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Long-term release of antibiotics by carbon nanotube-coated titanium alloy surfaces diminish biofilm formation by *Staphylococcus epidermidis*

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Abstract

Bacterial biofilms cause a considerable amount of prosthetic joint infections every year, resulting in morbidity and expensive revision surgery. To address this problem, surface modifications of implant materials such as carbon nanotube (CNT) coatings have been investigated in the past years. CNTs are biologically compatible and can be utilized as drug delivery systems. In this study, multi-walled carbon nanotube (MWCNT) coated TiAl6V4 titanium alloy discs were fabricated and impregnated with Rifampicin, and tested for their ability to prevent biofilm formation over a period of ten days. Agar plate-based assays were employed to assess the antimicrobial activity of these surfaces against *Staphylococcus epidermidis*. It was shown that vertically aligned MWCNTs were more stable against attrition on rough surfaces than on polished TiAl6V4 surfaces. Discs with coated surfaces caused a significant inhibition of biofilm formation for up to five days. Therefore, MWCNT-modified surfaces may be effective against pathogenic biofilm formation on endoprostheses.

Key words: Multi-walled carbon nanotubes; Drug delivery system; Biofilm; *S. epidermidis*; Antibiotics; Prosthetic joint infection

Bacteria commonly attach to natural and artificial surfaces within the host organism to form biofilms consisting of extracellular polysaccharides. Microbial adhesion to gut epithelium, teeth or skin is a physiological process that is strictly controlled by host defense mechanisms. These are, for example, epithelial shedding or bacterial killing by antimicrobial peptides,

Abbreviations: AB, antibiotic; PJI, prosthetic joint infection; CNT, carbon nanotube; MWCNT, multi-walled carbon nanotubes; PECVD, plasma enhanced chemical vapor deposition; VLS, vapor–liquid-solid.

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thus, preventing overgrowth or a shift toward pathogenicity.[1,2] Artificial surfaces such as prosthetic implants, however, are not well protected against colonization by biofilms and their overgrowth. One of the most frequently isolated bacteria from prosthetic devices is Gram-positive coagulase-negative *Staphylococcus epidermidis* (*S. epidermidis*), a prevalent microorganism inhabiting the skin and mucosal surfaces.[3]

Bacteria gain access to prosthetic devices either during the surgery procedure, for instance after incomplete skin disinfection or via the blood stream, which they may enter through micro-injuries, a process termed bacteremia.[4,5] Importantly, microorganisms within biofilms are resistant to antibiotics (AB) and more difficult to eliminate.[6] Prosthetic joint infection (PJI) is a relevant and serious complication of prosthetic joint implantation being associated with pain, loss of mobility and a mortality rate up to 2.5%.[7] The relative incidence of PJI in the United States from 2001 to 2009 ranged between 2.0% and 2.4% of total hip arthroplasties and total knee arthroplasties. The annual cost of surgical revisions to US hospitals increased from $320 million to $566 million during this time range and was projected to exceed $1.62 billion by 2020. As the demand for joint arthroplasty is expected to increase by up to 673% until 2030, the economic burden of PJI may equally increase.[8,9]

A possible approach to target PJI is the functional redesign of implant surfaces using nano technologies or antimicrobial coatings. Surfaces should simultaneously respond to various biological and mechanical requirements and minimize bacterial adhesion and biofilm formation.[10] In the past years, a range of nanocarriers has been proposed for the delivery of bioactive agents and for the inhibition of bacterial growth.[11] Carbon nanotubes (CNT) were shown to be suitable structures for prolonged drug delivery, e.g. of anti-inflammatory drugs or growth factors.[12,13] They demonstrably can limit biofilm formation when anchored to a surface,[14,15] in suspension[16] or embedded in polymer nanocomposites.[17] Furthermore, CNT structures were found to bind a range of antibiotics.[18–22] Previously, our own experiments have shown that multi-walled carbon nanotubes (MWCNTs) are capable of stimulating the growth of stem cells and their differentiation into osteoblasts,[23] whilst other groups have demonstrated an overall enhancement of osteoblast function by MWNT.[24,25] These effects were achieved by the MWCNTs alone without the addition of growth-promoting drugs.

Although AB have very limited effects against existing biofilms, they can successfully prevent their formation, when a continuous supply of AB is provided. Here, we present a method to reduce in vitro biofilm formation by *S. epidermidids* using AB impregnated MWCNT-modified TiAl6V4 titanium alloy surfaces. The novelty of this approach is the assessment of the liquid holding capacity and effectiveness against biofilm formation of these surfaces over time, thus providing further insight into medically relevant features of MWCNT-modified surfaces.

Methods

MWCNT-coating of titanium alloy discs and scanning electron microscopy (SEM)

Vertically aligned MWCNTs were grown on roughened TiAl6V4 titanium alloy disc surfaces via plasma enhanced chemical vapor deposition (PECVD).[23] The roughness of the surfaces is specified with R_z = 10 μm. Prior to the MWCNT growth, a 10 nm thin layer of nickel was deposited via electron beam evaporations that forms Ni droplets upon melting at the PECVD process temperature of approximately 750 °C. These liquid Ni droplets act as catalysts in a tip growth type vapor–liquid–solid (VLS) mechanism where NH_3 and C_2H_2 are the gaseous precursors and the resulting vertically aligned MWCNTs the solid product. For details on the synthesis of MWCNTs via PECVD see reference.[26] The majority of the utilized MWCNTs had an approximate length of 700 nm with closed tube ends encapsulating nickel catalyst particles. The tube diameters ranged from approximately 10 nm to 200 nm and inter-MWCNT distances were in the same range.

Bacterial culture

As a model bacterium and biofilm forming microbe, *S. epidermidis* (ATCC 35984) was used in this study. *S. epidermidis* was maintained on tryptic soy agar (TSA) (BD, Heidelberg, Germany). Before each experiment, overnight cultures were prepared in tryptic soy broth (TSB) (BD, Heidelberg, Germany) at 37 °C on an orbital shaker. Next, the overnight culture was diluted 100× and grown to late logarithmic phase as monitored by optical density measurement (OD_{600} = 0.9–1.0).

Preparation of titanium alloy discs

As a rule, discs were handled using forceps to avoid damages of the MWCNT surface. Discs were immersed in 10 μg/mL Rifampicin (Sigma Aldrich, Seelze, Germany), which was assessed as the minimal inhibitory concentration (MIC), for 4 h at 4 °C. Moreover, MIC_{50}, at which 50% of bacteria are inhibited, was measured at 5 μg/mL. No bacterial inhibition was observed at 1 μg/mL. Rifampicin was shown to be effective against *Staphylococcus* species.[27] Next, discs were washed in sterile PBS for 30 s using a squirt bottle in order to fully remove excess Rifampicin solution. Immediately after washing and shaking off excess liquid, the discs were placed into 1 mL of sterile PBS in 24-well plates for 1, 3, 5 or 10 days and kept at 4 °C. By these means, the AB diffused into the PBS for various amounts of time (AB diffusion time) and the retention time for the liquid containing the AB could be assessed. As controls, rough discs without MWCNT modification as well as MWCNT discs were prepared in the same manner. From the geometric dimensions of the MWCNT coating the loading of the Rifampicin samples can be approximated to 250 pg/disc. This was calculated using the following parameters: MWCNT had an approximate length of 700 nm, closed tube ends and an average tube diameter and inter-MWCNT distance of 10 nm to 200 nm (arithmetic average = 105 nm). Each disc had an MWCNT-coated surface of 60 mm^2, where approximately 4.5×10^7 nanotubes were present per mm^2. The total volume of MWCNTs (0.018 mm^3) was substracted from the total volume of the surface (0.042 mm^3). Therefore, the average volume of all MWCNT inter-spaces was 0.024 mm^3 and the approximate liquid absorption capacity 25 nL/disc.