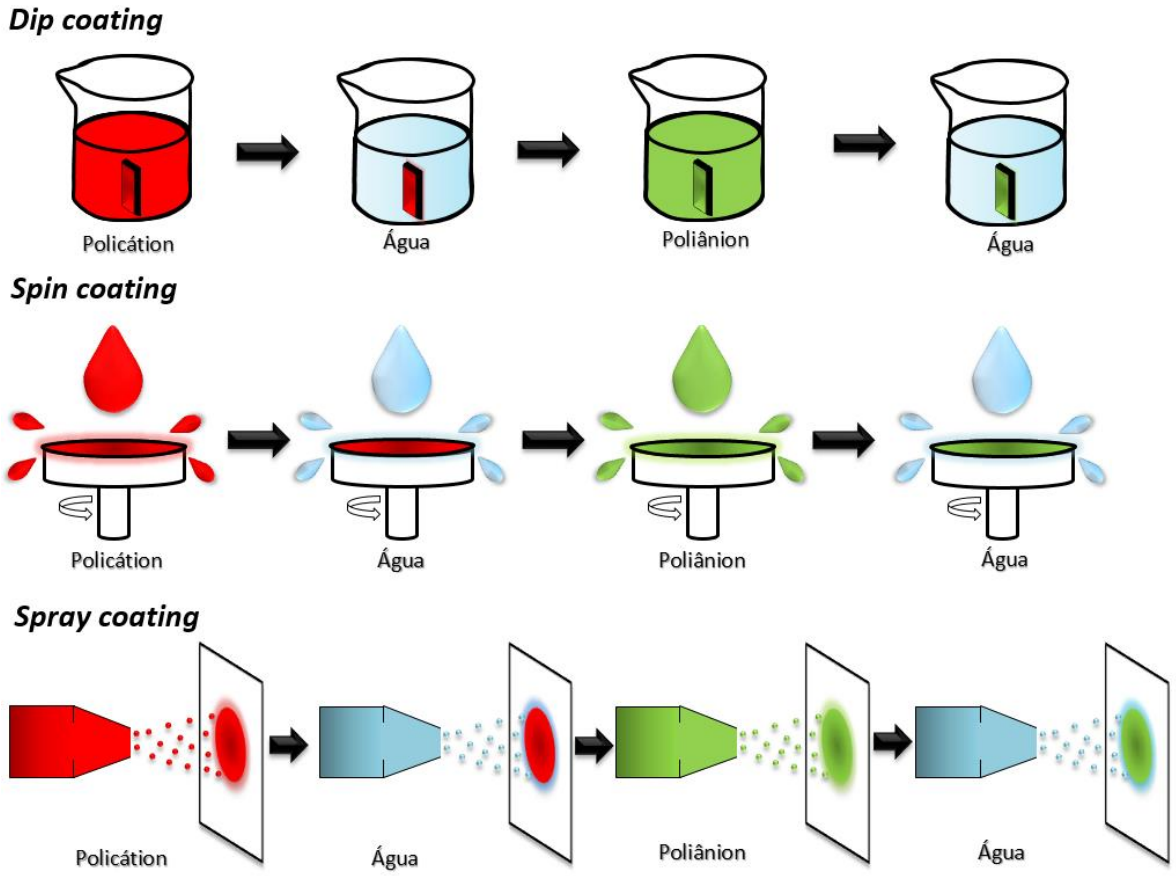
3.4 IMOBILIZAÇÃO DE MOLÉCULAS EM NANOTUBOS DE DIÓXIDO DE TITÂNIO

Com o objetivo de melhorar o desempenho em implantes, os TNTs têm sido funcionalizados com diferentes moléculas, tais como a proteína morfogenética óssea 2 (BMP-2) (LEE et al., 2015), o peptídeo de crescimento osteogênico (OGP) (LAI; JIN; SU, 2017), a quitosana e o poli(ácido lático-co-glicólico) (GULATI et al., 2012b). O efeito dos implantes funcionalizados pode ser potencializado pois as moléculas a serem imobilizadas nos TNTs podem modificar a rugosidade dessas superfícies tornando-as mais favoráveis para a adesão celular ou exercer efeito antimicrobiano, por exemplo. Inclusive, essas nanoestruturas também têm sido utilizadas para exercer a entrega de drogas no local onde o implante é colocado (WU et al., 2014).

Duas metodologias comumente empregadas para promover a funcionalização molecular dos TNTs são o *spin* e *dip coating*. O *spin coating* baseia-se na aplicação de uma solução em um substrato rotativo enquanto o *dip coating* é fundamentado no revestimento de uma superfície por imersão da mesma numa solução (OLIVEIRA; LAIA; BRANCO, 2012). A quitosana, por exemplo, já foi usada para promover o revestimento dos TNTs por ambas as técnicas (CHEN et al., 2013; KUMERIA et al., 2015). TNTs previamente funcionalizados através de sua imersão em soluções poliméricas, tais como (3-aminopropil)-trietoxisilano (APTES) e polidopamina, foram úteis para promover a imobilização dos peptídeos RGDC (Arginina-Glicina-Ácido Aspártico-Cisteína) e OGP, respectivamente (CAO et al., 2012; LAI; JIN; SU, 2017). Há uma outra técnica que poder ser empregada para o revestimento de substratos, a chamada *spray coating*. Neste método, o revestimento de uma superfície ocorre por pulverização, permitindo um controle preciso do fluxo de aerosol que é gerado por um aerógrafo e produção de filmes finos uniformes após ajustes adequados da pressão do ar, do ângulo e distância do *spraying* (ALEKSANDROVA; ANDREEV; KOLEV, 2015).

Ademais, diferentes polímeros têm sido imobilizados nos TNTs, tais como quitosana e gelatina, pelo método *Layer-by-Layer* (LbL) que inclusive tem sido considerado uma estratégia promissora de modificação de superfícies em implantes de Ti (ZHANG et al., 2016; LAI et al., 2017; SHI et al., 2017). O LbL é uma técnica versátil de automontagem usada para formular multicamadas de polieletrólitos onde estes apresentam cargas opostas, que se atraem eletrostaticamente, para serem depositados em um determinado substrato. Geralmente, após a deposição de cada polieletrólito é feita lavagem para remover o que não se ligou ou que ficou fracamente ligado, inclusive, é importante para evitar a contaminação cruzada entre os polieletrólitos de cargas opostas (SHI et al., 2017). Assim como está ilustrado na Figura 7, o LbL pode ser realizado através de três principais tecnologias: *dip coating* (montagem do LbL por imersão), *spin coating* (montagem do LbL por rotação) e *spray coating* (montagem do LbL por pulverização) (ABU-THABIT; HAMDY, 2016).

Figura 7 – Montagem do LbL por *dip*, *spin* e *spray coating*.



Fonte: O Autor (2017).

Os polieletrólitos positivos frequentemente utilizados no LbL são o hidrocloreto de polialilamina (PAH), cloreto de dialildimetilamônio, e polietilenoimina; e os de carga negativa comumente aplicados são o ácido poliacrílico (PAA), poliestireno sulfonato e poilivinil-sulfonato (SRIVASTAVA; KOTOV, 2008).O LbL tem sido considerado como uma estratégia ascendente para a montagem de multicamadas de filmes finos, possuindo simplicidade em seu processo e controle preciso da espessura desses filmes em escala nanométrica (FARIA et al., 2014; XIAO et al., 2016).

Portanto, a funcionalização dos TNTs tem sido aplicada para usufruir das ações que determinadas moléculas biocompatíveis podem exercer quando imobilizadas nos TNTs, por exemplo, impedir a formação de biofilmes em sua superfície, evitando consequentemente prováveis infecções; revestimentos poliméricos capazes de prolongar a liberação de drogas carregadas nos TNTs; e estimular a diferenciação osteogênica de MSCs e/ou exercer ação mitogênica em osteoblastos para tornar mais rápida a osseointegração (OLIVEIRA et al., 2017).

4 ARTIGO I

TITANIUM DIOXIDE NANOTUBES FUNCTIONALIZED WITH *CRATYLIA MOLLIS* SEED LECTIN, CRAMOLL, ENHANCED OSTEOBLAST-LIKE CELLS ADHESION AND PROLIFERATION

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Titanium dioxide nanotubes functionalized with *Cratylia mollis* seed lectin, Cramoll, enhanced osteoblast-like cells adhesion and proliferation

Weslley F. Oliveira[a], Germana M. M. Silva[b], Paulo E. Cabral Filho[c], Adriana Fontes[c], Maria D. L. Oliveira[a], César A. S. Andrade[a], Marcia V. Silva[a], Luana C. B. B. Coelho[a], Giovanna Machado[b], Maria T. S. Correia[a,*]

[a]Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife, PE, Brazil

[b]Laboratório de Nanotecnologia, Centro de Tecnologias Estratégicas do Nordeste, Recife, PE, Brazil

[c]Departamento de Biofísica e Radiobiologia, Universidade Federal de Pernambuco, Recife, PE, Brazil

*Corresponding author address: Departamento de Bioquímica, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, s/n, Cidade Universitária, CEP: 50670-910, Recife, PE, Brazil. Tel.: +55 81 21268540, e-mail address: mtscorreia@gmail.com.

Abstract

An alternative to accelerate the osseointegration of titanium dioxide nanotubes (TNTs) used in osseointegrated implants is through the functionalization of these nanostructured surfaces with biomolecules. In this work, we immobilized a lectin with recognized mitogenic activity, the Cramoll lectin, extracted from *Cratylia mollis* seeds, on surfaces modified by TNTs. For the immobilization of Cramoll on TNTs surfaces, we used the Layer-by-Layer technique (LbL) by forming five alternate layers of poly(allylamine) hydrochloride (PAH) and poly(acrylic) acid (PAA) and lastly we incubated the lectin, at different concentrations, with the TNTs-LbL. Before and after the immobilization procedures, the substrate surfaces were characterized by scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), atomic force microscopy (AFM), and, electrochemical impedance spectroscopy (EIS). We also evaluated Cramoll activity after immobilization on TNTs by using the lectin interaction with ovalbumin. Through this test, it was showed that the lectin did not lose its biological activity, even after immobilization onto nanotubular arrays. In addition, we observed an increase osteoblast-like cell adhesion on the TNTs-LbL-Cramoll system when compared to the bare TNTs surfaces. Moreover, a significative cell proliferation was identified on the substrates when Cramoll was immobilized at concentrations of 80, 160 and 320 μg/mL after 48 h of incubation by using the resazurin assay. Our results suggest that Cramoll was efficiently immobilized on a nanotubular array and this new platform presents a great potential for use in implantology by promoting a fast process of osseointegration.

Keywords: Titanium dioxide nanotubes; implant; Cramoll; osseointegration.

1. Introduction

Titanium (Ti) and its alloys are widely used in the manufacture of dental and orthopedic implants. This metal has high efficacy as biomaterial due to its excellent biocompatibility and high resistance to corrosion, characteristics resulting from the passivation phenomenon, that is the spontaneous formation of a stable layer of titanium dioxide (TiO_2), with a nanometric thickness, on the surface of Ti [1–3]. However, native TiO_2 layer on the surface of these metal implants is bioinert and does not promote a direct bond with the bone, but it develops bone formation in the early stage of the osseointegration [4,5]. Ineffective osseointegration in the bone/implant interface may allow bacterial adhesion and colonization for subsequently favor the biofilm formation on the surface of these implants [6].

Several techniques to modify the roughness, topography, charge, energy and chemical composition of the Ti surface have been used to accelerate osseointegration and to ensure the direct anchorage of the implant with the host bone tissue [7,8]. Among these modifications, stand out the use of TiO_2 nanotubes (TNTs) obtained by the electrochemical anodization process, that are capable of share similar dimensions to the structures present in nanoscale on the human bone. Such nanotopography obtained by TNTs can increase the roughness and the surface area of implants, being able to increase protein adsorption, important for the regulation of cellular interactions with the implant [9]. It is known that TNTs allow an increase adhesion and proliferation of osteoblasts on these nanostructured surfaces compared to non-anodized Ti surfaces, as well as can be used to carry drugs [10–12].

Osteogenic properties of TNTs can be enhanced by functionalization of these TiO_2 nanotubular arrays with biomolecules, such as the arginine-glycine-aspartic acid-cysteine (RGDC) sequence peptide, and the bone morphogenetic protein-2 (BMP-2),

since certain biomolecules can be responsible for accelerating bone formation and consequent adhesion of neoformed bone tissue to implanted material [13–16]. Plant secondary metabolites, such as the flavonoids quercetin and icariin, were loaded onto TNTs and could be used as alternatives of bioactive molecules to improve osseointegration of Ti implants [17,18]. In this context, plant resources can exert significant beneficial effects on health by providing natural bioactive substances, such as lectins [19].

Carbohydrate-binding proteins, lectins, which have been shown extensive biotechnological applications and uses for medical purposes because of their involvement in various biological phenomena. The recognition and subsequent binding of lectins to cell surface glycans without producing any structural changes can trigger events in cells, such as stimulation of cell proliferation [20,21]. Studies focusing on the biochemical bioprospection of bioactives of plants from the Caatinga, an exclusively Brazilian biome, including lectins, have been explored by scientific communities since they have great biotechnological potential, such as Cramoll 1,4, from now on named as Cramoll [22,23].

A glucose/mannose binding lectin, Cramoll, was extracted from seeds of a legume native from the northeast of Brazil, *Cratylia mollis* popularly known as camaratu bean [24]. This lectin is a molecule with highly versatile biological properties with great applicability in various areas of the biotechnology such as mitogenic action on different cell lines. The high affinity of Cramoll to fetuin has enabled the development of a biosensor for the differentiation between prostate cancer and benign prostatic hyperplasia through the immobilization of this lectin on carbon nanotubes [25–27].

To promote the immobilization of biomolecules, such as proteins and DNA, on the surface or modify surface properties of materials for use in several areas, such as

the manufacture of sensors and biomaterials, the Layer-by-Layer (LbL) assembly technique has been shown satisfactory applications [28,29]. LbL is based on the formation of polyelectrolyte multilayers (PEMs) by a self-assembling process that occurs by adsorption of oppositely charged polyelectrolytes electrostatically attracted, on a determined surface [30–32]. Poly(allylamine hydrochloride) (PAH) polycation and poly(acrylic acid) (PAA) polyanion have been considered as promising polyelectrolytes for use in biomaterials due to hemocompatibility and osteogenic properties of PAH/PAA films [33–35].

The aim of this study was to promote Cramoll immobilization on the TNTs surfaces by intermediate of five alternating layers of PAH/PAA to investigate the action of these functionalized TiO_2 nanotubular arrays on the proliferation and adherence of osteoblast-like cells. Cramoll immobilized on TNTs stimulated the proliferation of osteoblast-like cells and, thus can be used to improve the performance of metal implants inducing faster osseointegration. To our knowledge, this is the first work that used lectins to promote the proliferation of cells on TNTs surfaces opening new perspectives in the investigation of Cramoll potential in implants.

2. Materials and methods

2.1. Materials

Cramoll was purified as described previously by Correia and Coelho, 1995 [24]. Ti foil (99.6 % of Ti with a thickness of 0.5 mm) was supplied by Realum (São Paulo, BRA). Ethylene glycol was obtained from Química Moderna (São Paulo, BRA). Ammonium fluoride (NH_4F), Dulbecco's modified Eagle's medium with high glucose (DMEM), streptomycin, penicillin, trypsin-EDTA, and resazurin were purchased from

Sigma-Aldrich (St. Louis, USA). Fetal bovine serum (FBS) was obtained by Gibco (Life Technologies, São Paulo, BRA). Poly(allylamine hydrochloride) (PAH, M_W = 70,000 g/mol) was provided by Alfa Aesar (Ward Hill, USA) and poly(acrylic acid) (PAA, M_W > 200,000) by Polysciences (Warrington, USA). Human osteosarcoma cell line (HOS) was obtained from the Bank of Cells of Rio de Janeiro (BCRJ code: 0339, Rio de Janeiro, BRA). The fluorescent probes 4',6-diamidino-2-phenylindole (DAPI) and rhodamine-phalloidin were acquired from Invitrogen and Life Technologies Corporation, respectively.

2.2. Synthesis of TiO_2 nanotubes

Ti foils (1 x 1 cm) were first cleaned with a neutral detergent, distilled water, followed by immersion in an ultrasonic bath with acetone for 10 min, and dried at room temperature (RT). TNTs were fabricated by anodization process in an electrolyte of ethylene glycol containing 10 wt% water and 0.7 wt% NH_4F carried out at 30 volts (V) and 2 amperes (A) using the Supplier AC Power Source for 30 min in an ultrasonic bath. After anodization, samples were rinsed with acetone, water, and dried at RT. In order to crystallize the TNTs, samples were annealed in a furnace EDG 10P-S at 400°C for 3 h in atmospheric air.

2.3. Immobilization of Cramoll on TNTs

The functionalization of TNTs with Cramoll occurred by alternating deposition of cationic and anionic thin layers on the surface of TNTs. The self-assembled films (SAFs) were prepared with a StractoSequence VI (NanoStrata Inc., USA) dip coating robot. Briefly, TNTs were immersed in an aqueous solution of 10^{-2} M PAH for 12 min and then withdrawn from the solution and rinsed in deionized water by dipping for 1, 1,

and 1 min. Afterward, the nanotubes were immersed in an aqueous solution of 10^{-2} M PAA for 12 min and then rinsed in deionized water as described above. Both PAH and PAA aqueous solutions were prepared at pH 7.5 and 4.5, respectively. The polyelectrolytes were used as received without further purification and then the pH of polyelectrolyte solutions was adjusted to the desired pH by adding HCl or NaOH solution. Five layers of the polyelectrolytes were formed at the end of LbL, which started and ended with PAH. TNTs surfaces with the self-assembled films (TNTs-LbL) were immersed in the Cramoll solution with a volume of 0.5 mL in different concentrations (10, 20, 40, 80, 160, and 320 µg/mL) for 90 min, followed by washing once with 0.5 mL of phosphate buffered saline (PBS) buffer for removal of non-binding protein, obtaining at the end the TNTs-LbL-Cramoll system.

2.4. Characterization of samples

The surface morphology of the samples was observed by scanning electron microscopy (SEM) using a FEI QUANTA 200F microscope. The crystal structure of TNTs was evaluated by X-ray diffraction (XRD) (Bruker D8 Advance; Karlsruhe, GER). The chemical groups were identified by Fourier-transform infrared spectroscopy (FTIR) that was conducted on a Vertex 70 (Bruker Optics, GER) spectrometer in attenuated total reflectance (ATR) mode. Spectra were scanned between 4000 and 600 cm^{-1} using 16 scans at a resolution of 4 cm^{-1}.

Surface characterization of the self-assembly was performed using also an atomic force microscope (SPM-9500J2; Shimadzu, Tokyo, JPN). Cantilevers with a silicon atomic force microscopy (AFM) probe (Tap190Al-G, 190 kHz resonant frequency, 48 N/m force constant) were used for the noncontact mode in air at RT (approximately 25 ºC). Lateral resolution was set to 512 × 512 pixels in a scan area of

5 × 5 μm. At least three areas in each sample were macroscopically separated for analysis. The AFM Gwyddion software was used to analyze the images [36].

2.4.1. Electrochemical characterization

Electrochemical data was obtained using a PGSTAT potentiostat/galvanostat (MicroAutolab, Eco-Chemie, NL) interfaced with an analyzer controlled by a computer. Electrochemical analyses were performed using a three-electrode system composed of a Ti surface (working electrode), a platinum electrode (counter electrode) and Ag/AgCl saturated with KCl (reference electrode). We used an electrochemical impedance spectroscopy (EIS) frequency ranging from 100 MHz to 100 kHz, with the amplitude of the applied sine wave potential at 10 mV. $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ solution at 10 mM in a proportion of 1:1 was used as a redox probe in PBS at pH 7.4. EIS measurements were realized to cleaned Ti, TNTs, TNTs-LbL and TNTs-LbL-Cramoll modified surfaces. Cramoll was evaluated at different concentrations in PBS (10, 20, 40, 80, 160, and 320 μg/mL).

2.5. Bioactivity evaluation of Cramoll immobilized on the TNTs

Bioactivity of Cramoll after its immobilization on the TNTs was evaluated by ovalbumin assay; the glycidic moiety of ovalbumin interacts with Cramoll. An ovalbumin aqueous solution (100 μg/mL) was placed in a 24-well plate and, subsequently, each substrate with the TNTs functionalized with the lectin, in concentrations of 10, 20 and 40 μg/mL was added in the plate well containing the glycoprotein. After 1 h of incubation at RT, substrates were removed from each well; residual protein concentrations were determined through BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, USA).

2.6. Cell culture

HOS cells (osteoblast-like cells) were cultured in DMEM supplemented with 10% FBS, and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% of CO_2. When osteoblast-like cells reached 70% or more of the confluence in the culture flask, they were detached with 0.25% (w/v) trypsin-EDTA solution. For the next steps, osteoblast-like cells were seeded onto the samples in 24-well plates at a density of 1 x 10^5 cells/mL and incubated for different periods.

2.6.1. Cell adhesion analysis

Osteoblast-like cells adhesion on the surface of the substrates (TNTs, TNTs-LbL and TNTs-LbL-Cramoll) was evaluated by fluorescence microscopy. Samples with the adhered cells after 24 h of incubation were washed with PBS and then fixed in 3.7% (v/v) of formaldehyde in PBS for 10 min. Cells fixed on the samples were permeabilized with 0.1% (v/v) Triton X-100 and then the cytoskeleton actin filaments were stained with rhodamine-phalloidin for 20 min. Finally, the cell nuclei were stained with DAPI for 5 min. Adhered and properly stained cells were visualized in ten different fields for each sample by the ZEISS Axio Observer.Z1 fluorescence microscope. It was used for red emission the excitation band pass (BP) filter 546/12 nm and the emission BP was 575-640 nm; while for the DAPI emission it was used the excitation in 365 nm and the emission BP 445/50 nm.

2.6.2. Evaluation of cell proliferation

The proliferation percentage of osteoblast-like cells was performed by the resazurin assay. This analysis is based on the ability of mitochondrial enzymes in living cells to reduce resazurin to resorufin, which exhibit absorption peaks at 600 nm and

570 nm, respectively [37]. Cells were incubated with the different substrates (TNTs, TNTs-LBL and TNTs-Cramoll) in 24-well plates for 24, 48 and 72 h; after each time the medium DMEM was removed and followed by twice washing with PBS in each well. Then, 300 µL of resazurin (3 µg/mL) was added followed by incubation at 37 °C by 1 h. Then, 200 µL of each well were transferred to a 96-well plate and absorbance was measured in a BioTek µQuant MQX200 microplate reader spectrophotometer. Cell proliferation was calculated according to Equation 1, been reproduced by three independent experiments:

$$\text{Cell Proliferation } (\%) = \frac{(A_{570\text{ nm}} - A_{600\text{ nm}}) \text{ of treated cells}}{(A_{570\text{ nm}} - A_{600\text{ nm}}) \text{ of control cells}} \text{ x } 100 \qquad \text{Equation 1}$$

.

The resazurin assay was used because it has some advantages when compared to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, as following described. Cells on substrates keep alive and can be reused in another experiment, such as microscopy images, if necessary; only is required the supernatant containing resorufin and substrates will not interfere with the absorbance reading.

Results were plotted in GraphPad Prisma 5.0 software show the average ± standard error bars associated. The statistical analysis was performed with Mann-Whitney test by BioEstat 5.0 software, because the data did not follow a normal distribution $p > 0.05$, according to the Shapiro-Wilk test.

3. Results and discussion

3.1. Morphology and crystallinity of TNTs

Anodization resulted in the formation of TNTs (Fig. 1a) with average value internal diameter of 70.9 ± 7.9 nm and an average wall thickness of 10.1 ± 2.1 nm as observed in the histograms of Figs. 1b and 1c, respectively. These two parameters were determined by measurements performed in 200 nanotubes, through the images obtained by SEM, using ImageJ software. The highly ordered layers of TNTs obtained are in agreement with previous studies that showed the influence of electrolytic conditions on the anodization, especially fluoride ions for the well-organized growth of TNTs [38–40].

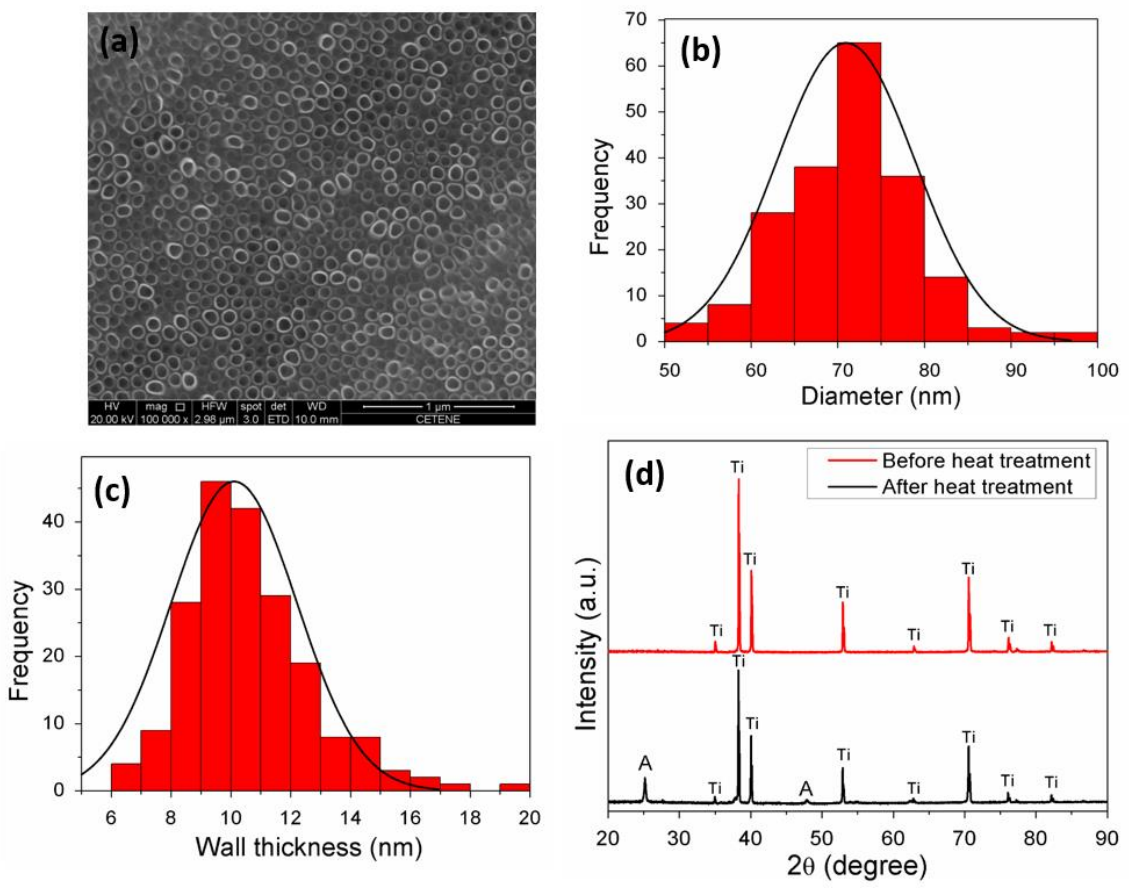

Fig. 1. SEM image of the top view (a), histograms of the diameter (b) and wall thickness (c) of TNTs. XRD patterns of Ti substrate with TNTs before (red line) and after (black line) heat treatment (d). Ti refers to the Ti peak and A indicates the anatase phase of TiO_2. The scale bar for SEM image corresponds to 1 μm.

Cells activity may be influenced by TNT crystallinity since proliferation and mineralization of osteoblasts are significantly enhanced in TNTs with crystalline phase than in amorphous structure [11]. TNTs obtained by anodization process were subjected to thermal treatment at 400 °C during 180 min to promote crystallization of TNTs into anatase phase, as shown by XRD diffractograms in Fig. 1d. TNTs, before and after the heat treatment, show diffraction peaks corresponding to Ti according to the diffractogram of the JCPDS file No. 44-1294. Furthermore, after the annealing process, it was observed Bragg reflections for crystal planes (101) and (200) corresponding to values of 2θ equal to 25 and 48, respectively; these reflections are characteristic of the anatase phase of TiO_2 according to the JCPDS file No. 21-1272. We used the anatase phase of TiO_2 because studies showed that this crystalline structure promotes the nucleation of hydroxyapatite providing an adequate atomic arrangement for the growth of this mineral on TiO_2 surface [41,42]. Such findings are of great importance since the hydroxyapatite crystals promote mineralization of the osteoid in the process of bone formation [43].

3.2. Characterization of TNTs functionalized with Cramoll

Polyelectrolytes coated TNTs by LbL occurred without any modification of the morphology of TNTs as shown in Fig. 2a. Polymer charge density (ionization) can be altered with adjustments in the solution where they are found. Studies have shown that the degree of ionization of PAA carboxylate groups at pH 4.0 increases in the process of forming multilayers to about 63% and the degree of PAH ionization at pH 7.0 is approximately 95% [44,45]. In the pH conditions of our study with PAH/PAA presenting values 7.5/4.5, respectively, there was the neutralization of partially protonated $-COO^-$ (carboxylates) groups of PAA with $-NH_3^+$ (amine) groups in the protonated condition of

PAH. In these conditions with the LbL system finalization was possible to form NH_3^+ groups available to interact with carboxyl groups of the lectin through electrostatic interactions, promoting, thus, immobilization by adsorption of the lectin similarly to a previous study [46].

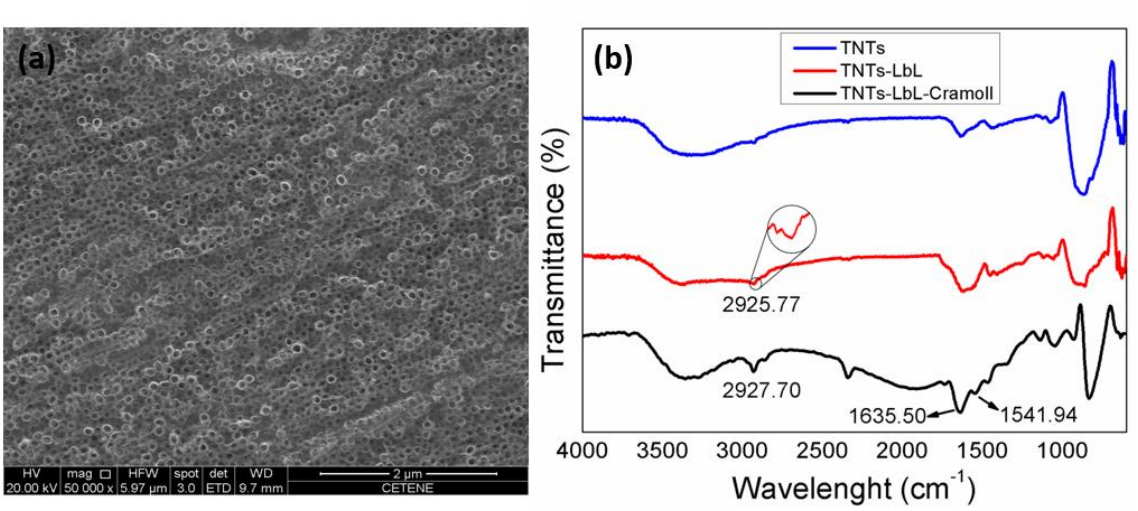

Fig. 2. SEM image of the top view of TNTs–LbL (a). FTIR of the TiO_2 nanotubular arrays before and after functionalization with Cramoll (b). Scale bar for SEM image: 2 µm.

Different compounds have been employed to promote immobilization of peptides and proteins on TNTs surface. For example, the glycine-arginine-glycine-aspartic acid-serine (GRGDS) peptide was immobilized on TNTs surface functionalized with (3-aminopropyl)-triethoxysilane (APTES), while the osteogenic growth peptide (OGP) and BMP-2 were conjugated onto TNTs through an intermediate layer polydopamine [47–49]. PAA and PAH showed efficiency not only for the ability to immobilize molecules but also for their potential use in biomaterials; for example, both polyelectrolytes induced mesenchymal stromal cells to differentiate into osteoblasts and consequently biomineralization [35].

In order to characterize the immobilization of CramoII on TNTs surface, comparative spectra of modified and non-modified TNTs were evaluated by FTIR, as shown in Fig. 2b. A broad band observed between 3600-3000 cm^{-1} in the samples is related to the O-H stretching mode of hydroxyl group, indicating moisture in the samples [50]. Hydrophilicity of nanotubes is related to the presence of hydroxyl groups on their surfaces in the form of $Ti(OH)_4$ after anodization and over time the OH^- groups are transferred to the air in order to reach surface hydroxylation/dehydroxylation equilibrium. The anatase crystalline structure prevents better the establishment of this equilibrium compared to amorphous TiO_2 [51]. Hydroxyl group was evidenced in the TNTs (blue line) in our study and is known that such groups are released from surfaces of the anodically oxidized Ti improves the nanomechanical properties (hardness and elastic modulus) of the mineralized tissues. Hydroxyl radicals can oxidize lysine residues of immature collagen generated by osteoblasts, thus promoting a cross-linked collagenous matrix which together with the expression of specific genes promotes the ossification [52]. The absorption peaks occurring in the range of 800-400 cm^{-1} represent the Ti-O vibrations in TiO_2 [53,54].

PAH absorption bands in FTIR overlap with the PAA bands, in PAH/PAA multilayers [44]. Choi and Rubner (2005) also reported the intensity of the NH_3^+ band of PAH centered around 3016 cm^{-1}; these NH_3^+ groups were fully protonated at pH 1.97 and partially protonated at pH 7.5. Only at pH 12 the amine groups were fully deprotonated in NH_2 form [44]. An absorption peak around 2900 cm^{-1} can be evidenced in the present study as shown in the red line of Fig. 2b and such band is still enhanced in the immobilized lectin (black line) since the amino acids that make up proteins have amine groups.

Furthermore, evidence of lectin presence on the surface of TNTs-LbL-Cramoll substrate was obtained by the two major bands of the proteins, the amide I band (1720-1600 cm^{-1}) referring mainly to the peptide bond C=O stretching vibration and the amide II band (1600-1500 cm^{-1}) resulting from N-H and C-N bonds [55–57]. Amide I and amide II bands of Cramoll FTIR spectra also were identified when this lectin was immobilized on carbon nanotubes and galactomannan film [27,58].

Fig. 3 shows representative AFM images of the topography from these surfaces. The scale (on the right side of each image) provides roughness information, which in fact represents the information about the depth of the dimples compared to the rest of the area. The surface roughness (Fig. 3b) is mainly due to the pronounced grains of TiO_2 film along with the nanoporous layer. As we can see from these images, the overall roughness was increased. Fig. 3 shows changes in roughness of Ti surface after the self-assembly process. The molecules organize themselves after modification with TNTs, in the form of a self-assembled monolayer (SAM) over the Ti surface favored by the intermolecular forces (Fig. 3a). An effective Ti dense film modifying the surface topography with a height of about 0.5 µm is observed in Fig. 3b. A distinctive change in height to 1.0 µm is observed after the LbL adsorption on the TNTs (Fig. 3c). Following Cramoll immobilization, the average height increased to 1.1 µm (Fig. 3d). This slight increase of surface roughness after the functionalization process was observed also in the immobilization of a peptide on TNTs through polydopamine, which showed this behavior after nanobiology surface modification [49].

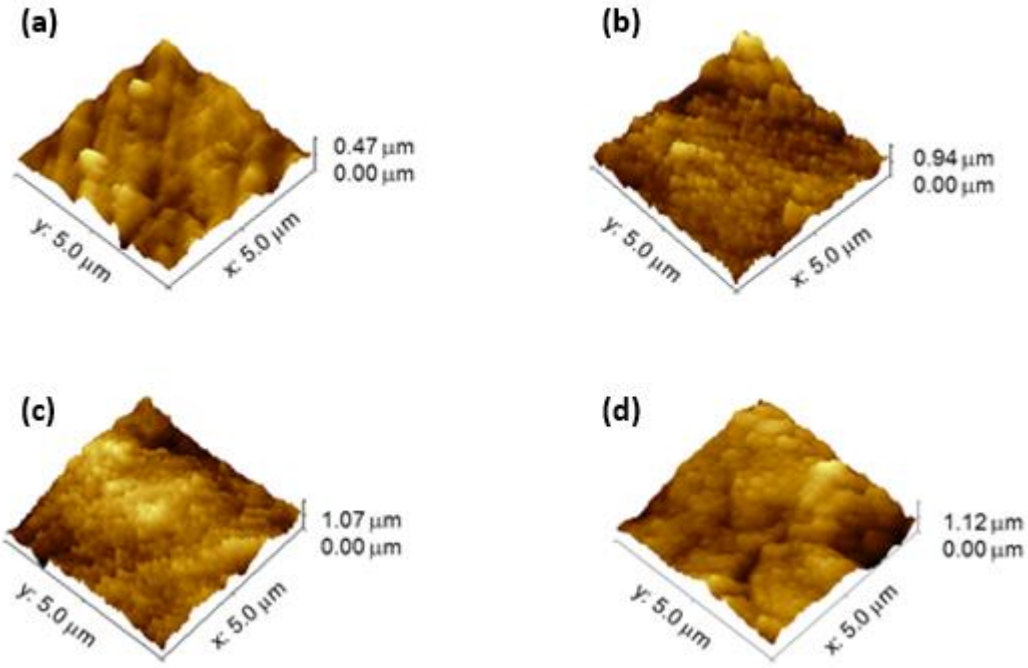

Fig. 3. AFM topographic images of cleaned Ti (a), TNTs (b), TNTs-LbL (c) and TNTs-LbL-Cramoll (d) modified surfaces.

The impedimetric response of the cleaned Ti electrode is shown in Fig. 4. There is a very small semicircle domain, implying very low resistance (R_{CT} = 0.3 kΩ) to the redox-probe dissolved in the electrolyte solution. Subsequently, TNTs growth on cleaned surface resulted in assembled layers with a higher interfacial R_{CT} = 3.7 kΩ, indicating that the nanosystem partially inhibited the electron transfer of the electrochemical probe. The interfacial resistance of TNTs-LbL modified surface decrease (R_{CT} = 2.0 kΩ) associated with the electric properties of the PAH/PAA. In addition, Fig. 4 shows the impedance responses of TNTs-LbL-Cramoll modified surface in 10 mM $K_4[Fe(CN)_6]^{4-}/K_3[Fe(CN)_6]^{3-}$ PBS solution at different concentrations of Cramoll to evaluate the adsorption process of this lectin. RCT increases after Cramoll immobilization since the probe molecules block the electron transfer at the electrode surface/electrolyte interface.

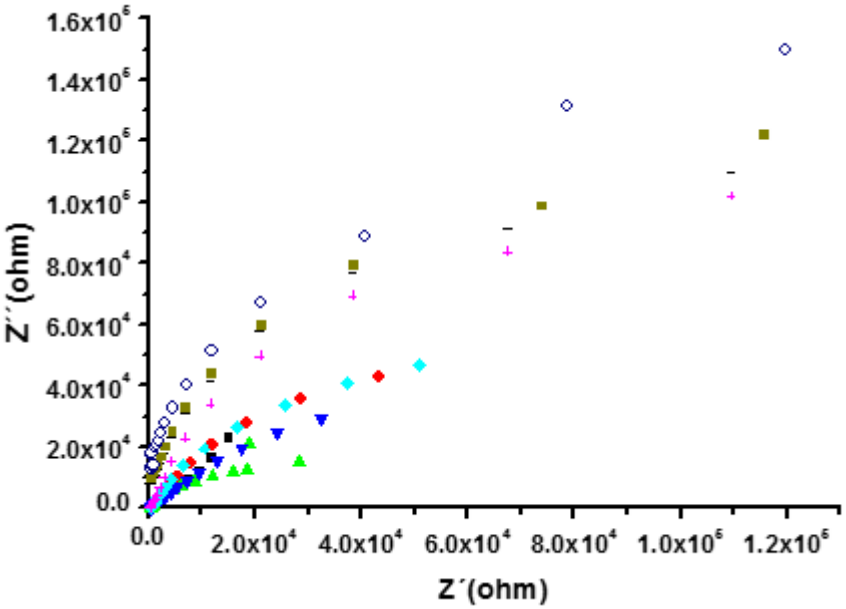

Fig. 4. Nyquist plots of the different steps of functionalization: Ti (■), TNTs (●), TNTs-LbL (▲), TNTs-LbL-Cramoll 10 µg/mL (▼), TNTs-LbL-Cramoll 20 µg/mL (♦), TNTs-LbL-Cramoll 40 µg/mL (+), TNTs-LbL-Cramoll 80 µg/mL (–), TNTs-LbL-Cramoll 160 µg/mL (■), and TNTs-LbL-Cramoll 320 µg/mL (◊), in the presence of the redox pair from $K_4[Fe(CN)_6]^{4-}/K_3[Fe(CN)_6]^{3-}$ 1:1 in 10 mM PBS, pH 7.4.

The total impedance is determined by important parameters such as electrolyte resistance (R_Ω), constant phase element (Q), charge transfer resistance (R_{CT}), and Warburg element (Zw). To providing more detailed information on the impedimetric behavior of the biosensor, a modified Randles equivalent circuit was adopted. We list the parameters of the fitted R_{CT} element in Table 1. The sequence of measurements shows an additional blockage of the interface after each step, confirming that the amount of material immobilized on the electrode surface directly correlates with the impedance. Similar behavior was obtained using Cramoll lectin adsorbed at nanosystems based on polyvinylbutyral, polyaniline and gold nanoparticles [59,60]. Furthermore, this research revealed that Cramoll adsorption provokes a blockage of

electron transfer at work electrode interface due to roughness increasing [59,60].

Table 1. Values of the resistance charge transfer from fitted impedance.

Samples	**R_{CT} (kΩ)**
Ti	0.3
TNTs	3.7
TNTs-LbL	2.0
TNTs-LbL-Cramoll 10 µg/mL	4.7
TNTs-LbL-Cramoll 20 µg/mL	15.4
TNTs-LbL-Cramoll 40 µg/mL	39.5
TNTs-LbL-Cramoll 80 µg/mL	40.1
TNTs-LbL-Cramoll 160 µg/mL	40.8
TNTs-LbL-Cramoll 320 µg/mL	42.5

3.3. Bioactivity of Cramoll immobilized on TNTs

Bioactivity confirmation of Cramoll on TNTs-LbL-Cramoll system was done with the assay using ovalbumin due to the ability of lectins to recognize carbohydrates; the ovalbumin glycoprotein glycidic moiety binds to Cramoll. Residual concentrations of ovalbumin after 1 h of incubation with the substrates are presented in Table 2 as the average of triplicate wells. It was observed that the higher the Cramoll concentration on TNTs-LbL, the lower the residual ovalbumin concentration, that is, the higher the concentration of ovalbumin bound to the lectin in a dose-dependent relationship. It can be inferred that even immobilized on the nanotubular arrays, Cramoll did not lost bioactivity.

Table 2. Protein dosage of ovalbumin assay.

Initial concentration of ovalbumin (µg/mL)	Concentration of Cramoll on the TNTs-LbL-Cramoll (µg/mL)	Average of residual ovalbumin concentration after 1 h of incubation (µg/mL)
100	10	80.1
100	20	67.0
100	40	6.8

The lectin has about 82% homology with Concanavalin A differing only in 42 from 236 Cramoll amino acid residues; both lectins have identical binding sites for monosaccharide, Ca^{2+} and Mn^{2+} [61]. These lectins recognize a fraction of ovalbumin containing Man7 and Man8 oligomannose chains [62]. Cramoll continued with its bioactivity by interacting with ovalbumin even after immobilized on TNTs-LbL. Similar result using Cramoll immobilized on the surface of gold electrode already has been reported [59].

3.4. Cell adhesion

One of the first responses after implantation of a biomaterial in a recipient organism is cell adhesion to its surface and such interaction can affect cellular functions like proliferation and differentiation, since adhesion of osteoblasts is a necessary prerequisite for the later functions of these cells, for example, for calcium deposition in bone formation [63,64]. Fig. 5 shows images of osteoblast-like cells adhesion with nuclei visualized in blue, stained with DAPI dye, and actin filaments stained with rhodamine-phalloidin, in red. An increasing of cell adhesion was observed on TNTs in the presence of polyelectrolytes (Fig. 5b) and then with Cramoll immobilized in the concentrations of 10, 20 and 40 µg/mL (Figs. 5c, 5d, 5e,

respectively) comparing to the surfaces with bare TNTs (Fig. 5a). The appearance of interconnected cells that can be visualized in the fluorescence images is due to the filopodia phenomenon as explained in a previous study where osteoblasts were seeded on TiO_2 nanotubes grown on titanium surfaces and in Ti6Al4V and Ti6Al7Nb alloys [65].

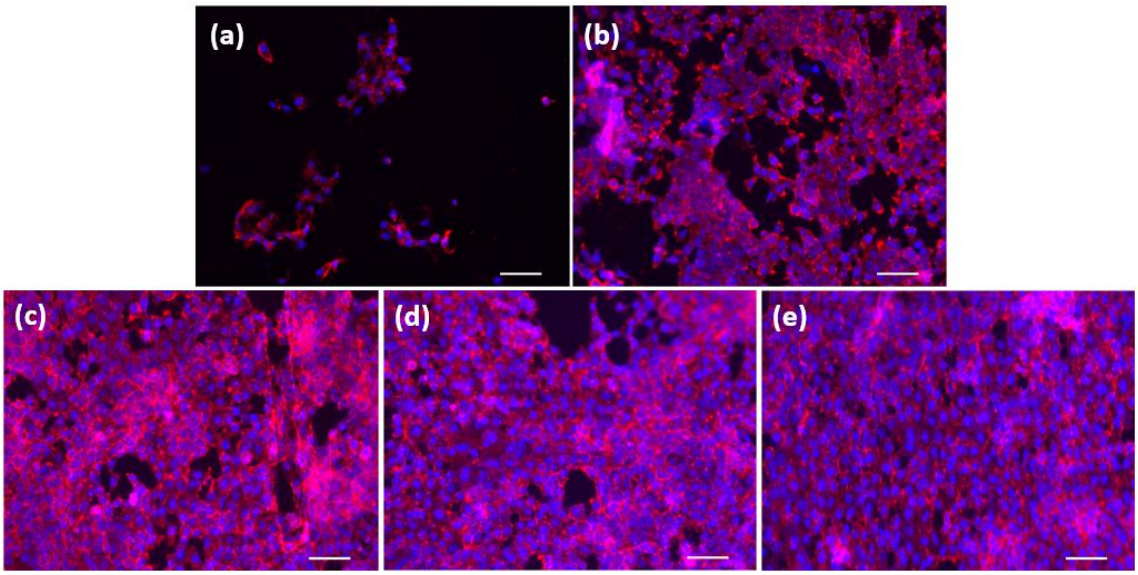

Fig. 5. Fluorescence microscopy images of osteoblast-like cells after 24 h of incubation with the different samples: (a) TNTs, (b) TNTs-LbL, (c) (d) and (e) TNTS-LbL-Cramoll in the concentrations of 10, 20 and 40 μg/mL, respectively. In red is the cytoskeleton and in blue the nuclei stained by rhodamine-phalloidin and DAPI, respectively. Scale bar: 100 μm.

Previous studies demonstrated an effective cell adhesion on TNTs surfaces in the presence of self-assembled PAH/PAA films by using these polyelectrolytes in pH conditions similar to the values used in our study. Furthermore, they showed that in low thicknesses formed films (around 4 or 5 layers) there was a greater cell propagation on substrate surfaces by anchoring of the actin filaments; on the other

hand, cell adhesion was diminished by increasing the number of layers [34,35,66]. We observed on TNTs-LbL and TNTs-LbL-Cramoll surfaces that the cells were scattered randomly and homogeneously overlapping each other resulting in a dense cell layer. Therefore, our results demonstrated a good biocompatibility of both functionalized nanotubular arrays.

3.5. Osteoblast-like cells proliferation

Osseointegration can be achieved by the creation of a favorable microenvironment where the osteogenic cell lines are capable of proliferating, and consequently synthesizing bone matrix [67]. The cell proliferation assay is important because it can determine the best surface in which more cells proliferate to promote host bone formation faster around the implant. The analysis of cell proliferation on the different substrates was performed and the results after 48 h of incubation are represented in Fig. 6.

Osteoblast-like cells after 24 h of incubation, indicated to have a higher proliferation in the TNTs-LbL-Cramoll systems, independent of concentrations applied, but not significantly when compared to the other systems (24 well plates, bare TNTs, and TNTs-LbL). Additionally, after 48 h (Fig. 6) a significative cell proliferation was observed on the substrates with Cramoll at 80, 160 and 320 μg/mL, when compared to the surfaces without the lectin and with the systems containing immobilized Cramoll in low concentrations (10, 20 and 40 μg/mL). However, we observed after 72 h of incubation a similar percentage of viable cells among all Cramoll concentrations. This probably occurred because the cells incubated with high immobilized Cramoll concentrations (80, 160 and 320 μg/mL) on TNTs-LbL reached a maximum confluence after 48 h and the excess of cells was withdrawn by the washes performed during the

72 h assay. Moreover, cells incubated with Cramoll in smaller concentrations (10, 20 and 40 μg/mL) also reached the maximum confluence in the well after 72 h.

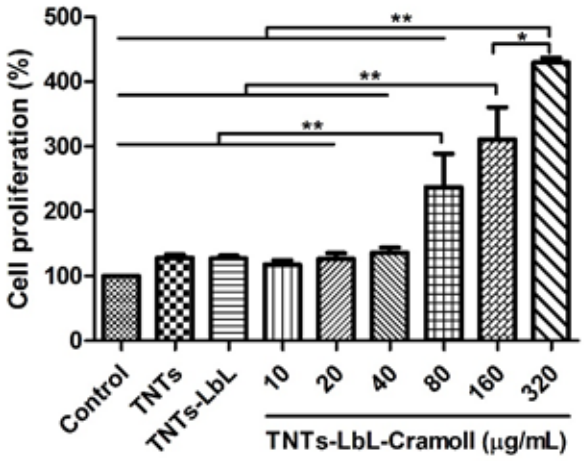

Fig. 6. Osteoblast-like cells proliferation seeded on the different substrates after 48 h. *p < 0.05, **p < 0.01.

TNTs functionalized with bioactive molecules, such as icariin, gelatin, chitosan and GRGDS peptide also promoted proliferation of osteoblasts, as well as Cramoll, immobilized on TNTs surfaces [17,48,68,69]. Therefore, biomolecules can improve the biocompatibility of implants manufactured by TNTs as well as the Cramoll lectin.

Bone cells can adhere directly to the surface of an implant or indirectly, binding proteins found in the surrounding fluids to the surface, followed by binding of those biomolecules to receptors on cell surface and such binding can culminate in cellular signals resulting in better adhesion and proliferation [70]. Lectins can bind to cell surface carbohydrates and trigger various cellular events, such as stimulation of cell proliferation. Mitogenic effects of Cramoll on murine and human lymphocytes are known and both actions were related to carbohydrate lectin binding sites [25,71,72].

Cramoll also has immunomodulatory activity and is capable of stimulating the proliferation of splenocytes by induction of these cells to the S phase of cell cycle [26].

Previous studies indicated that Cramoll, both in its native and recombinant form, maintained the viability of peritoneal exudate cells obtained in the peritoneal cavity of rats; such cells include macrophages, lymphocytes, dendritic cells, granulocytes and natural killers [73]. Due to the ability of Cramoll bind to carbohydrate residues present on the cell surface and to promote a mitotic stimulus, this can be a factor that stimulates the proliferation of osteoblasts and contributes even more with the fast osseointegration through TNTs-LbL-Cramoll surfaces.

Conclusions

The deposition of self-assembled PAH/PAA films on the surface of TNTs was effective for binding with Cramoll; this lectin remained bioactive even after its immobilization on the TNTs-LbL. A new nanotube surface coated with PAH/PAA and the immobilized lectin favored a better cell adhesion when compared with bare TNTs. Cramoll stimulated the osteoblast-like cells proliferation; therefore this lectin showed to be a promising bioactive molecule for a faster healing with better osseointegration of implants.

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5 ARTIGO II

FUNCTIONALIZATION OF TITANIUM DIOXIDE NANOTUBES WITH BIOMOLECULES FOR BIOMEDICAL APPLICATIONS

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Review

Functionalization of titanium dioxide nanotubes with biomolecules for biomedical applications

Weslley F. Oliveira[a], Isabel R.S. Arruda[b], Germana M.M. Silva[b], Giovanna Machado[b], Luana C.B.B. Coelho[a], Maria T.S. Correia[a,*]

[a] Departamento de Bioquímica, Universidade Federal de Pernambuco (UFPE), Av. Prof. Moraes Rego, s/n, Cidade Universitária, CEP: 50670-420, Recife, PE, Brazil
[b] Laboratório de Nanotecnologia, Centro de Tecnologias Estratégicas do Nordeste (CETENE), Av. Prof. Luiz Freire, 01, Cidade Universitária, CEP: 50740-540 Recife, PE, Brazil

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ABSTRACT

Titanium (Ti) and its alloys are extensively used in the manufacture of implants because they have biocompatibility. The production of a nanostructured surface can be achieved by means of titanium dioxide nanotubes (TNTs) which can have dimensions equivalent to the nanometric components of human bone, in addition to increasing the efficiency of such implants. The search is ongoing for ways to improve the performance of these TNTs in terms of their functionalization through coating these nanotubular matrices with biomolecules. The biocompatibility of the functionalized TNTs can be improved by promoting rapid osseointegration, by preventing the adhesion of bacteria on such surfaces and/or by promoting a more sustained local release of drugs that are loaded into such TNTs. In addition to the implants, these nanotubular matrices have been used in the manufacture of high-performance biosensors capable of immobilizing principally enzymes on their surfaces, which has possible use in disease diagnosis. The objective of this review is to show the main techniques of immobilization of biomolecules in TNTs, evidencing the most recent applications of bioactive molecules that have been functionalized in the nanotubular matrices for use in implants and biosensors. This surveillance also proposes a new class of biomolecules that can be used to functionalize these nanostructured surfaces, lectins.

1. Introduction

Titanium (Ti) and its alloys have been a raw material for the manufacture of biomaterials because of their biocompatibility and resistance to corrosion [1]. The passivation phenomenon contributes to these characteristics and makes Ti suitable for use in dental and orthopedic implants, without promoting adverse reactions locally or systemically. Importantly, this metal upon exposure to air or aqueous electrolytes forms a passive and stable layer of titanium dioxide (TiO_2) which can reach a thickness of 2–10 nm on its surface in 1 s, providing resistance to the release of metal ions [2,3]. TiO_2 can also be used for the construction of biosensors, because as a semiconductor it allows for the rapid transport of electrons from reactions on its surface to the Ti substrate, improving the performance of these important tools for the diagnosis of diseases [4].

According to the dimensions of the surface characteristics, the roughness of the surface of the implants can be of macro- (varies from millimeters to microns), micro- (1–10 μm) or nano- (1–100 nm) scales [5]. Nanoscale surface topography is preferred for implant making. The fact that bone tissue presents nanoscale structures, such as collagen, allows this nanotopography, with surface energy higher than the other texture scales, to improve the adhesion of matrix proteins, such as fibronectin and vitronectin, and to stimulate cellular migration and proliferation, important steps in the process of osseointegration, *i.e.* the formation of bone around the implant [6,7]. Functionalized and modified nanostructured devices for biomedical applications have become increasingly investigated in the hybrid science field named nanobiotechnology [8]. The metallic, ceramic, polymeric and composite nanomaterial properties may be integrated with biomolecules to promote combined and synergistic effects from the hybrid systems, such as biomolecule-nanoparticles [8,9]. The hybrid nanomaterials combining inorganic and organic or even bioactive components into a single material are promising for using in biomedicine, for example, the organic-inorganic hybrid hydrogel, polymers and the magnetic Janus particles that have physical properties on their two or more dissimilar faces for drug delivery [10–12]. Inorganic/organic hybrid materials can be obtained by atomic layer deposition to promote the modification of polymer surfaces [13].

* Corresponding author.
E-mail address: mcorreia@ufpe.br (M.T.S. Correia).

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Drugs may be delivered with release in a target site of the organism, even at the cellular level for delivery of genes into cells using nanocarriers, such as inorganic nanoparticles and quantum dots, for example, carbon dots developed with glucose and polyethyleneimine [14–16]. Specific nanomaterials to use as vehicles depending of the characteristics of the therapeutic biomolecule; for that the delivery and expression of optogene used in the optogenetics may to stimulate or inhibit the neural activity should be taken into account the optical, electrical, thermal properties and proper bio-functionalization of nanomaterials [17]. Upconversion nanoparticles, luminescent materials that can absorb near-infrared light and emit UV–visible light, covalently conjugated with a photosensitizer has been a highly specific and targeted treatment option in the photodynamic therapy studies [18]. Gold nanostructures can be synthesized with size controlled using reduction of copper; the properties of nanostructures depend on their morphology and are widely used in biomedicine, such as drug delivery and biosensing systems [19,20]. Proteins and peptides from the eggshells participate of the nucleation of calcium carbonate crystals and play an important role in the eggshell biomineralization; such biomolecules can be used in the future with TiO_2 nanostructured for manufacturing of implants [21,22].

TiO_2 nanotubes (TNTs) are tubular and self-organized nanostructures that have attracted considerable attention in implant manufacturing because of their mechanical stability, low cost of preparation and better biocompatibility compared to TiO_2 film [23–25]. TNTs are able to form well-defined nanostructured platforms with favorable transport pathways, good adhesion to the substrate and high surface area, *i.e.*, having a large number of atoms on their surface, available to interact with many biomolecules and allowing their use as an electrode in the manufacture of biosensors [26]. TNTs can be obtained in large quantities by various synthesis techniques, such as the sol-gel method, hydrothermal treatment and electrochemically by anodization [27]. Anodic oxidation (or anodization) is a simple and versatile technique that synthesizes TNTs with controlled structure and morphology, being aligned perpendicularly and easily to the Ti substrate [28,29].

Therapeutic failure in the use of implants may occur due to insufficient bone formation in the tissue surrounding the biomaterial, as ineffective bone fixation may lead to bacterial infection [30]. Research has been carried out with the aim of improving the functionality of these implants made of TNTs, for example, by the immobilization of biomolecules on the surface of these nanotubular matrices. The administration of growth factors such as bone morphogenetic protein 2 (BMP2) in TNT implants may improve their osteoinductive capacity [31,32]. TNTs can be used as matrices for the immobilization of proteins and enzymes for use in biosensors, such as the enzyme glucose oxidase (GOx) on the surface of TNTs for the preparation of an enzymatic biosensor capable of detecting glucose [33].

The objective of this review is to address some techniques used for the immobilization of biomolecules in TNTs, since its synthesis, to make the applicability of these functionalized TNTs in biomedicine more efficiently, either as implants or as biosensors.

2. Immobilization of biomolecules in TNTs

The growth of TNTs by electrochemical anodization occurs in aqueous electrolytes with fluoride ions and organic electrolytes such as glycerol or ethylene glycol. This method is based on an oxidation-reduction reaction that occurs in an electrochemical cell in which titanium is used as the anode and at the cathode is an inert material such as platinum [29,34]. An electrical potential from a power source is applied in the process to promote an electrical field and thus the diffusion of oxygen ions present in the electrolyte to form an oxide layer on the surface of the anode [35].

Fig. 1 shows the scanning electron microscope (SEM) image of TNTs at a magnification of 100,000 × (Fig. 1a), showing an ordered layer of the nanotubes obtained by the anodization process. Fig. 1b is at 7000 × and bacterial *Staphylococcus aureus* cells can be seen forming clusters on the surface of the TNTs.

Fig. 2 presents a schematic showing the formation of TNTs and their functionalization with biomolecules. The reaction that occurs on the anode describes the growth of oxide on the surface of Ti (Fig. 2a), in which the oxidized species of the metal react with the O^{2-} ions, provided by the water molecules, to form the TiO_2 layer (Fig. 2b). The fluoride ions, present in the electrolyte, have the ability to form $[TiF_6]^{2-}$ complexes, which are soluble in water and promote a chemical attack, that is, the dissolution of the TiO_2 formed. As soon as the oxide layer is obtained, there is decay of the current applied in the anodization and then the nanopores begin to grow on the surface of the metal (Fig. 2c). The equilibrium state is reached when the growth rate of the nanopores at the oxide-metal interface is the same as the rate of dissolution of the oxide, allowing continuous growth of the nanotubes (Fig. 2d) on the Ti surface [36]. Depending on the application, the TNTs can undergo a heat treatment to convert their amorphous structure into nanocrystalline structures such as anatase and rutile [34].

The reaction that leads to the synthesis of TNTs in Ti (a) begins with the formation of the TiO_2 layer on the metal (b); then this oxide undergoes dissolution by fluoride ions that leads to the appearance of nanopores (c); these nanopores become deeper and deeper until they form an orderly and compact layer of TNTs (d). Nanotubes can still have their functionality enhanced by the immobilization of biomolecules by loading and/or coating them (e).

The biomolecules can be immobilized in the nanotubular matrices by coating the surface of the TNTs and/or by loading them (Fig. 2e). The adsorption of biomolecules in TNTs can occur by physical methods such as hydrophobic interactions, hydrogen bonds and electrostatic interactions, or by chemical methods such as the covalent attachment with formation of ether, amide and thioether linkages, for example.

The sol-gel coating methods have been classified into two types: dip coating and spin coating [37]. These techniques are being applied to promote the coating of TNTs with biomolecules, such as the coating of these nanotubular matrices with chitosan biopolymer [38,39]. Spin coating is based on the application of a solution on a rotating substrate with subsequent ejection and evaporation of the solvent. Dip coating consists of immersing a substrate in a solution followed by gravitational drainage and evaporation of the solvent. Both methods allow the formation of a homogeneous film on the surface of the substrate [40].

Spin coating and dip coating can also be used for Layer-by-Layer (LbL) assembly, which is a technique capable of forming Polyelectrolyte Multilayers (PEMs) by adsorption of oppositely charged polyelectrolytes attracting by electrostatic interaction, on the surface of a given substrate [41]. TNTs could be coated by LbL using the polysaccharides chitosan and sodium hyaluronate, with positive and negative charges, respectively [42].

Spin coating is a fast method to obtain films homogenous with thickness easily changed mainly by changing spin speed, or the viscosity of the solution to be deposited [40,43]. A limiting factor to the spin coating technique is the substrate size because large substrates cannot be spun at a sufficiently high rate in order for formation thin film [43]. Besides, dip coating does not need sophisticated apparatus characterizing a low cost solution deposition; a thin film of solution onto a plate, cylinder, or irregular shaped substrate, that is, without defined geometry, is a distinguishing feature of this technique [44–46]. There are those who consider one drawback the fact that in the dip coating process occurs the film formation in both sides of the substrate [45]. However, this factor may be favorable for the immobilization of biomolecules on the TNTs, since in certain anodization conditions the TNTs formation occurs in both sides of the Ti foil, so, unlike spin coating that promotes the immobilization on a single side, the dip coating may to allow the functionalization in the two sides of the anodized Ti foil, that is, more functionalized biomolecules on the TNTs may be obtained.

Biomolecules are also covalently immobilized on TNTs. BMP2 can

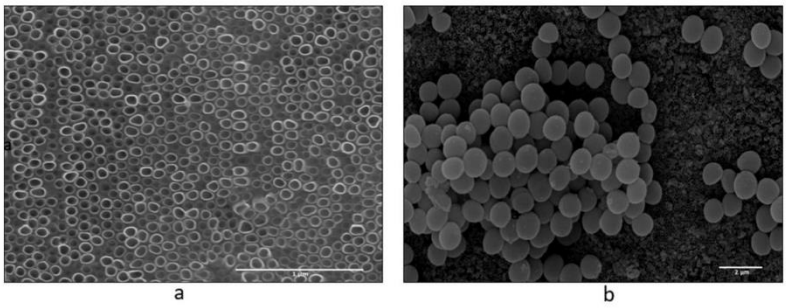

Fig. 1. SEM top view images of TNTs at different orders of magnification: (a) 100,000 × and (b) 7000 × with evidence of *S. aureus* colonies.

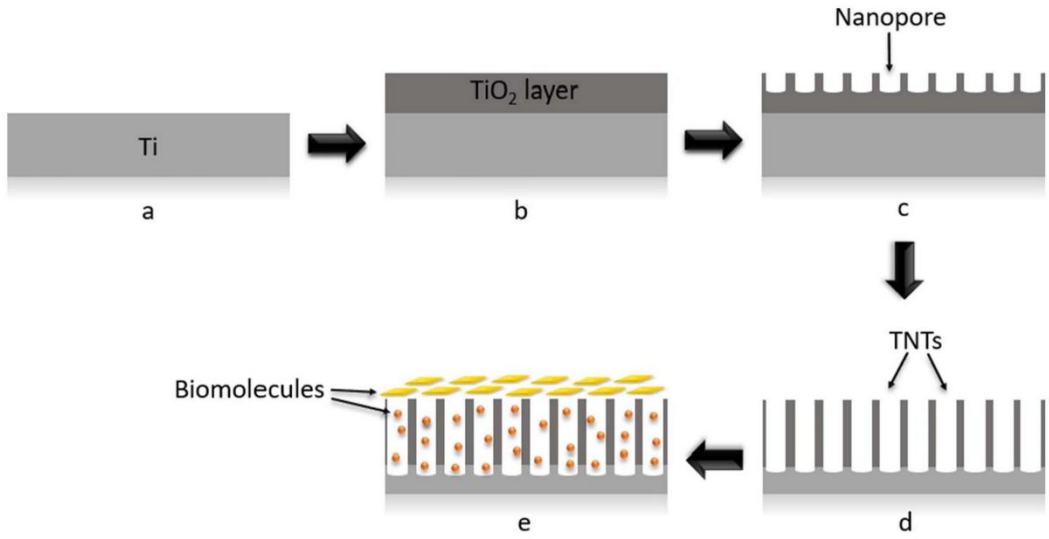

Fig. 2. Synthesis of TNTs and their functionalization with biomolecules.

functionalize TNTs through a covalent bond of this protein to the catechol and quinine groups derived from dopamine polymerization [31]. In another approach, a TNT/polypyrrole hybrid matrix was prepared for the covalent immobilization of GOx, where the chemical bond −CH═N− was formed between the enzyme and polypyrrole through the glutaraldehyde cross-linker [47].

TNTs can be loaded with bioactive molecules to promote localized release of them into a specific body compartment. Lyophilization has been a technique used to fill TNTs, for example, with connective tissue growth factor (CNN2) and the antimicrobial peptide cyproterin B (CecB) [42,48]. Lyophilization or freeze drying is a process that removes a solvent, usually water, from a product frozen by sublimation (primary drying) and desorption (secondary drying) obtaining biomolecules that can be labile with purity and high stability [49,50].

3. Functionalized TNTs with biomolecules

In order to increase the biocompatibility of TNTs used in implants, these matrices have been functionalized with cytocompatible biomolecules (Fig. 3) that may be able to stimulate the migration of cells to the implant site, increase cell adhesion, promote osteogenic differentiation of mesenchymal stem cells or even stimulate the proliferation of osteoblastic cells when the biomolecule is endowed with mitogenic activity. Another approach used in studies is the use of bactericidal or bacteriostatic biomolecules or those that only help prevent the adherence of planktonic (free-floating) bacterial cells on the surfaces of the TNTs in order to consequently avoid the formation of biofilm that can culminate with a peri-implant infection and implant loss.

The immobilized biomolecules may be able to render the surface of TNTs attractive for cell adhesion, such as mesenchymal stromal cells (MSCs) and mature osteoblasts. Another artifice that can be exploited of some biomolecules is the possibility of them preventing bacterial adherence, thus avoiding the formation of biofilm in these functionalized nanotubular matrices.

Table 1 summarizes some biomolecules that functionalize TNTs and their main applications. Different peptides and proteins were immobilized in the TNTs mainly aiming at the osseointegration process in comparison to the nonfunctionalized nanotube matrices. However, enzymes were also immobilized in these matrices and were successful for the detection of a specific analyte for use in bioenergetics. Table 1 shows the use of chitosan for the functionalization of TNTs. This bioactive polymer, besides being biocompatible and able to be used as a polyelectrolyte for surface coating, also has antimicrobial property which can act in synergism with the functioning of drugs that are loaded in TNTs [51]. The flavonoids quercetin and icariin were used as alternative, natural compounds capable of improving the biocompatibility of biomaterials (Table 1).

4. Mechanisms through which the functionalizing biomolecules of TNTs improve the use of biomaterials

The definition of biomaterials developed by the National Institutes

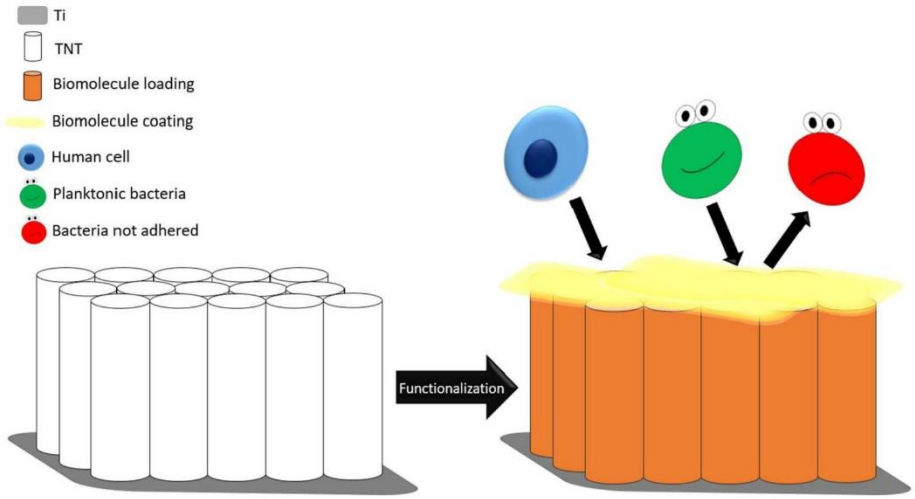

Fig. 3. Use in implants of TNTs functionalized with biomolecules improving cell adhesion for better osseointegration and preventing bacterial adhesion as a way to avoid possible infection.

of Health Consensus of 1982 is still used, which considers biomaterials as any substance or combination of substances, of natural or synthetic origin, that can be used for any period of time, as a whole or a part of a system in order to treat, increase or replace any tissue, organ or body function [74]. Biomaterials can be classified as metals, crystalline elements when solids, characterized by their opacity, ductility, conductivity and unique brightness; ceramics, hard, brittle materials, resistant to heat and corrosion, generally made by combining metal elements with oxygen or carbon; and polymers, high molecular weight compounds, derived from addition or condensation of many smaller molecules with elimination of water, alcohol or the like [75].

In relation to the biological reaction of the tissue to the biomaterials, these can be classified into three distinct categories: biotolerant, such as stainless steel and polymethyl-methacrylate, are substances that become surrounded by fibrous connective tissue after implantation and, although they promote the release of substances in non-toxic concentrations, they are not necessarily rejected; bioinert, such as alumina and zirconia, which allow bone apposition on its surface despite being susceptible to encapsulation in fibers; and bioactive, such as hydroxyapatite and bioglass, which are able to promote the formation of new bone on the surface, and interdiffusion of ions and chemical bonds with tissue [3,76,77]. Bioactive biomaterials can further be classified into osteoconductors, which are capable of binding to hard tissue, for example tricalcium phosphate; and osteoproductive biomaterials, such as Ti and niobium, which are those that bind spontaneously to the cells of the bone tissue and stimulate the growth of a new bone on its surface [76].

The main factors needed to achieve direct bone fixation of implants

Table 1
Biomolecules and their applications after functionalizing TNTs.

Biomolecules	Applications	References
Antimicrobial peptides	Possess antimicrobial activity for use in localized drug delivery	[52,53]
Arg-Gly-Asp peptide	Promote initial attachment and proliferation of human mesenchyme stem cells (MSCs) and improve adhesion of rat bone marrow stromal cells (BMSCs) from rat and osteogenic gene expression	[54,55]
Gly-Arg-Gly-Asp-Ser peptide	Stimulate cell spreading and proliferation of the osteoblast-like cell	[56]
Lys-Arg-Ser-Arg peptide	Increase preosteoblast adhesion and spreading on TNTs	[57]
Epidermal growth factor	Promotes rat MSCs proliferation and prevents cellular apoptosis induced by TNTs with a diameter of 100 nm	[58]
Bone morphogenetic protein-2	Osteoinductive action, reduces inflammatory responses, and promotes enhanced bone remodeling *in vivo*	[31,32,58–61]
Gelatin	Stabilizes gold nanoparticles improving osteoblast adhesion and propagation; gelatin is used mainly as coating to control drug release profile	[60,62–64]
Hemoglobin	Detection of hydrogen peroxide	[65]
Glucose oxidase	Detection of glucose	[26,66–68]
Urate oxidase	Detection of uric acid	[26]
Trehalose	Together with BMP2 on TNTs have osteogenic potential on BMSCs and anti-inflammatory properties	[32]
Chitosan	Controls the release of drugs, has antimicrobial action, good osteoconductivity, and manufacturing nanoparticles	[38,39,42,60,63,64,69–72]
Hyaluronic acid/hyaluronate	Fabrication of bacteria triggering antibacterial and manufacturing nanoparticles	[42,72]
Palmitoyl-oleoyl phosphatidyl-choline	Used as a barrier for controlling and sustaining release of drug	[53]
Quercetin	Loads TNTs and its release into the environment as an alternative for the treatment of post-operative infection, inflammation and quick healing with better osseointegration	[73]
Icariin	Enhances bioactivity of osteoblasts	[63]
Small interfering RNA (siRNA) targeting tumor necrosis factor alpha (TNF-α)	Suppresses inflammation and improves osteogenesis	[72]

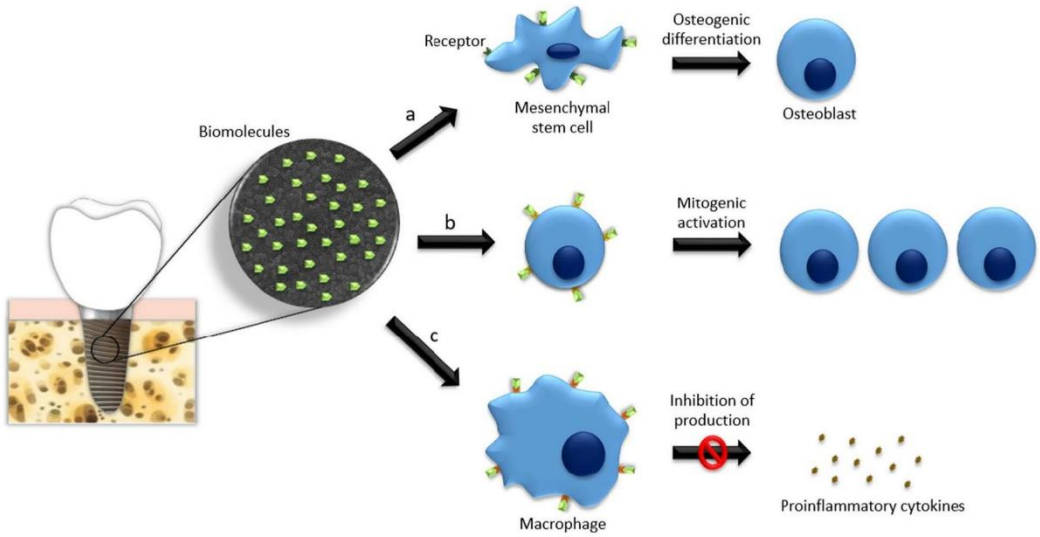

Fig. 4. Endosteal implantation of TNTs functionalized with biomolecules and their interaction with cells to accelerate osseointegration.

used in the areas of dentistry and orthopedics include surface properties and implant design, the quality of the host bone, the preparation of the surgical site, loading conditions and the prevention of initial and chronic infections [78]. Orthopedic implants include temporary ones such as plaques and screws, and permanent ones that are used to replace the hip, knee, spinal column and finger, for example [79]. The two main types of dental implants are subperiosteal, inserted into the top of the bone that lies below the periosteum over the bone cortex, and the endosteal, inserted into the cortical/basal bone usually in the maxilla or mandible and typically has a screw format to mimic the root of the tooth [79–81].

Fig. 4 shows an endosteal implant, with biomolecule-functionalized TNTs, inserted into the bone and replacing a dental root. In the conventional process, after the placement of these implants a period, generally three to six months, is expected for osseointegration and to be able to place a crown prosthesis. However, there is also the immediate loading of these implants with the prosthesis to shorten the time of the complete treatment and guarantee aesthetics [82,83]. The interactions that may occur between the functionalizing biomolecules of the TNTs with the cells in the peri-implant microenvironment are also elucidated in Fig. 4 as mechanisms necessary to accelerate osseointegration.

After implant insertion, immobilized biomolecules in TNTs can enhance osseointegration through their binding to cell surface receptors culminating in the following: differentiation of mesenchymal stromal cells into osteogenic lineage (a); stimulation of osteoblast proliferation (b); and inhibition of the production of proinflammatory cytokines by leukocytes, such as macrophages (c).

4.1. Cellular response in bone formation

The first biological component to come into contact with the implant after its insertion is blood, resulting in a series of biological processes on the surface of the material [84]. Some extracellular matrix proteins that are adsorbed on the surface of the implant contain the Arg-Gly-Asp tripeptide that is capable of interacting with adhesion proteins (integrins) present in the cell membrane, culminating in cell adhesion [85,86]. The peptide Lys-Arg-Ser-Arg linker of heparan sulfate and a constituent of transmembrane proteoglycans, has also been used for the adhesion of osteoblasts [87].

Early interactions of blood cells and fibrin on the surface of implants influence platelet activation and clot formation [88]. Platelets contain in their granules growth factors, such as vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF), which contribute to the recruitment of osteogenic cells [89]. Therefore, the fibrin matrix acts as a scaffold (osteoconduction) for the migration of mesenchymal cells and eventual differentiation (osteoinduction) of these cells in the osteoblastic lineage [84,90]. Fig. 4a illustrates these osteoconduction processes with the consequent osteogenic differentiation, since the functionalized biomolecules in the TNTs can interact with cell surface receptors of the MSCs resulting in cascades of intracellular signaling and activation of transcription factors that culminates with osteoblastogenesis [91]. It is known that some transcription factors, such as runt-related transcription factor 2 (Runx2) and osterix (Osx, also known as Sp7), are fundamental for the differentiation of MSCs in osteoblasts, since these factors regulate the expression of genes related to osteoblasts including that of osteocalcin [91,92].

Bone morphogenetic proteins (BMPs) are a group of proteins that can play a vital role in cell stimuli, such as differentiation, proliferation and inhibition of growth of various cell types, depending on the cellular microenvironment and other regulatory factors [93]. There are approximately 20 known BMPs, but BMP2 and BMP4 act as the main triggers for osteogenic differentiation [94]. BMP2 has been widely used to functionalize TNTs (see Table 1), including a recombinant human form, to make osseointegration faster.

Stem cells differentiate into osteoprogenitors with limited self-renewal capacity, after osteoinduction; they become pre-osteoblasts with limited proliferation, until they mature into mature osteoblasts that synthesize the osteoid which is the non-mineralized, organic component of the bone matrix. The osteoid is then mineralized to form the trabecular bone that eventually restructures into lamellar bone in direct contact with the surface of the implant [90,95]. The optimal implant surface nanostructure for osteoblast proliferation is yet to be established [96].

Cells may proliferate or remain quiescent in response to the cellular environment; while the progression of the G1 phase to the S phase of interphase is considered the point of no return, since in the absence of stress such as DNA damage, the cell is committed to complete the cell cycle and divide [97]. The stimulus of biomolecules for the proliferation of osteoblasts is illustrated in Fig. 4b; the p38 mitogen-activated protein kinase (MAPK), extracellular, signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) can be activated, resulting in the stimulation of osteoblast proliferation. BMPs, for example, are capable of

activating these three pathways [98].

Biomaterials capable of modulating the response of osteoblasts and osteoprogenitor cells may be crucial for the mechanical fixation of implants. Biomolecules may be options for making these biomaterials able to do such modulation, as in icariin immobilized in TNTs, which has been found to promote the proliferation of osteoblasts by regulating the expression of genes related to osteogenesis [63,95].

4.2. Anti-inflammatory action

Surgical injury caused by the insertion of an implant may result in an inflammation that typically occurs in the absence of microorganisms and is called sterile inflammation. The monocytes and neutrophils that circulate in the capillaries surrounding the implant are attracted to and activated in the peri-implant space due to cytokines released by the platelets; such activated leukocytes produce proinflammatory cytokines and chemokines such as interleukin 1 (IL-1) and tumor necrosis factor (TNF), which can induce bone resorption by osteoclasts [84,99].

Surfaces of TNTs with a diameter of about 78 and 80 nm have been able to promote *in vitro* adhesion and proliferation of macrophages by reducing the expression of the mRNA of proinflammatory cytokines, even in a lipopolysaccharide (LPS)-induced inflammation of *Escherichia coli*, used to mimic a stimulus of inflammation caused by bacterial infection [100,101]. Matching the characteristics of nanotopography with bioactive molecules capable of attenuating the inflammatory response may increase the chances of success in post-implant rehabilitation. Expression of BMP2 protein can be stimulated by proinflammatory cytokines [102]. TNTs functionalized with BMP2 and the carbohydrate trehalose, in addition to enhancing osseointegration, also promoted inhibition of IL-1 and TNF-α production following stimulation with LPS [32]. Fig. 4c demonstrates the recognition and binding of biomolecules to their specific receptors on the surface of cells involved in the inflammatory response, such as macrophages, which can trigger signal transduction and lead to inhibition of proinflammatory cytokine production. For example, activated transcription factor Nrf2 (NF-E2-related factor-2) inhibits the transcription of genes expressing IL-6 and IL-1β in macrophages [103].

It is known that quercetin has an anti-inflammatory action by the rat-paw edema test induced by carrageenan and also by the ability to reduce the production of proinflammatory cytokines by mast cells [104,105]. Icariin reduced the expression and secretion of IL-1, IL-6 and TNF-α in a model of osteolysis in rat calvaria induced by Ti particles [106]. Therefore, both flavonoids may have an additional, anti-inflammatory effect in their use when immobilized on TNTs in addition to their action on peri-implant bone formation.

4.3. Drug delivery

Osteoblasts can compete with bacteria for adherence to implant surfaces. When bacteria win this competition, they soon secrete extracellular polymeric substances (EPS), forming microcolonies until they develop into a mature biofilm. This leads to an infection that is one of the most recurrent causes of implant loss [107,108].

Usually peri-implant infections are treated by the administration of systemic drugs, but these are distributed throughout the body rather than to specific sites of interest which entail a series of complications and limitations such as poor biodistribution and low selectivity [109]. Localized drug delivery systems have been the most promising methods to promote a controlled and persistent release of drugs from TNTs to treat specific sites not only for infections but also inflammation and even cancer [110–114].

In order that the therapeutic agents used to load the TNTs may have extended release, they have been encapsulated into micelles and/or the nanotubular matrices have been coated by biopolymers so that the TNTs do not elute rapidly. Fig. 5 illustrates how such micelles and coatings are being manufactured by biomolecules such as D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) and chitosan, respectively [39,112–115].

TPGS is a natural derivative of vitamin E conjugated with polyethylene glycol with amphiphilic and nonionic properties; its voluminous structure and large surface area makes it an excellent emulsifier and solubilizer of hydrophobic drugs. One such example is TPGS micelles encapsulating indomethacin and itraconazole to load TNTs [114,116,117]. The antibacterial property of chitosan occurs due to electrostatic interaction between its NH_3^+ groups and the phosphoryl groups of the phospholipid components of the membrane of bacterial cells, leading to damage in the latter [118]. Therefore, chitosan may converge with the action of antibiotics in preventing bacterial adhesion on the surfaces of TNTs, in addition to prolonging the release of these drugs and favoring better adherence of osteoblasts as mentioned in Table 1.

5. TNTs *versus* biosensors

Biosensors are analytical devices composed of a specific bioelement (bioreceptor), such as an enzyme, which recognizes a specific analyte and a transducer sensor element that converts the biological response into an electrical signal [119,120]. Bioreceptors can be of two types: catalysts such as enzymes, microbes and organelles, as well as those of the affinity type, which include antibodies and nucleic acids, for example [121].

Currently, enzymatic biosensors are valuable tools for qualitative and quantitative analyses for markers used in disease diagnosis and environmental monitoring, as well as in biological and biomedical research [122]. The amount and activity of the immobilized enzymes, as well as the conductivity of the immobilization supports are key factors in the elaboration of a biosensor with high performance, such as high selectivity, sensitivity and reproducibility [33,123].

TNTs have been successful in the immobilization of biomolecules for the manufacture of electrochemical biosensors [124]. The matrices of TNTs have large specific surface area, high uniformity and their semiconductor characteristic increases the electron transport of the surface reaction to the Ti substrate, thus improving the performance of biosensors [125,126].

Several enzymes have been immobilized in these nanotubular matrices (see Table 1), with emphasis on the GOx that catalyzes the oxidation of glucose in the presence of oxygen in glucuronic acid and hydrogen peroxide (H_2O_2), which allows a redox electrochemical on the TiO_2/Ti electrode. The intensity of the generated current reflects mainly the amount of interfacial electron transfer in the TNTs/Ti electrode [127,128]. The enzyme fructosyl-amino acid oxidase has also been immobilized in TNTs for the detection of glycated hemoglobin (HbA1c), which is a biomarker of extreme importance for the monitoring of diabetic patients [129].

In addition to enzyme biosensors, TNTs have been used to construct an immunosensor, such as nanobodies, a distinct type of antibody fragment, functionalizing the TNTs capable of recognizing cystatin C through an antigen-antibody-like binding on the surface of TNTs. Further uses in the future may include determining the rate of glomerular filtration and diagnosis of different diseases [130]. Therefore, in addition to being able to be applied in the immobilization of enzymes, other biomolecules can also be used to functionalize the TNTs in order to recognize biomarkers and thus generate high performance biosensors, be they catalytic or the affinity type.

6. Lectins as promising biomolecules to functionalize TNTs

Lectins are proteins or glycoproteins of non-immunological origin and are characterized by binding to soluble carbohydrates or to a portion of sugar present in a glycoprotein or glycolipid [131]. These bioactive proteins are ubiquitous in nature, being found in viruses, microorganisms, plants and animals. Lectins have been the subject of

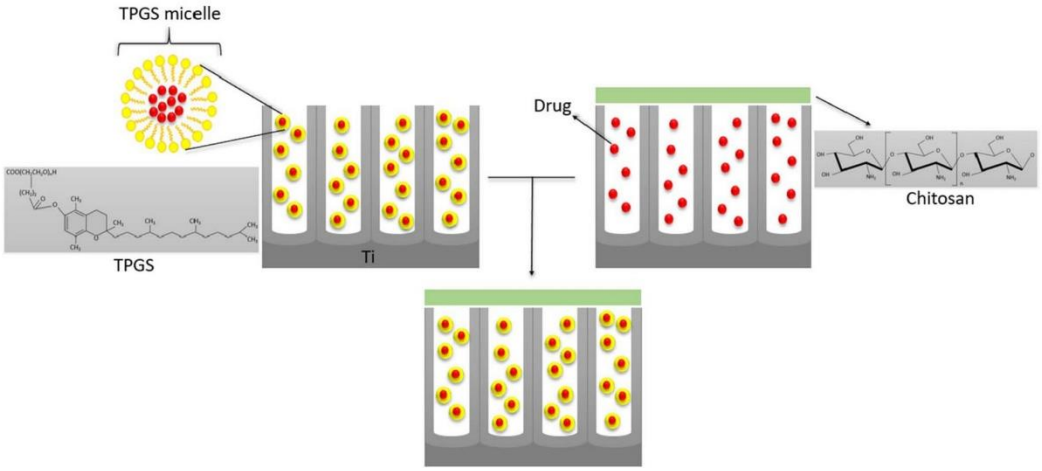

Fig. 5. Drug release from TNTs can be extended by encapsulating them in micelles, coating the TNTs with biopolymers or using the two mechanisms at the same time.

intense investigations in recent decades due to their great diversity of biological properties [132,133].

The ability to bind to glycoconjugates present on the cell surface makes lectins capable of exerting different effects on cells, such as agglutination, mitogenic stimulation, inhibition of bacterial and fungal growth, immunomodulation, among others [134]. Lectins harbor other characteristics that offer them advantages in their use in biomedical research, such as stability, low concentration activity, commercial availability and the ability to test subtle structural differences on the cell surface [135].

Some lectins have antimicrobial action, such as *Pp-Lec* purified from the hemolymph of the crab *Portunus pelagicus*, which showed antibacterial activity for gram positive and negative bacteria, besides having antibiofilm activity [136]. However, even lectins which do not exert a bactericidal or bacteriostatic effect may prevent the formation of biofilm. This is the case of the lectin obtained from *Bothrops jararacussu* venom which is able to inhibit biofilm formation without affecting the viability of *S. aureus* and *S. epidermidis*, the bacteria most commonly involved in biomaterial infections [137]. One possibility of using lectins for immobilization in TNTs is to render such matrices repellent to bacterial cells to prevent biofilm formation that might compromise treatment with those biomaterials.

A lectin that has presented a great versatility of biotechnological applications is Cramoll, which is obtained from seeds of the legume *Cratylia mollis*, popularly known as camaratu bean. This species belongs to the tribe Phaseoleae, subgroup Dioclinae, which also includes the species *Canavalia ensiformes*, botanically related to *C. mollis*, and the source of Concanavalin A (Con A). Con A is the most widely studied lectin and has 82% homology in the sequence of amino acids with Cramoll [138–140]. It has been found that Cramoll has immunomodulatory action and is also able to stimulate proliferation of lymphocytes and splenocytes [141–143]. The mitogenic activity of Cramoll and other lectins that have this action may be exerted when these bioactive proteins are immobilized in TNTs and thus stimulate the proliferation of osteoblasts to accelerate osseointegration in implants. Cramoll has also been immobilized on carbon nanotubes in the preparation of a biosensor to differentiate prostate cancer and benign prostatic hyperplasia, so the matrix of TNTs may be an alternative to electrodes offering biomolecule recognition in biosensors [144].

7. Conclusions

Biomolecules can be immobilized by coating and/or loading TNTs to make them more effective in their applications. Functionalizing biomolecules may be able to attract and induce osteogenic differentiation of MSCs, as well as promote better adhesion of osteoblasts and stimulate them to cell proliferation. In addition to rapid bone formation around implants, other important mechanisms to increase the biocompatibility of these biomaterials result from the ability of these biomolecules to inhibit the production of proinflammatory cytokines produced by leukocytes and by preventing the adhesion of bacteria on the surface of functionalized TNTs. These nanotubular matrices have also been able to provide large surface area for the immobilization of biomolecules, mainly enzymes, in the manufacture of biosensors. Lectins may have potential for immobilization in TNTs and thus improve the biocompatibility of implants and even serve in the manufacture of biosensors for the detection of glycosylated biomarkers necessary for the diagnosis of diseases.

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Competing interests

The authors declare that no competing interests exist.

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6 CONCLUSÕES

- Os TNTs revestidos com os filmes automontados de PAH/PAA pela técnica *Layer-by-Layer* (TNTs-LbL) permitiram a imobilização de Cramoll 1,4 nessas superfícies nanotubulares (TNTs-LbL-Cramoll);
- A bioatividade da lectina foi mantida após sua imobilização nas matrizes nanotubulares;
- Os TNTs revestidos com os polieletrólitos e com a lectina nas concentrações de 10, 20 e 40 µg/mL favoreceram maior adesão celular do que as superfícies com apenas os TNTs;
- A superfície TNTs-LbL-Cramoll com a lectina na concentração de 320 µg/mL estimulou maior proliferação das celulas comprovando a ação mitogênica desta lectina quando imobilizada nas superfícies nanotubulares;
- A funcionalização dos TNTs com Cramoll na presença de PAH/PAA mostrou ser uma plataforma promissora na elaboração de implantes metálicos pela capacidade desta lectina estimular a proliferação de células semelhantes a osteoblastos, o que pode acelerar o processo de osseointegração;
- Biomoléculas podem ser imobilizadas nos TNTs através do carregamento ou revestimento dessas superfícies nanoestruturadas, tal funcionalização pode melhorar a biocompatibilidade de implantes e ser uma alternativa para a fabricação de biossensores.

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ANEXO A – COMPROVAÇÃO DE SUBMISSÃO DO ARTIGO I

Manuscript Details

Manuscript number	MSEC_2017_3053
Title	Titanium dioxide nanotubes functionalized with Cratylia mollis seed lectin, Cramoll, enhanced osteoblast-like cells adhesion and proliferation
Article type	Research Paper

Abstract

An alternative to accelerate the osseointegration of titanium dioxide nanotubes (TNTs) used in osseointegrated implants is through the functionalization of these nanostructured surfaces with biomolecules. In this work, we immobilized a lectin with recognized mitogenic activity, the Cramoll lectin, extracted from Cratylia mollis seeds, on surfaces modified by TNTs. For the immobilization of Cramoll on TNTs surfaces, we used the Layer-by-Layer technique (LbL) by forming five alternate layers of poly(allylamine) hydrochloride (PAH) and poly(acrylic) acid (PAA) and lastly we incubated the lectin, at different concentrations, with the TNTs-LbL. Before and after the immobilization procedures, the substrate surfaces were characterized by scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), atomic force microscopy (AFM), and, electrochemical impedance spectroscopy (EIS). We also evaluated Cramoll activity after immobilization on TNTs by using the lectin interaction with ovalbumin. Through this test, it was showed that the lectin did not lose its biological activity, even after immobilization onto nanotubular arrays. In addition, we observed an increase osteoblast-like cell adhesion on the TNTs-LbL-Cramoll system when compared to the bare TNTs surfaces. Moreover, a significative cell proliferation was identified on the substrates when Cramoll was immobilized at concentrations of 80, 160 and 320 µg/mL after 48 h of incubation by using the resazurin assay. Our results suggest that Cramoll was efficiently immobilized on a nanotubular array and this new platform presents a great potential for use in implantology by promoting a fast process of osseointegration.

Keywords	Titanium; dioxide nanotubes; implant; Cramoll; osseointegration.
Corresponding Author	Maria Tereza Dos Santos Correia
Corresponding Author's Institution	Universidade Federal de Pernambuco
Order of Authors	Weslley Oliveira, Germana Silva, Paulo Cabral Filho, Adriana Fontes, Maria Danielly Lima de Oliveira, Cesar Andrade, Márcia Silva, Luana Cassandra Breitenbach Barroso Coelho, GIOVANNA MACHADO, Maria Tereza Dos Santos Correia

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ANEXO B – ARTIGO ACEITO PARA PUBLICAÇÃO NA REVISTA JOURNAL OF HOSPITAL INFECTION

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Review

Staphylococcus aureus and *Staphylococcus epidermidis* infections on implants

W.F. Oliveira [a], P.M.S. Silva [a], R.C.S. Silva [b], G.M.M. Silva [b], G. Machado [b], L.C.B.B. Coelho [a], M.T.S. Correia [a,*]

[a] *Departamento de Bioquímica, Centro de Biociências, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil*
[b] *Laboratório de Nanotecnologia, Centro de Tecnologias Estratégicas do Nordeste, Recife, Pernambuco, Brazil*

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SUMMARY

Infections are one of the main reasons for removal of implants from patients, and usually need difficult and expensive treatments. *Staphylococcus aureus* and *Staphylococcus epidermidis* are the most frequently detected pathogens. We reviewed the epidemiology and pathogenesis of implant-related infections. Relevant studies were identified by electronic searching of the following databases: PubMed, ScienceDirect, Academic Google, and CAPES Journal Portal. This review reports epidemiological studies of implant infections caused by *S. aureus* and *S. epidermidis*. We discuss some methodologies used in the search for new compounds with antibiofilm activity and the main strategies for biomaterial surface modifications to avoid bacterial plaque formation and consequent infection. *S. aureus* and *S. epidermidis* are frequently involved in infections in catheters and orthopaedic/breast implants. Different methodologies have been used to test the potential antibiofilm properties of compounds; for example, crystal violet dye is widely used for in-vitro biofilm quantification due to its low cost and good reproducibility. Changes in the surface biomaterials are necessary to prevent biofilm formation. Some studies have investigated the immobilization of antibiotics on the surfaces of materials used in implants. Other approaches have been used as a way to avoid the spread of bacterial resistance to antimicrobials, such as the functionalization of these surfaces with silver and natural compounds, as well as the electrical treatment of these substrates.

Introduction

Biomaterials are natural or synthetic materials, including polymers and metals, used to replace any living tissue that has undergone some accidental damage or destruction due to some pathology or even plastic surgery repair [1]. Researches and technological advances in the development of biomaterials have shown rapid growth in order to maintain a demand at the population level; the biomaterials can replace or restore the shape and function of a compromised tissue, improving people's quality of life and longevity [2].

Cytocompatibility and preservation of the differentiated phenotype of the cells surrounding the implants are fundamental properties of the biomaterials designed to be integrated to tissues, such as orthopedic implants, whose main objective is the osseointegration [3]. Despite the benefits that implants can offer, they are susceptible to several problems such as lack of integration, inflammatory process, total

* Corresponding author. Address: Departamento de Bioquímica, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, s/n, Cidade Universitária, CEP: 50670-910, Recife, PE, Brazil. Tel.: +55 81 21268540.
E-mail address: mtscorreia@gmail.com (M.T.S. Correia).

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rejection by the receiving individual, and bacterial infection, which is the main cause of implant loss [4]. Bacteria may adhere to form biofilms in foreign bodies placed in patients, such as central and peripheral venous catheters, and in breast/orthopaedic implants, thus establishing infection [5]. In addition, bacteria embedded in biofilms may exhibit greater resistance to environmental conditions as result of the high degree of horizontal gene transfer among them, including antibiotic resistance genes, favouring the infection [6].

Implant infections are most usually caused by staphylococci (about four cases in five). Two species, *Staphylococcus aureus* and *S. epidermidis*, account for around two-thirds of infections [7]. A strategy to eradicate implant infections is prolonged treatment with high doses of antibiotics, often using antimicrobials that act through different mechanisms [8]. However, in clinical practice, infected implants usually require their surgical removal in addition to long-term antibiotic therapy. These problems have stimulated advances in implant surface engineering research, in order to produce implants more resistant to bacterial colonization [9]. One of the most widely adopted strategies is the coating of surfaces with antibiotics; however, this is potentially a hazardous approach due to the risk of selection of drug-resistant micro-organisms, such as meticillin-resistant *S. aureus* (MRSA) [10].

This review aims to clarify mechanisms of *S. aureus* and *S. epidermidis* pathogenicity in implant infections, and to highlight some alternative approaches to preventing infections related to modifications on implant surfaces that could be used in the manufacture of biomaterials.

Methods

Studies were searched in electronic databases according to article titles, abstract contents, and relevance in the field of staphylococcal implant infections. The databases used in this research were PubMed, ScienceDirect, Academic Google and CAPES Journal Portal. The main terms applied were *S. aureus*, *S. epidermidis*, catheter, orthopedic implant, breast implant, infection, biofilm, antibiofilm activity and antibacterial implant surfaces. Articles were sought that provided new knowledge about the epidemiology of implant infections, the pathogenicity of *S. aureus* and *S. epidermidis* in these infections, and approaches to the prevention of implant-related infections. Each publication identified in the electronic searches was evaluated against these criteria by four authors (W.F.O., P.M.S.S., R.C.S.S. and G.M.M.S.); the selected articles were finally verified and approved by the other authors (G.M., L.C.B.B.C., and M.T.S.C.).

Microbial epidemiology of infections in intravascular catheter and orthopaedic/breast implants

The incidence of local or bloodstream infections associated with intravascular catheters is generally low. However, infections are important, because they are inconvenient to treat, and because serious infectious complications may occur, including sepsis and septic shock, infective endocarditis, and other metastatic infections [11].

Santarpia *et al.* studied 172 patients who had a total of 238 central venous catheters (CVC) used for home parenteral nutrition. Ninety-four of the catheters were associated with catheter-related bloodstream infection (CRBSI). Coagulase-negative staphylococci (CoNS) were the most frequent causative agents (52.8%); Gram-negative bacteria accounted for 18.6% of infections; 7.1% were caused by fungi, and 15% were by polymicrobial infections [12]. In another study of 85 patients receiving parenteral nutrition, 19% developed CRBSI. Again, *Staphylococcus* spp. (44%) were the most frequent species, followed by *Candida* spp. (25%) [13]. In a recent study by Wu *et al.*, 8% of patients with CVC following gastrointestinal surgery developed CRBSI, and once again CoNS were the most frequent cause of infection [14].

The main micro-organisms that cause infections in orthopaedic implants are Gram-positive bacteria such as *S. aureus*, *S. epidermidis*, and less frequently, *Propionibacterium acnes*; streptococci and enterococci tend to occur in later infections, and Gram-negative bacteria are seen far less frequently [15]. Montanaro *et al.* studied the microbial aetiologies of infections in 242 orthopaedic patients with infections, to investigate their aetiology. Overall, staphylococci accounted for ~75% of all isolates. *S. epidermidis* was the main pathogen in patients with knee and hip arthro-prostheses, whereas *S. aureus* was the main pathogen in patients with infections associated with internal and external fixation systems and in patients without implants [16]. A study of 163 patients aged 19–94 years with infected implants in the main joints or long bones of the lower limbs showed a predominance of *S. epidermidis* (51.5%), with 43.6% caused by *S. aureus* (43.6%), and both pathogens isolated from 4.9%. Older patients had a higher mortality rate and higher frequency of infection with meticillin- or multidrug-resistant bacteria [17]. In another study of 115 patients with *S. aureus* orthopaedic implant infections, those who had implants for bone fixation had a lower rate of MRSA infection than those who had arthroplasties. Other risk factors for MRSA were having an open fracture, nursing home residence, renal failure and hospitalization in an intensive care unit. This research raised the possibility of adapting antimicrobial prophylaxis for these higher-risk groups of patients [18].

The majority of isolates from breast-implant infection cases are *Staphylococcus* spp., particularly *S. aureus* and *S. epidermidis* [19,20]. A study with 37 cases of breast implant infection (81% silicone implants and 19% saline implants) showed that the most frequent aetiological agent was *S. aureus* (18 cases) [21]. However, Darragh *et al.* performed two retrospective audits: one with 86 patients undergoing 106 implant-based reconstructions, and another with 89 patients who underwent 105 implant-based reconstructions. In the first audit, bacteria were isolated in three cases, all of which were Gram negative (*Escherichia coli*, two cases; *Pseudomonas aeruginosa*, one case). In the second audit there were five infections, three caused by Gram-negative bacteria and one each caused by *S. aureus* and *S. epidermidis* [22].

Adhesion and biofilm formation: pathogenicity in medical devices

Multidrug-resistant nosocomial pathogens are the most common micro-organisms in medical device infections. They colonize the external and internal region of the catheters and proliferate at a rate of 0.5 cm of surface area per hour, being able to form a thick biofilm in 24 h on the surface of these

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plastic devices, from an inoculum with a small number of bacteria [23].

CoNS are important endogenous pathogens of intravascular catheters. Infections tend to be subacute or chronic, whereas infections with *S. aureus* are more likely to be acute due to its ability to stimulate an acute immune response in the host [24]. Figure 1 shows the routes of infection via CVCs, and the mechanism of biofilm formation. The most usual route of infection in short-duration catheters is by migration of micro-organisms from the skin at the insertion site to reach the catheter tip [25]. Catheter hub contamination by contact with contaminated hands, fluids or devices may also lead to an intraluminal colonization of the device [25,26]. More rarely the catheter may be contaminated via the haematogenous route; occasionally, contaminated infusate may introduce micro-organisms into the catheter lumen [26].

Biofilm growth occurs through a series of physical, chemical, and biological processes [27]. The ability of *S. aureus* to adhere to eukaryotic cells and abiotic surfaces through the proteins of its cell wall with subsequent biofilm formation are characterized as important virulence factors in infections associated with implanted biomaterials. The cell-to-cell attachment in the biofilm is known as cohesion [27,28]. Biofilm formation is divided into three steps (Figure 1): initial adhesion to a surface, microcolony formation and biofilm maturation with detachment of bacterial cells. The initial micro-organism adhesion to surfaces in which the planktonic cells become sessile (Figure 1a) strongly depends on the conditioning layer formed by the adsorption of (macro)molecules on the substrate. The composition of this biofilm favours bacterial adhesion and varies according to the environment to which those surfaces are exposed [29,30]. *S. aureus* and *S. epidermidis* express several microbial surface components that recognize and bind to extracellular matrix molecules, such as fibrinogen and fibronectin proteins, acting in the first stage of biofilm formation in the human body. The matrix proteins are also adsorbed on the surface of medical devices after implantation and may be targets for specific binding to the surface components of staphylococci [31,32]. The initial bacterial adhesion to surfaces is mediated by reversible interactions whose associated physical forces are van der Waals forces and steric–electrostatic interactions [24,27]. Subsequently the bacterial cells adhere irreversibly to the substrates through hydrogen bonds, ionic bonding, and dipole–hydrophobic interactions. Bacterial cell surface structures such as lipopolysaccharides (LPS) and exopolysaccharides also participate in these irreversible interactions [24]. For example, the production of slime exopolysaccharide by *S. epidermidis* is indispensable for its direct adhesion on implants [33].

The secretion of an extracellular polymeric substance (EPS) consisting of extracellular DNA, proteins, lipids and mainly polysaccharides (homo- and heteropolysaccharides) facilitates

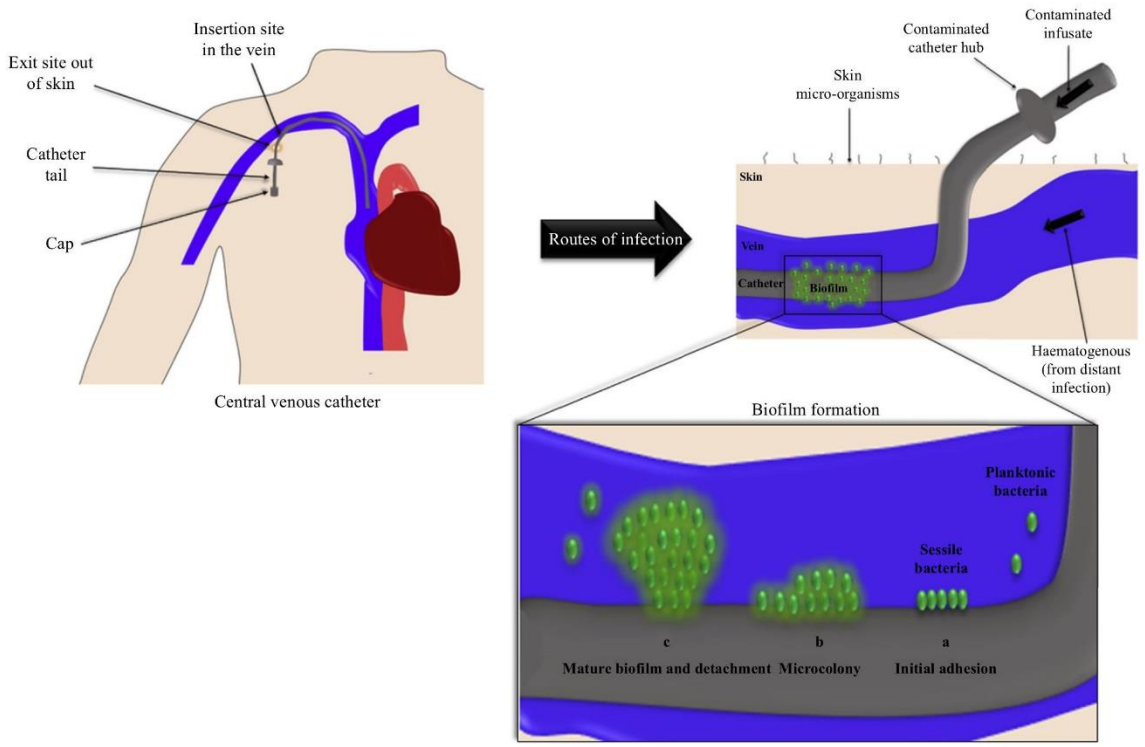

Figure 1. Schematic illustration of central venous catheter infection, showing the main access routes of micro-organisms to cause infection and biofilm formation.

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the adhesion between cells and surfaces [24,30]. Bacteria adsorbed on surfaces grow in microcolonies (Figure 1b) and secrete EPS, becoming encapsulated in a hydrogel layer that forms a physical barrier between the microbial community and the extracellular environment [30]. In biofilm development, microcolonies increase progressively and when several layers of cells accumulate on the surface the third stage of formation is reached, indicated by the presence of a mature biofilm (Figure 1c) which is characterized by the presence of macrocolonies surrounded by channels that help to distribute nutrients and signalling molecules. Finally, in order to survive when there is a limitation of nutrients or to spread and colonize other niches, some cells detach from the biofilm individually or in agglomerates [24]. EPS from staphylococci biofilms are composed of extracellular DNA, proteins, amyloid fibrils and polysaccharides such as the polysaccharide intercellular adhesin (PIA) known as poly-β(1–6)-*N*-acetylglucosamine (PNAG), which is the main component responsible by intercellular adhesion in staphylococci. PIA is the primary polysaccharide involved in the formation of *S. aureus* and *S. epidermidis* biofilms, contributing significantly to infections in medical devices and to the evasion of host immune responses [31,34,35]. PIA is synthesized by enzymes encoded by the *ica*ADBC locus consisting of four genes, the first and the second being *ica*A and *ica*D, respectively, which together synthesize a transmembrane enzyme with *N*-acetylglucosaminyl transferase activity, since this enzyme is only catalytically active with the junction of the products of these two genes. The *ica*C product appears to translocate PIA to the bacterial surface and the *ica*B product promotes deacetylation of the molecule. Another gene, *ica*R, known as the intercellular adhesin locus regulator, encodes a product that regulates negatively the *ica*ADBC locus [35]. The deacetylation of the *N*-acetylglucosamine residues in PIA is of great biological importance, since its free amine group confers positive charge to the molecule. This is then electrostatically attracted to the negative charge on the bacterial cell surface, mainly due to the presence of teichoic acids, contributing to the staphylococci adhesion in biofilms on certain surfaces [31]. The auto-inducing molecule called AI-2, which is a product of the LuxS gene and belongs to the LuxS quorum sensing system, regulates negatively the expression of the *ica* gene at the transcriptional level in *S. epidermidis* [36]. In *S. aureus* the Spx protein (global regulator of stress response genes) induces *ica*R gene expression, which promotes downregulation of *ica*ADBC expression, whereas the protein Rbf (protein regulator of biofilm formation) inhibits the expression of *ica*R, leading to an upregulation of *ica*ADBC [37]. Bacteria in the biofilm may be 500–5000 times more antimicrobial resistant than planktonic bacteria. Though biofilm initiates the antigenic response in the host by stimulating the production of antibodies, these communities are not yet affected by the host immunogenic response [38,39]. Several mechanisms contribute to biofilm resistance to antimicrobials, such as low penetration of the antimicrobial agent due to biofilm matrix barrier function, presence of persistent dormant cells, and small, highly resistant variant colonies. Reduction of antibiotic susceptibility also occurs: stress-adaptative responses of the bacterial cells in the biofilm may lead to delayed drug penetration or slow cell growth, to changes in the chemical microenvironment within the biofilm, and to upregulation of several biofilm-specific resistance genes [40–42].

Assays applied in the investigation of biofilm formation

Several methods are used to evaluate biofilm formation by bacteria and therefore may be applied to evaluate new compounds with antibiofilm action which may have potential value in implant functionalizations. Among these methods we highlight the tissue culture plate (TCP), tube method (TM), Congo Red agar method (CRA), bioluminescence assay, and fluorescent microscopic examination [43]. Christensen *et al.* were pioneers in investigating the formation of *S. epidermidis* biofilms on smooth surfaces as plastic tubes (by the TM method) and 96-well tissue culture plates (by the TCP method) [44,45]. The TCP method using microtitre plates is one of the most used to evaluate the formation of bacterial biofilms [46]. Knobloch *et al.* carried out a comparative study to evaluate the biofilm formation by *S. aureus* using the TCP, TM, and CRA methods. They verified that among 128 strains analysed, around 57% showed biofilm formation by the TCP method, and the addition of glucose and/or sucrose to the media (brain–heart infusion and tripticase soy broth) strongly influenced biofilm production among the strains. This study also showed that the CRA method is not indicated to evaluate the biofilm formation by *S. aureus*, and the TM method yielded a good correlation with the TCP test, but the classification by TCP test was difficult for biofilm-forming weak strains [47].

Biofilm formation may be quantified by different methods, including the use of dyes such as crystal violet. Dyes are often employed due to the low cost and good reproducibility. Crystal violet binds to negative charges, revealing the total biofilm biomass by the affinity to the bacteria and the EPS [48]. In addition to the total biomass quantification methods, there are assays that quantify the total number of bacterial cells, the number of viable cells, the amount of proteins and polysaccharides, presenting advantages and disadvantages that vary between cost and detection efficiency [48]. It is also possible to analyse the patterns of biofilm formation and to evaluate the activity of bioactive compounds through microscopy. Confocal laser scanning microscopy (CLSM) enables observation of whether the adhered cells forming the biofilm are alive or dead, using dyes such as SYTO-9, which is a fluorescent green dye that binds to nucleic acids and stains live and dead cells. Propidium iodide, which is a fluorescent red dye that penetrates damaged cells, stains dead cells [49].

Trentin *et al.* tested the antibiofilm activity of different plant extracts against *S. epidermidis* at concentrations of 4 and 0.4 mg/mL and evaluated the minimum inhibitory concentration (MIC) that kills 100% of the bacteria [50]. It was observed that the extracts actively inhibited the biofilm formation and that for the *Commiphora leptophloeos* extract the highest concentration tested was also bactericidal, suggesting that the biofilm inhibition at this concentration was due to the death of bacteria [50]. It is important to analyse the effect of the active agent on planktonic bacteria concomitantly to determine whether the compound acts to impede bacterial adhesion and/or biofilm destruction, or whether it kills bacteria, thereby reducing biofilm production. The nematode *Caenorhabditis elegans* is often used as an experimental model for in-vivo assay. Bakkiyaraj and Pandian tested the in-vivo and in-vitro antibiofilm activity of a coral-associated actinobacteria (extract CAA-3) against *S. aureus* [51]. In their study, *C. elegans* was infected

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with different strains of *S. aureus* and submitted to the treatment with CAA-3. Intestinal colonization of *C. elegans* was observed by CLSM and measured by Z-stack analysis. The conclusion was that intestinal colonization by *S. aureus* was reduced to ~70% when compared with the control.

The functionalization of nanostructured surfaces with bioactive compounds has attracted considerable interest for several applications. In a study by Qi *et al.*, multiple-wall carbon nanotube surfaces (MWNTs) were covered with a peptide with antimicrobial potential known as nisin, in order to observe *S. aureus* biofilm formation. A decrease of up to 95% in *S. aureus* biofilm formation was observed, compared with a reduction of only 37% for MWNTs alone. In this context, the surface modification used in implants, as functionalization with compounds that have antibiofilm activity, may be an effective alternative in the prevention of post-surgical infections [52].

Strategies for prevention of staphylococcal infections: catheter and implants

Some implant coatings have been used together with antibiotics in preoperative patients to prevent staphylococcal infections [53]. However, studies have also been carried out in order to add antibiotics or other biomolecules to the implants as a preventive action of possible postoperative infections [54]. The surface of implants may be functionalized with compounds having antibiofilm activity to promote a local effect of preventing bacterial adhesion and biofilm formation (Figure 2). This modification of surfaces in biomaterials has been an innovative strategy to be applied in the manufacture of implants.

A film composed of chitosan and gentamicin on titanium (Ti) nanotubes was studied by Feng *et al.* to evaluate the antibiotic effect against *S. aureus* [55]. Antibacterial effect was not observed with the Ti nanotubes without the coating on the bacterial colony. The results indicated that nanotubes coated with gentamicin and chitosan have high resistance to adherence of *S. aureus*, showing an antibacterial activity close to 100%. The coating of chitosan and gentamicin showed excellent antibacterial activity and may be applied to implants [55].

The use of synthetic and natural compounds as an alternative to antibiotics on biomaterial surfaces has also been investigated. Kuehl *et al.* examined the preventive effect on biofilm production of an implant using a silver-coated titanium–aluminium–niobium metal alloy. The in-vivo tests showed that the compound formed by the Ag-coated alloy was effective in the prevention of postoperative infection by *S. epidermidis*, especially in conjunction with perioperative antibiotic prophylaxis. On the other hand, such silver-coated implants showed only limited effect in the prevention of *S. aureus* infections [9]. Ti alloys are suitable for various requirements of implants [2]. Silver has bactericidal and bacteriostatic properties, and then its controlled release can be obtained with the use of silver nanoparticles. These nanoparticles have been immobilized on medical implant surfaces such as Ti and diamond-like carbon, enabling the prevention of *S. aureus* and *S. epidermidis* biofilm formation, including at the transcriptional level [56,57].

The biomolecules polymethyl methacrylate and polystyrene are widely used in the composition of biomaterials. When these materials were coated with the essential oil from the plant *Ocimum tenuiflorum*, the prevention of bacterial adhesion and *S. aureus* biofilms formation on these substrates was observed [58]. The enzyme deoxyribonuclease I (DNase I), capable of degrading extracellular DNA, was immobilized on Ti surfaces and showed a preventive role for *S. aureus* adhesion and its consequent biofilm formation [59].

Venous catheters can be treated with electrical conduction in order to prevent *S. aureus* biofilm formation, but there are no in-vivo assays due to the lack of patients willing to perform the tests. The applied electrical parameters are safe, avoiding the emergence of arrhythmias. Catheters were submitted to an electric current in units ranging from 4 to 8 μA and a reduction of 90% of viable bacteria was observed when the current was 4 μA for a 24 h period. However, the application of a current of 8 μA did not show a reduction proportional to the current increase [60]. The results appeared encouraging for developing solutions that avoid the contamination of catheters.

Conclusion

Staphylococcus aureus and *S. epidermidis* are frequently involved in infections in catheters and orthopaedic/breast implants. Planktonic bacteria become sessile cells capable of forming microcolonies after adhering to surfaces, secreting biomolecules that make up the EPS where they are embedded.

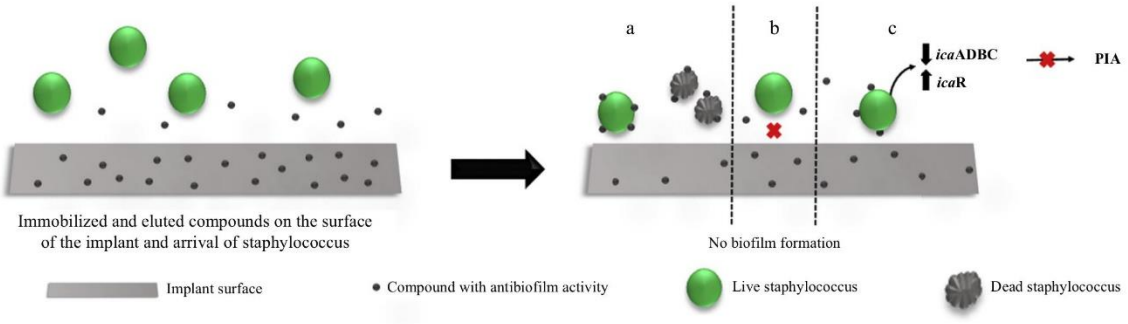

Figure 2. Compounds with antibiofilm activity can be immobilized on the surface of implants. The composites prevent the formation of biofilm, (a) stopping the growth or causing the death of the bacterium; (b) inhibiting the bacterial adhesion on the surface without exerting bacteriostatic/bactericidal effect; (c) and repressing the expression of the genes located in the icaADBC locus and stimulating the expression of icaR genes which consequently does not promote the production of polysaccharide intercellular adhesin (PIA).

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S. aureus and *S. epidermidis* synthesize PIA by the expression of genes located in the *ica*ADR locus, and the deacetylation of this adhesin promotes the adhesion of these bacteria to the biomaterial surfaces and the consequent infection. In the mature biofilm, bacteria can establish communication with one another, receive nutrients and water through channels, contributing to their survival on the biomaterial surfaces until they detached and become free-living cells capable of contaminating other locations. Bacteria in biofilm achieve greater resistance to antibiotics and to the immune system of the infected host. Due to the difficulty in treating implant infections, different methodologies have been used to test the potential antibiofilm of compounds; for example, crystal violet dye for in-vitro biofilm quantification offers low cost and good reproducibility. Changes in the surface biomaterials are necessary to prevent biofilm formation. Some studies have investigated the immobilization of antibiotics on the surfaces of materials used in implants. Other approaches have also been used as a way to avoid the spread of bacterial resistance to antimicrobials, such as the functionalization of these surfaces with silver and natural compounds, as well as the electrical treatment of these substrates.

Conflict of interest statement
None declared.

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