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Pre-adsorption of serum proteins regulates bacterial infections and subsequent macrophage phagocytosis on biomaterial surfaces

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Abstract

Bacterial infection has been one of the main obstacles for extensive biomedical applications of biomaterial films. Understanding the interactions among macromolecules, cells, and bacteria in the microenvironment located on the film surface at the molecular level is essential for developing anti-bacterial films. Here we report the distinct influence of several key serum proteins adsorbed on DLC and traditional Ti films on initial bacterial adhesion, biofilm formation and corresponding immune

responses. Type I collagen, Fn, and IgG were selected as the typical serum proteins. Gram-positive bacterium *Staphylococcus epidermidis* and Gram-negative bacterium *Escherichia coli* were used as the model bacteria. Macrophage phagocytosis tests were carried out to examine the impact of adsorbed proteins on the ability of macrophages to clear the adhered pathogens. Results show that it was the specific molecular recognition between adsorbed proteins and bacteria, not the surface physiochemical properties such as surface wettability, surface roughness and surfaces charge, that decisively affected bacterial adhesion and following biofilm formation. Collagen resisted bacterial adhesion on both DLC and Ti films, even though the molecules exhibited distinct conformations on the two surfaces. Whereas for Fn and IgG, the specific molecular recognition was closely related to protein conformations. Fn molecules formed globular aggregates on Ti surfaces that greatly enhanced bacterial adhesion, but exhibited a fibril conformation on DLC surfaces that inhibited bacterial adhesion. IgG showed an end-on orientation with free F(ab)₂ domains on Ti surfaces, facilitating bacterial adhesion and biofilm formation, while the flattened orientation on DLC films showed little effect on bacterial behaviors. Furthermore, pre-adsorption of Fn and IgG significantly promoted the phagocytosis ability of macrophages against *S. epidermidis* and affected the corresponding secretion of inflammatory cytokine. These results would give insights into understanding protein-surface interactions for developing appropriate surface modification techniques for biomaterials with desired anti-inflammatory functions.

Keywords: Protein adsorption, bacterial adhesion, biofilm formation, macrophage phagocytosis, protein conformation.

Introduction

With the rapid development of biomedical technologies, the role of biomaterials have become increasingly important. However, biomaterial-associated infections have been a major obstacle that limits the further application of biomedical materials. It was reported that more than 70% of surgery failure resulted from biomaterial-associated

infections¹, which have to be taken into serious consideration. Therefore, a deep understanding of bacterial biofilm formation on biomaterial surfaces is crucial to the surface design of biomedical devices and implants.

When bacteria indwell and aggregate on biomedical devices, they usually produce a matrix of extracellular polymeric substances (EPS) composed of polysaccharides, proteins, lipids and extracellular DNA². Planktonic bacteria are embedded in the self-produced matrix layers and form biofilms, which will protect the bacteria from the attack of bactericides and the host immune system while promoting further biofilm growth, thus leading to serious infections and surgery failure³⁻⁵. Once infections occur, immune cells are recruited to the infected sites within minutes, phagocytize pathogens and activate cellular functions such as proliferation, secretion of proteins and cytokines, and respiratory burst to destroy phagocytized organisms⁶⁻⁷.

Nevertheless, when studying biomaterial-associated infections, a crucial but usually ignored issue is serum protein adsorption. After biomaterials are implanted into the human body, adsorption of proteins occurs prior to the attachment and colonization of bacteria or cells on materials surfaces⁸. Therefore, adsorbed proteins serve as the bridge between the material surface and adherent cells. Adsorbed proteins may influence cellular and bacterial growth by altering surface physicochemical properties, such as surface wettability, surface roughness and surface charge⁹. Moreover, the type, amount and conformation of adsorbed proteins could also affect the subsequent adhesion of bacteria and host cells¹⁰⁻¹². In an earlier study, we have demonstrated that the conformation of vitronectins affected cell adhesion¹³: osteoblast adhesion and integrin gene expression were greatly promoted when vitronectins formed multimers, but were hardly affected when vitronectins formed dimmers. Hence, it is of vital importance to study the influence of serum protein adsorption on biomaterial-associated infections, given that related studies are rare and often ignored by researchers.

In this study, to address the bacterial infection problems of biomedical films, we

proposed a research strategy of examining the adsorption behaviors of typical key serum proteins on the films, the recruitment and competitive adhesion of bacteria and immune cells, and the interaction mechanisms among the species. The traditional biomedical material Ti¹⁴ and a newly emerged biomaterial DLC were selected as model biomaterials. DLC films may serve as promising coatings for biomedical devices owing to their superior biological and mechanical properties¹⁵⁻¹⁷, yet bacterial infections are the major challenge limiting their extensive biomedical applications. In this work, three types of serum proteins: type I collagen, fibronectin (Fn), and immunoglobulin G (IgG), due to their rich abundance and great importance on cellular and bacterial behaviors¹⁷⁻¹⁹, were typically selected to investigate the influence of their adsorption behaviors on bacterial biofilm formation and immune responses. Gram-positive bacterium *Staphylococcus epidermidis* and Gram-negative bacterium *Escherichia coli* were typically chosen to assess bacterial infections. Throughout this study, we attempted to disclose the mechanisms of the influence of serum protein adsorption on biomaterial-associated infections on the molecular level and provide insights for biomaterial surface design.

Materials and methods

Materials.

DLC films were purchased from Star Arc Coating New Material Technology Co. Ltd. Suzhou, China. The films were fabricated by CVD deposition on silicon substrates. Ti films were prepared by PVD deposition on silicon substrates.

Culturing of bacteria.

S. epidermidis (CMCC(B)26069) and *E. coli* (ATCC25922) were cultured at 37°C overnight in Trypticase Soy Broth (TSB) and Luria-Bertani (LB) media, respectively. The bacteria were harvested by centrifugation at 1500 rpm for 10 min twice, then diluted in PBS to an OD₆₀₀ value of 0.1 ($\sim 1 \times 10^8$ cells/ml). For bacterial adhesion test, *S. epidermidis* and *E. coli* were seeded onto the surfaces of the DLC and Ti films

with/without prior protein adsorption. Morphologies of bacteria were acquired by using field emission scanning electron microscopy (FESEM, FEI Quanta FEG 250, USA). After incubation for 4 h, the samples were rinsed three times with PBS and then submerged in 4% paraformaldehyde to fix the adherent cells. Biofilm formation was evaluated by crystal violet staining analysis method. After incubation for 24 h, the samples were rinsed three times with PBS, and the biofilms were stained with 0.1 wt% crystal violet solution for 5 min in dark. After washing the samples twice with PBS, 95% ethanol solution was added onto the surfaces and remained for 30 min to dissolve the stained biofilms. Finally, biofilm formation on the samples was measured using microplate reader at OD₅₇₀.

Pre-adsorption of proteins.

Three key serum proteins, type I collagen (Solarbio, C8026), fibronectin (Fn, Solarbio, F8180), and IgG (Sigma, I4506), were typically selected to adsorb on material surfaces. For pre-adsorption, 200 µl protein solution (20 µg/ml) was added onto the DLC and Ti samples and remained for 30 min. The samples were subsequently rinsed with PBS for three times to remove unadsorbed protein molecules. Adsorbed proteins on the DLC and Ti films were characterized by atomic force microscopy (AFM) (Bruker, Bioresolve, Germany) in aqueous environment. Micro BCA protein assay (Sangon Biotech, China) was conducted to quantitatively assess protein adsorption. After the pre-adsorption of proteins and three-time rinsing, the samples were incubated in 300 µl 2% SDS solution at 37°C for 24 h.

Characterization of surface physicochemical properties.

The surface roughness and surface potential of the DLC and Ti films with/without protein adsorption were characterized by AFM (Bruker, Incon, Germany) in AM-PKFM mode. The surface wettability of the DLC and Ti surfaces with/without protein adsorption was assessed by static water contact angle measurement.

Macrophage culturing and phagocytosis tests.

Macrophage cell line (RAW 264.7, Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) was used to reveal the cellular responses of macrophages on the sample surfaces. Macrophages were cultured in Dulbecco's modified eagle media (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 2% penicillin-streptomycin (Gibco) in humidified atmosphere with 5% CO₂ at 37°C. The cells were counted and diluted to a concentration of 5×10^5 cells/ml. Cell adhesion was examined using immunofluorescence assay. Cells were seeded onto the surfaces of the DLC and Ti films with/without pre-adsorbed proteins. After incubation for 4 h, the cells were washed three times with PBS, then fixed with 4% paraformaldehyde. Subsequently, the cells were blocked by 1% BSA overnight. Actin filaments were labeled with FITC-conjugated phalloidin (Sigma) for 90 min. Nuclei were stained with DAPI (Sigma) for 30 min. The samples were rinsed with PBS three times and examined immediately with confocal laser microscopy (CLSM, Leica TCS SP5, Germany).

The phagocytosis tests were conducted after the adhesion of *S. epidermidis* and *E. coli* ($\sim 1 \times 10^8$ cells/ml) for 4 h on the DLC films with/without prior protein adsorption. The samples were washed with PBS three times to remove unadsorbed bacteria, and macrophages were then seeded. After incubated for 4 h, the cells were rinsed with PBS and fixed with 4% paraformaldehyde. Morphologies of bacteria and macrophages were characterized by FESEM.

Results and discussion

To gain a comprehensive understanding of the influence of pre-adsorbed serum protein layers on bacterial behaviors on the DLC and Ti films, initial adhesion of *S. epidermidis* and *E. coli* on the films were characterized using SEM (Fig. 1, 2). Surprisingly, pre-adsorption of proteins showed remarkable influence on bacteria adhesion behaviors. For *S. epidermidis*, the pre-adsorption of collagen could dramatically inhibit bacterial adhesion on both DLC and Ti films (Fig. 1 A-2, B-2), where the adherent density decreased from around 3.3×10^5 cells/cm² to around 1.5×10^5 cells/cm² on both two films (Fig. 1C). Two or three bacteria bound with each other and were evenly

distributed on the entire surfaces. However, Fn or IgG pre-adsorption inhibited *S. epidermidis* adhesion only on the DLC films (Fig. 1 A-3, A-4), and there was no significant difference in bacterial adhesion on the Ti films (Fig. 1 B-3, B-4). In addition, for *E. coli*, the bacterial cells formed bacterial clusters without protein pre-adsorption (Fig. 2 A-1, B-1), especially on the surface of DLC films. On the surfaces pre-coated with proteins, the *E. coli* cells showed well-dispersed colonization. Collagen pre-adsorption also significantly deterred *E. coli* adhesion, as the density of adherent bacteria decreased from 5.0×10^4 cells/cm² to approximately 2.0×10^4 cells/cm² on both two surfaces (Fig. 2 A-2, B-2, C). Noticeably, Fn and IgG showed different effect on *E. coli* adhesion on the Ti and DLC surfaces. On the DLC surfaces, pre-adsorption of Fn substantially inhibited the initial adhesion of *E. coli*, reducing bacterial density to about 1.9×10^4 cells/cm² (Fig. 2 A-3). Whereas on the Ti surfaces, Fn dramatically enhanced bacterial attachment, increasing bacterial density to around 7.0×10^4 cells/cm² (Fig. 2 B-3). Moreover, IgG pre-adsorption on the Ti surfaces significantly promoted *E. coli* adhesion, increasing bacterial density to four times that on the untreated surfaces (Fig. 2 B-4). On the DLC surfaces, however, IgG had no pronounced promotion effect (Fig. 2 A-4).

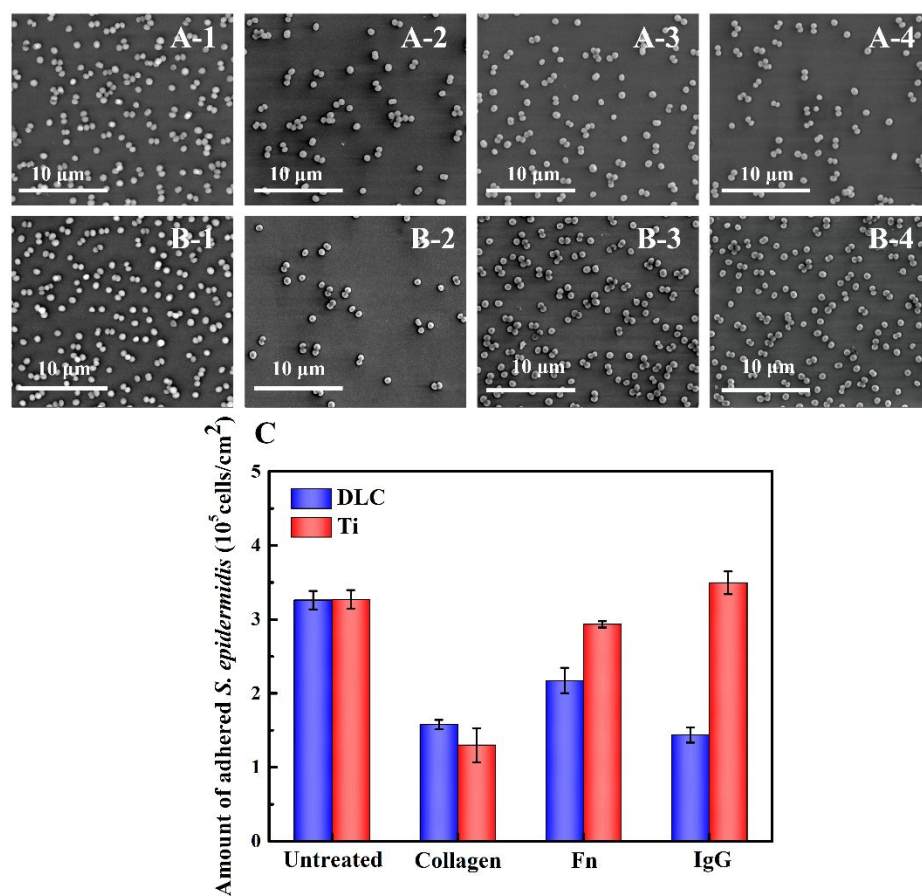


Fig. 1 A, B: FESEM images showing the initial adhesion of *S. epidermidis* on DLC (A) and Ti (B) films without (A-1, B-1) or with the pre-adsorption of type I collagen (A-2, B-2), Fn (A-3, B-3), and IgG (A-4, B-4). C: The density of adherent *S. epidermidis* on the DLC and Ti surfaces with/without protein adsorption.

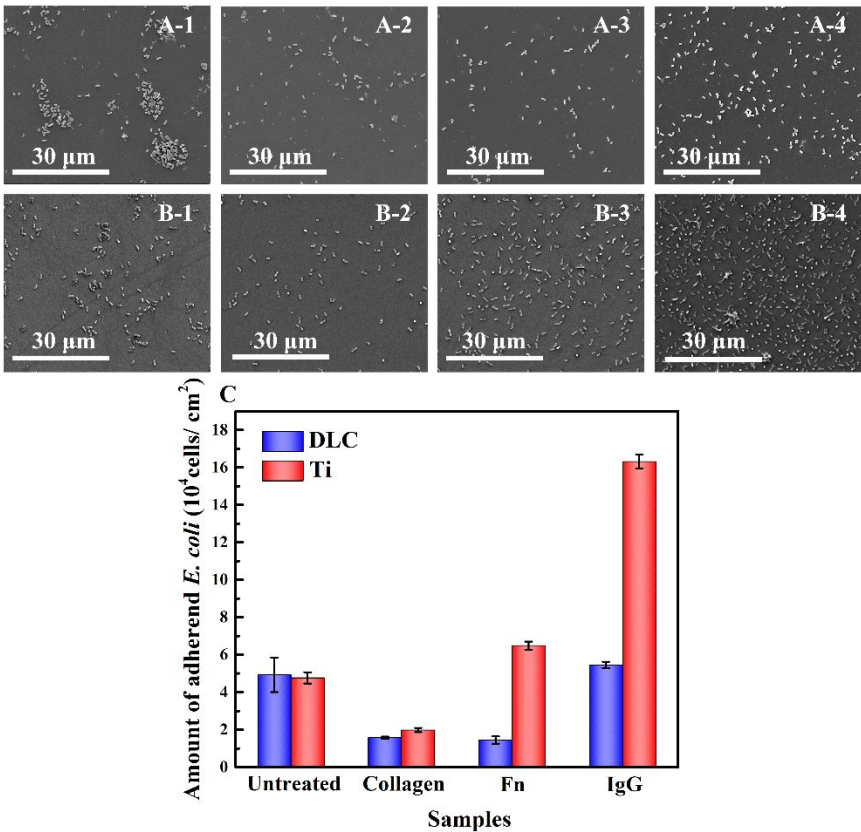


Fig. 2 A, B: FESEM images showing the initial adhesion of *E. coli* on DLC (A) and Ti (B) films without (A-1, B-1) or with the pre-adsorption of type I collagen (A-2, B-2), Fn (A-3, B-3), and IgG (A-4, B-4). C: The density of adherent *E. coli* on the DLC and Ti surfaces with/without protein adsorption.

These results suggest that protein adsorption could dramatically influence initial bacterial adhesion behaviors. Initial bacterial attachment may have considerable impact on the following biofilm formation and bacterial infection²⁰, thus the influence of protein adsorption on the subsequent biofilm growth of *S. epidermidis* (Fig. 3A) and *E. coli* (Fig. 3B) was quantitatively assessed. For *S. epidermidis*, pre-adsorption of collagen, Fn and IgG inhibited biofilm formation to different degrees, following a trend similar to that observed for initial adhesion. This indicates that the regulation of *S. epidermidis* biofilm formation by protein pre-adsorption was mainly achieved by regulating initial bacterial adhesion. For *E. coli*, however, pre-adsorption of collagen and Fn enhanced biofilm formation, and the promotional effect of Fn was more pronounced. In contrast, IgG exerted adverse effects on biofilm formation of *E. coli*.

This is different from the phenomenon observed for the initial *E. coli* adhesion, where bacterial adhesion was significantly promoted by IgG and inhibited by collagen. Thus, these results demonstrate that the biofilm formation of *E. coli* was only partly affected by the initial bacterial adhesion, and might also have been determined by the following bacterial growth factors, such as proliferation and EPS secretion, which were also influenced by protein adsorption. The underlying mechanisms should be further investigated.

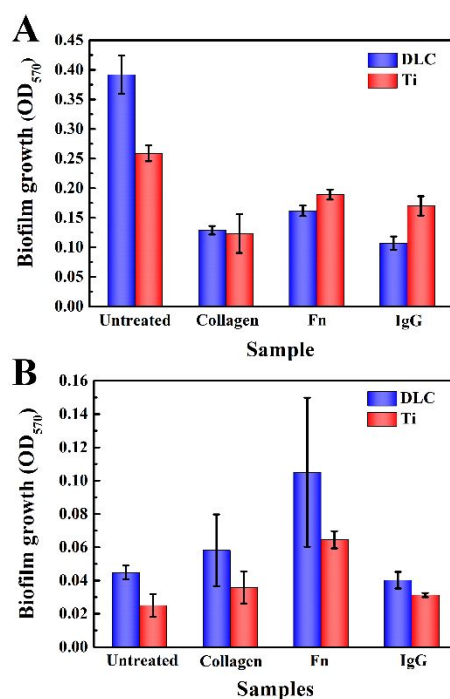


Fig. 3 Biofilm formation of the *S. epidermidis* (A) and *E. coli* (B) on the DLC and Ti surfaces with/without protein adsorption.

In order to elucidate the underlying mechanism as to how the pre-adsorbed proteins influenced bacterial adhesion and biofilm formation, we analyzed the possible explanations. Given that bacterial adhesion is a complex process, many research efforts have been devoted to clarifying the phenomenon from two possible perspectives: on the one hand, it is believed that the effect of adsorbed proteins on bacterial adhesion and biofilm formation is determined by surface physicochemical properties including wettability, roughness, and charge state²¹⁻²⁴, which could be altered by the protein adsorption process. On the other hand, some researchers believe that the specific

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4 molecular recognition between adsorbed proteins and bacterial cell wall receptors²⁵⁻²⁷
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6 plays the decisive role, thus the conformation of the adsorbed proteins was the key point.
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8 In this work, we analyzed the relationship between the altered surface physicochemical
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10 properties and bacterial adhesion and biofilm formation of *S. epidermidis* and *E. coli*.
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12 AFM was employed to evaluate the surface roughness and surface charge, and water
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14 static contact angle measurement was applied to assess the wettability. Results showed
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16 that after protein adsorption, the water contact angle decreased on both the DLC and Ti
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18 films, indicating that the surfaces became more hydrophilic. Next, we analyzed the
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20 bacterial density (Fig. 1C, 2C) and biofilm growth (Fig. 3) of *S. epidermidis* and *E. coli*
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22 as a function of the water contact angle. It was found that the bacterial growth of *S.*
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24 *epidermidis* and *E. coli* showed similar trends with the changes of the water contact
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26 angle, but there was no clear linear relationship (Fig. 4A). Besides, the relationship
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28 between surface roughness and bacterial growth was analyzed, but no noticeable
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30 correlation was found (Fig. 4B). Because the surface of the bacterial cell wall is
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32 negatively charged, some studies presumed that this electronegativity is the main factor
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34 affecting bacterial adhesion and growth on surfaces²⁸⁻²⁹. Therefore, we further
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36 characterized the surface potential of the surfaces before and after the adsorption of
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38 collagen, Fn and IgG to explore the influence of surface potential on *S. epidermidis* and
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40 *E. coli* adhesion and biofilm formation (Fig. 4C). Disappointingly, there was no
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42 significant linear relationship between initial bacterial attachment and subsequent
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44 biofilm formation and surface potential. These results suggest that the three surface
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46 physicochemical properties: surface wettability, surface roughness and surface charge
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48 were not the decisive factors affecting bacterial adhesion and biofilm formation.
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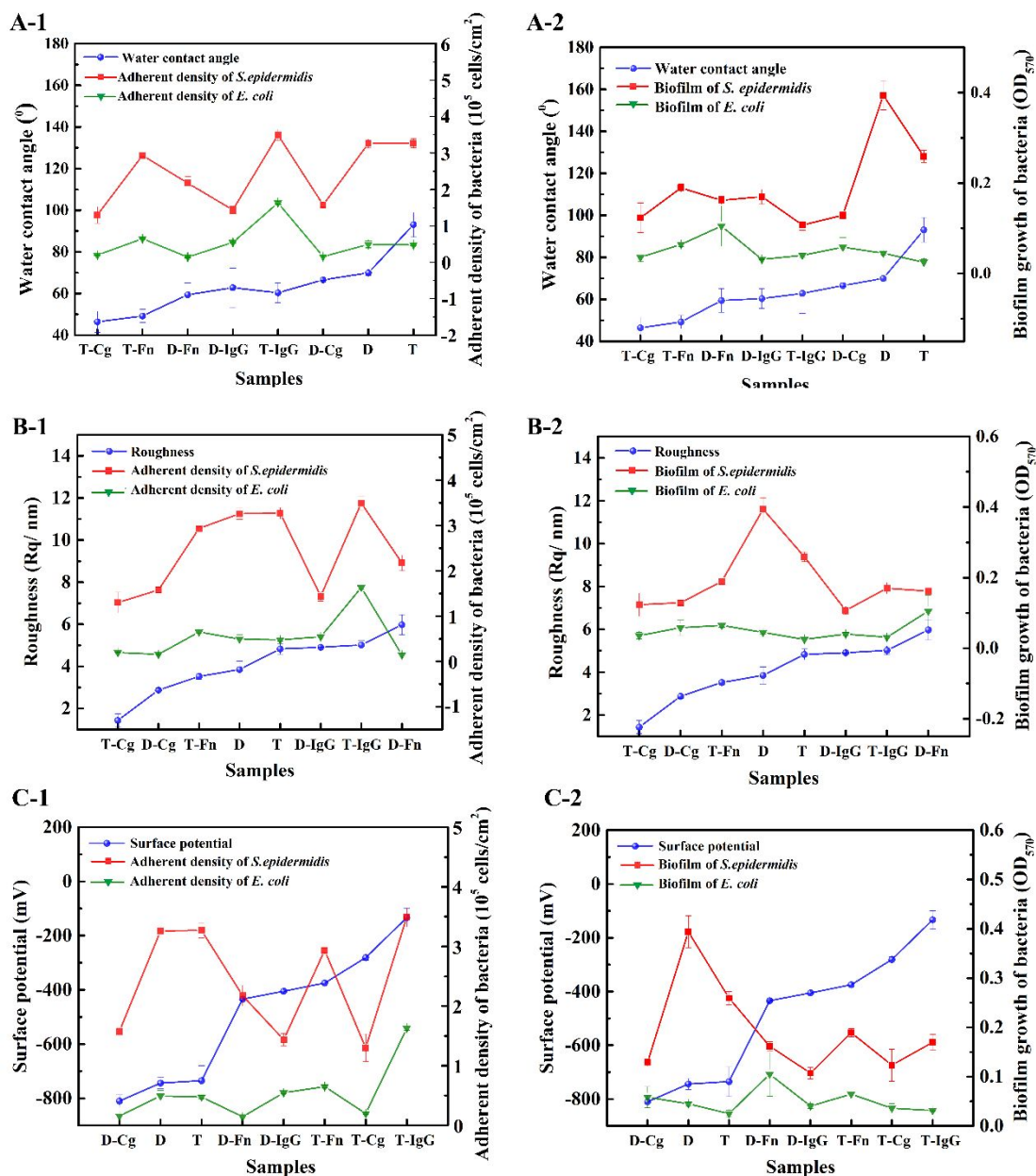


Fig. 4 The variation of the density of adherent bacteria (-1) and biofilm formation (-2) with surface wettability (A), surface roughness (B) and surface potential (C) on DLC and Ti surfaces with/without protein adsorption.

Given that bacterial growth was not dominated by non-specific interactions, we next studied the molecular specific recognition between adsorbed proteins and bacteria. AFM was employed to characterize the microstructure of the DLC and Ti surfaces. It was found that the DLC films displayed large island-like structures with diameters of

about 150~200 nm (Fig. 5A-1), while the Ti films possessed small grain-like structures with diameters of about 30~50 nm (Fig. 5A-2). The morphologies of collagen, Fn and IgG proteins adsorbed on the surfaces were then characterized. It was noted that collagen was apt to form interconnected folded fibril clusters with fibril diameters of approximately 15 nm on the surface of the DLC films (Fig. 5B-1), whereas quite long, thick fibrillar structures with diameters of around 30 nm were formed on the entire Ti film surface (Fig. 5B-2), indicating that collagen is easier to self-assemble to form fibrous structures on Ti surfaces. Fn exhibited fibril-contained nodules in a beads-on-a-string arrangement with an average height of 2 nm, and the nodules were evenly distributed on the island-like particles of DLC films in high density (Fig. 5C-1). On Ti surfaces, Fn molecules formed globular agglomerates with an average diameter of ~43 nm and an average height of ~2.5 nm (Fig. 5C-2). IgG molecules showed a globular conformation on both the DLC and Ti surfaces (Fig. 5D). After analyzing the heights of the observed molecules, it was noted that the majority of the IgG molecules adsorbed on the Ti surfaces possessed a higher height of 3.5 nm compared to that on the DLC surfaces (2.5 nm) (Fig. 5D), suggesting that IgG displayed distinct conformations on the two surfaces. In addition, micro-BCA assay was used to evaluate the amount of adsorbed proteins. It was found that the amounts of proteins adsorbed on the DLC surfaces were significantly higher than those on the Ti surfaces. The amounts of different types of proteins were basically the same on the same materials.

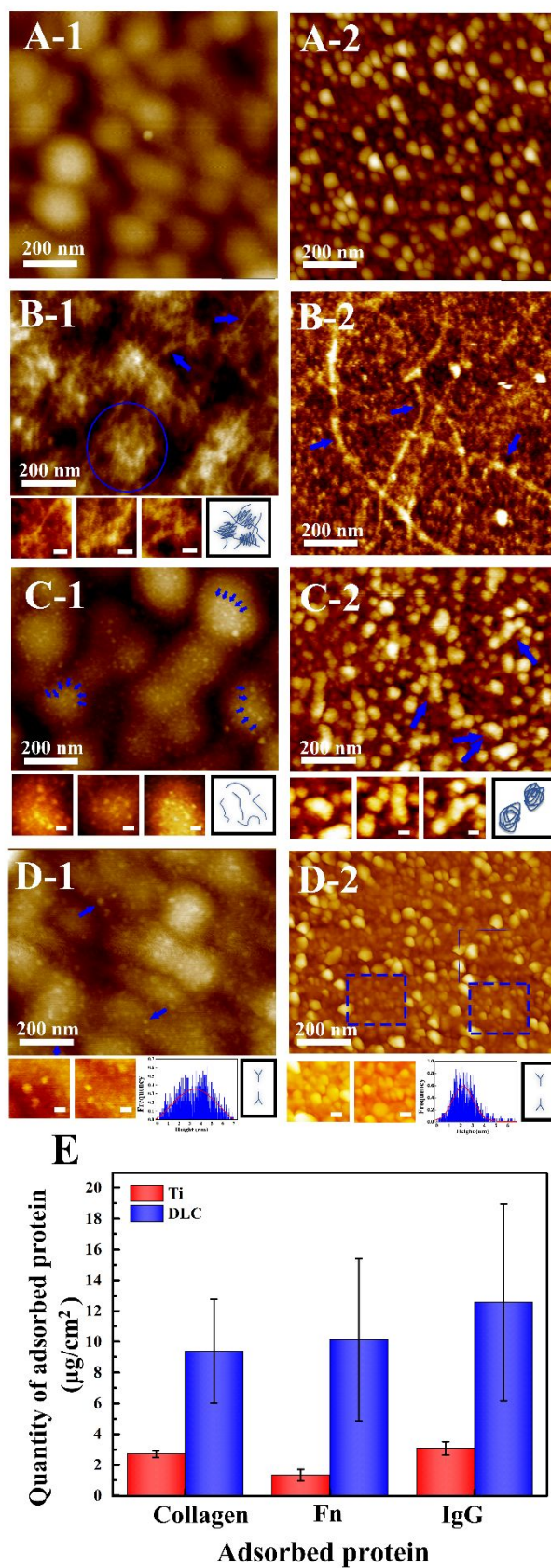


Fig. 5 A, B, C, D: AFM images showing the surface morphologies of the DLC (-1) and Ti (-2) films with/without protein pre-adsorption. The bare films (A) and the films pre-adsorbed with type

I collagen (B), Fn (C) and IgG (D). The typical molecules adsorbed on the films are highlighted in the framed and arrow-pointed areas. The magnified images of adsorbed proteins are on the bottom of B, C and D (the scale bar is 50 nm). Graphical depictions of the typical protein molecules are shown in the right-bottom corners of B, C and D. The height distributions of the adsorbed IgG particles are shown on the bottom of D-1 and D-2. E: The quantity of adsorbed proteins on DLC and Ti surfaces.

Combined with the results of initial bacterial adhesion tests on *S. epidermidis* and *E. coli*, it was found that despite collagen exhibited distinct conformations, the pre-adsorption of collagen inhibited bacterial adhesion on both two films, demonstrating that the inhibitory effect of collagen pre-adsorption is independent of the conformation of the molecules. However, the situation was quite different for IgG and Fn. IgG dramatically enhanced the initial adhesion of *S. epidermidis* and *E. coli* on the Ti films, whereas greatly inhibited bacterial adhesion on the DLC films. This phenomenon may correlate with the protein conformation of IgG molecules. The height analysis suggested that the average height of the adsorbed IgG was higher on the Ti surfaces than on the DLC surfaces. Our previous investigations on IgG adhesion have shown that when the adsorbed IgG molecules exhibited a flat conformation, the average height was ~2.5 nm; when the adsorbed IgG molecules exhibited a vertical conformation, the average height could reach ~ 3.5 nm³⁰. It is therefore likely that in this case, the IgG molecules adsorbed on the Ti surfaces showed a vertical conformation, while the molecules on the DLC surfaces had a flattened conformation. Moreover, taking the surface potential data into consideration, it was found that the pre-adsorption of IgG made the surface potential much higher on the Ti films (~160 mV) than on the DLC films (~400 mV) (Fig. 4C). An IgG molecule is composed of three domains: one Fc domain with negative charge and two Fab domains with positive charge. Therefore, the higher surface potential of Ti surfaces after IgG adsorption induced the protein molecules to display an end-on conformation, exposing the two Fab domains which could specifically recognize the receptors on the bacterial cell wall, thus promoting bacterial adhesion on the Ti surfaces. The IgG with the flattened conformation on the

DLC surfaces, however, had little effect on bacterial adhesion. For Fn, it was demonstrated that the pre-adsorption of Fn enhanced *E. coli* adhesion on the Ti films, but inhibited *E. coli* adhesion on the DLC films, suggesting that the distinct initial adhesion behaviors of the bacteria may have resulted from the different conformations of the adsorbed Fn molecules. From the AFM images, it was found that the Fn adsorbed on the Ti surfaces formed globular agglomerates, which was in consistence with previous findings reporting that Fn preferred to adsorb in a globular conformation with a mean width of 55 ± 9 nm and a mean height of 4.6 ± 1.6 nm on Ti-based materials, such as Ti, Ti compounds and Ti alloys³¹⁻³². Whereas on the DLC films, the Fn molecules displayed a fibril conformation containing nodules in a beads-on-a-string arrangement. The globular Fn nodules were 12~22 nm in diameter and around 2 nm in height. It is reported that these nodules were the result of Fn dimers aligning in a staggered manner with an overlap between the first seven Fn type III modules of each Fn dimer³³. The conformational changes of Fn molecules may induce the differences in exposed cell-binding domains, thus affecting the initial adhesion of bacteria. Based on the discussion above, it could be concluded that the molecular specific recognition between Fn/IgG proteins and bacteria was the major factor affecting the initial bacterial adhesion. But the detailed mechanisms of how the proteins interact with cell wall receptors and the inhibitory effect need to be further investigated.

To figure out the subsequent impact of the pre-adsorption of the proteins on bacterial infections, the inflammatory responses towards *S. epidermidis* and *E. coli* were assessed. Macrophages are one of the most predominant immune cells. They can reach the implant site immediately after implantation and remain at the biomaterial surface to detect bacteria via their surface receptors, thereby aiding to regulate inflammatory processes and rejection reactions³⁴⁻³⁵. After the 30-minute protein adsorption and the following 4-hour macrophage adhesion, macrophage cells were fixed and stained for further analyses. Results reveal that collagen substantially reduced macrophage adhesion, whereas Fn and IgG greatly promoted the attachment of macrophages, and the promotional effect of IgG was more pronounced (Fig. 6). This result suggests that

the types of the pre-adsorbed proteins on the films significantly affected macrophage adhesion.

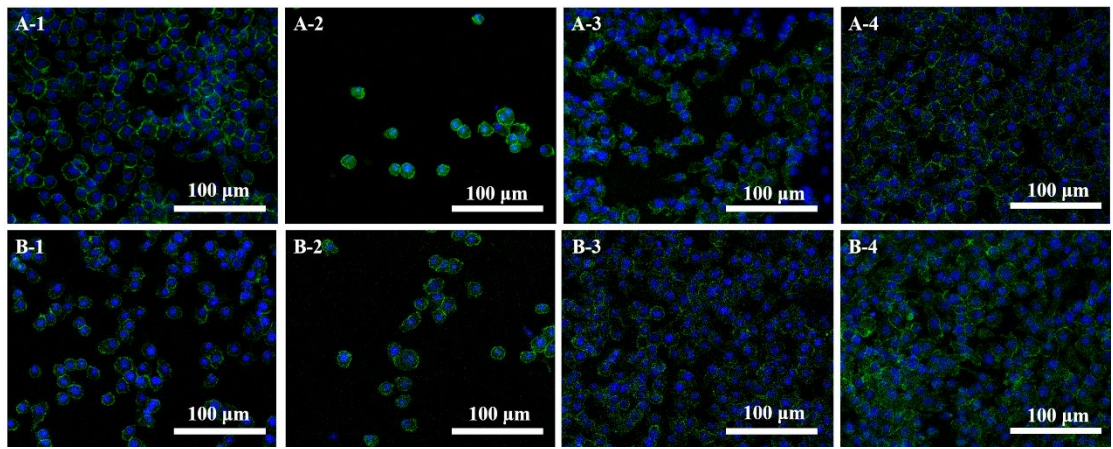


Fig. 6 CLSM images showing the macrophages adhered on the DLC (A) and Ti (B) films. The bare films (-1) and the films pre-adsorbed with collagen (-2), Fn (-3) and IgG (-4).

The impact of protein adsorption on macrophage phagocytosis was further investigated. After protein adsorption, *S. epidermidis* was allowed to adhere and grow on the film surfaces, then macrophages were introduced after colonization of the bacteria. The morphologies of the macrophages were characterized by SEM (Fig. 7). Compared with those attached on the bare DLC and Ti surfaces, the macrophages spread poorly and did not covered large surface area on the collagen-coated surfaces. The cells showed isolated circular shapes (Fig. 7A-2, B-2), and abundant unphagocytized *S. epidermidis* bacteria could be seen, implying the negative effects of the pre-adsorbed collagen on macrophage phagocytosis. In contrast, however, for both the Fn and IgG-coated surfaces, the cells spread extensively with strengthened vesicular membranes. On the IgG-coated surfaces, the cells possessed the largest spreading area, and the engulfing phenomenon at the edges of single macrophages was clear to see (Fig. 7A-4, B-4), suggesting activated cell states of macrophages and strong phagocytosis abilities. Furthermore, the production of inflammatory-associated cytokine during macrophage phagocytosis was assessed using TNF- α secretion assay (Fig. 7C). TNF- α is a vital pro-inflammatory factor that can be up-regulated at inflammatory sites, and it has been reported to promote macrophage phagocytosis³⁶. It was noted that the adsorbed

collagen and IgG had no significant effect on the secretion of TNF- α on the DLC and Ti surfaces, but Fn substantially promoted TNF- α secretion on the DLC surfaces. The promotional effect of Fn may be associated with the Fn conformation on the DLC surfaces, where they exhibited linear fibril shapes, exposing more cell-binding domains. Nevertheless, the detailed mechanisms should be further studied using biochemical characterization and structural biological analysis.

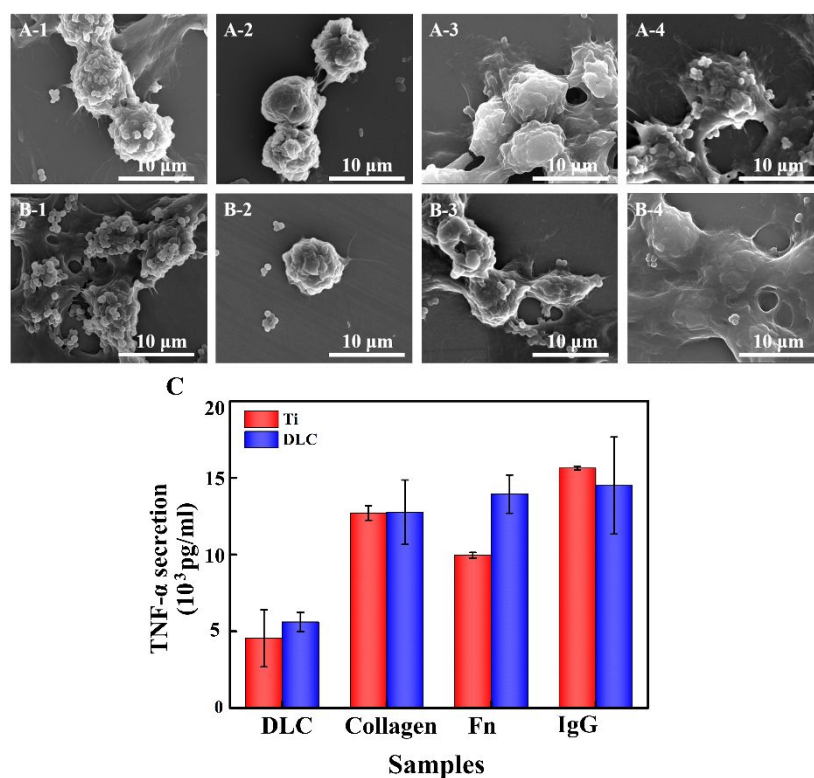


Fig. 7 A, B: FESEM images of macrophage phagocytosis against *S. epidermidis* on the DLC (A) and Ti (B) films. The bare films (-1) and the films pre-adsorbed with collagen (-2), Fn (-3) and IgG (-4). C: TNF- α secretion of macrophages with and without protein pre-adsorption.

Conclusion

Inflammatory responses on biomedical DLC and Ti films were investigated by examining the influence of pre-adsorbed type I collagen, Fn and IgG on initial bacterial adhesion and biofilm formation of *S. epidermidis* and *E. coli* and corresponding macrophage phagocytosis. It was disclosed that the specific molecular recognition between adsorbed proteins and bacteria was the decisive factor affecting bacterial

adhesion and following biofilm formation, and this process was partly attributed to the conformation of adsorbed protein molecules. Fn molecules formed globular aggregates on Ti surfaces that greatly enhanced bacterial adhesion, but exhibited a fibril conformation on DLC surfaces that inhibited bacterial adhesion. IgG showed an end-on orientation with free F(ab)₂ domains on Ti surfaces, facilitating bacterial adhesion and biofilm formation, while the flattened orientation on DLC films showed little effect on bacterial behaviors. Further macrophage phagocytosis assessment revealed that the phagocytosis against colonized bacteria was mainly determined by the types of pre-adsorbed proteins. Fn and IgG strongly promoted macrophage adhesion and phagocytosis on both two surfaces, but collagen pre-adsorption showed an adverse effect. The underlying specific molecular mechanisms, such as related signal paths and bacteria binding domains should be further investigated in future work. The results would give insights into understanding biomaterial-associated bacterial infections and shed light on the application of surface modification by proteins in designing anti-bacterial surfaces.

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References

(1) Hall-Stoodley, L.; Costerton, J. W.; Stoodley, P. Bacterial Biofilms: From the Natural Environment to Infectious Diseases. *Nat. Rev. Microbiol.* 2004, 2 (2), 95-108.

(2) Flemming, H.-C.; Wingender, J.; Szewzyk, U.; Steinberg, P.; Rice, S. A.; Kjelleberg, S. Biofilms: An Emergent Form of Bacterial Life. *Nat. Rev. Microbiol.* 2016, 14 (9), 563-575.

(3) Van Houdt, R.; Michiels, C. W. Role of Bacterial Cell Surface Structures in *Escherichia coli* Biofilm Formation. *Res. Microbiol.* 2005, 156 (5-6), 626-633.

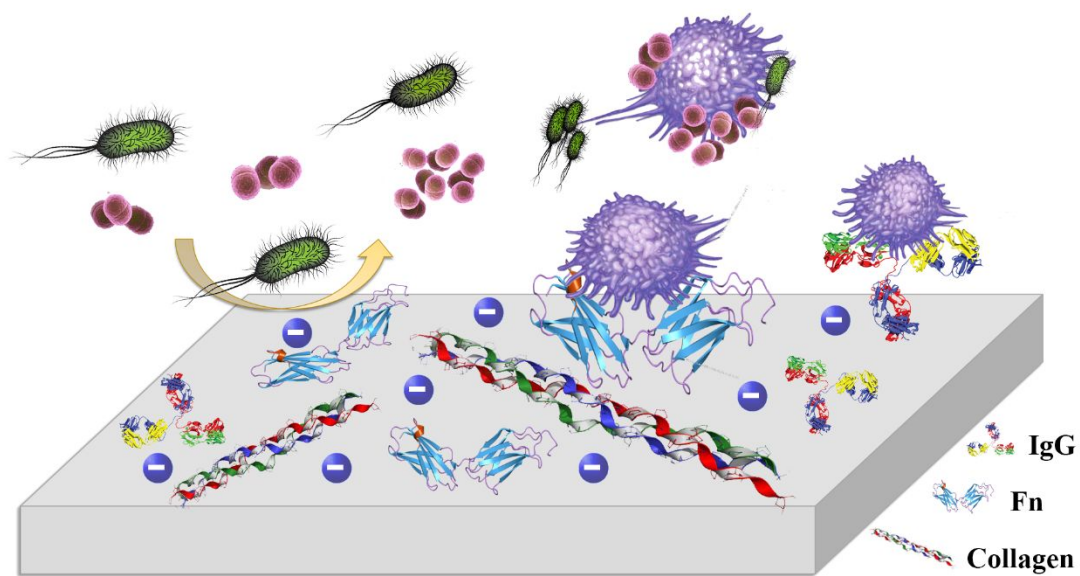
(4) Christner, M.; Franke, G. C.; Schommer, N. N.; Wendt, U.; Wegert, K.; Pehle, P.; Kroll, G.; Schulze, C.; Buck, F.; Mack, D.; Aepfelbacher, M.; Rohde, H. The Giant Extracellular Matrix-Binding Protein of *Staphylococcus epidermidis* Mediates Biofilm Accumulation and Attachment to Fibronectin. *Mol. Microbiol.* 2010, 75 (1), 187-207.

(5) Costa, A. R.; Henriques, M.; Oliveira, R.; Azeredo, J. The Role of Polysaccharide Intercellular

- Adhesin (PIA) in *Staphylococcus epidermidis* Adhesion to Host Tissues and Subsequent Antibiotic Tolerance. Eur. J. Clin. Microbiol. 2009, 28 (6), 623-629.
- (6) Le, K. Y.; Park, M. D.; Otto, M. Immune Evasion Mechanisms of *Staphylococcus epidermidis* Biofilm Infection. Front. Microbiol. 2018, 9, 1-8.
- (7) Sheikh, Z.; Brooks, P. J.; Barzilay, O.; Fine, N.; Glogauer, M. Macrophages, Foreign Body Giant Cells and Their Response to Implantable Biomaterials. Materials 2015, 8 (9), 5671-5701.
- (8) Hobley, L.; Harkins, C.; MacPhee, C. E.; Stanley-Wall, N. R. Giving Structure to the Biofilm Matrix: An Overview of Individual Strategies and Emerging Common Themes. Fems Microbiol. Rev. 2015, 39 (5), 649-669.
- (9) Spriano, S.; Chandra, V. S.; Cochis, A.; Uberti, F.; Rimondini, L.; Bertone, E.; Vitale, A.; Scolaro, C.; Ferrari, M.; Cirisano, F.; di Confiengo, G. G.; Ferraris, S. How Do Wettability, Zeta Potential and Hydroxylation Degree Affect the Biological Response of Biomaterials? Mat. Sci. Eng. C-Mater. 2017, 74, 542-555.
- (10) Barrias, C. C.; Martins, M. C. L.; Almeida-Porada, G.; Barbosa, M. A.; Granja, P. L. The Correlation Between the Adsorption of Adhesive Proteins and Cell Behaviour on Hydroxyl-Methyl Mixed Self-Assembled Monolayers. Biomaterials 2009, 30 (3), 307-316.
- (11) Battiston, K. G.; Labow, R. S.; Santerre, J. P. Protein Binding Mediation of Biomaterial-dependent Monocyte Activation on a Degradable Polar Hydrophobic Ionic Polyurethane. Biomaterials 2012, 33 (33), 8316-8328.
- (12) Eroshenko, D.; Morozov, I.; Korobov, V. The Role of Plasma, Albumin, and Fibronectin in *Staphylococcus epidermidis* Adhesion to Polystyrene Surface. Curr. Microbiol. 2015, 70 (6), 846-853.
- (13) Hou, W.; Liu, Y.; Zhang, B.; He, X.; Li, H. Electron Microscopy Visualization of Vitronectin Adsorbed on -COOH and -NH₂ Functionalized Surfaces: Distinctive Spatial Alignment and Regulated Cellular Responses. Adv. Mater. Interfaces 2017, 4 (23), 1-12.
- (14) Ferraris, S.; Spriano, S. Antibacterial Titanium Surfaces for Medical Implants. Mat. Sci. Eng. C-Mater. 2016, 61, 965-978.
- (15) Lubwama, M.; Corcoran, B.; Sayers, K. DLC Films Deposited on Rubber Substrates: A Review. Surf. Eng. 2015, 31 (1), 1-10.
- (16) Mohanty, M.; Anilkumar, T. V.; Mohanan, P. V.; Muraleedharan, C. V.; Bhuvaneshwar, G. S.; Derangere, F.; Sampeur, Y.; Suryanarayanan, R. Long Term Tissue Response to Titanium Coated with Diamond Like Carbon. Biomol. Eng. 2002, 19 (2-6), 125-128.
- (17) Myllymaa, K.; Levon, J.; Tiainen, V.-M.; Myllymaa, S.; Soininen, A.; Korhonen, H.; Kaivosoja, E.; Lappalainen, R.; Kontinen, Y. T. Formation and Retention of Staphylococcal Biofilms on DLC and Its Hybrids Compared to Metals Used as Biomaterials. Colloid Surface B 2013, 101, 290-297.
- (18) Zuyderhoff, E. M.; Dupont-Gillain, C. C. Nano-Organized Collagen Layers Obtained by Adsorption on Phase-Separated Polymer Thin Films. Langmuir 2012, 28 (4), 2007-2014.
- (19) Overdijk, M. B.; Verploegen, S.; Buijsse, A. O.; Vink, T.; Leusen, J. H. W.; Bleeker, W. K.; Parren, P. Crosstalk Between Human IgG Isotypes and Murine Effector Cells. J. Immunol. 2012, 189 (7), 3430-3438.
- (20) Flemming, H. C.; Wingender, J. The Biofilm Matrix. Nat. Rev. Microbiol. 2010, 8 (9), 623-633.
- (21) MacKintosh, E. E.; Patel, J. D.; Marchant, R. E.; Anderson, J. M. Effects of Biomaterial Surface Chemistry on the Adhesion and Biofilm Formation of *Staphylococcus epidermidis* in Vitro. J. Biomed. Mater. Res. A 2006, 78A (4), 836-842.
- (22) Banerjee, I.; Pangule, R. C.; Kane, R. S. Antifouling Coatings: Recent Developments in the Design

- of Surfaces That Prevent Fouling by Proteins, Bacteria, and Marine Organisms. *Adv. Mater.* 2011, 23 (6), 690-718.
- (23) Gittens, R. A.; Scheideler, L.; Rupp, F.; Hyzy, S. L.; Geis-Gerstorfer, J.; Schwartz, Z.; Boyan, B. D. A Review on the Wettability of Dental Implant Surfaces II: Biological and Clinical Aspects. *Acta Biomater.* 2014, 10 (7), 2907-2918.
- (24) Darmanin, T.; Guittard, F. Recent Advances in the Potential Applications of Bioinspired Superhydrophobic Materials. *J. Mater. Chem. A* 2014, 2 (39), 16319-16359.
- (25) Mahmoud, H.; Williams, D. W.; Hannigan, A.; Lynch, C. D. Influence of Extracellular Matrix Proteins in Enhancing Bacterial Adhesion to Titanium Surfaces. *J. Biomed. Mater. Res. B* 2012, 100B (5), 1319-1327.
- (26) Yang, Y.; Wikiel, A. J.; Dall'Agnol, L. T.; Eloy, P.; Genet, M. J.; Moura, J. J. G.; Sand, W.; Dupont-Gillain, C. C.; Rouxhet, P. G. Proteins Dominate in the Surface Layers Formed on Materials Exposed to Extracellular Polymeric Substances from Bacterial Cultures. *Biofouling* 2016, 32 (1), 95-108.
- (27) Birkenhauer, E.; Neethirajan, S.; Weese, J. S. Collagen and Hyaluronan at Wound Sites Influence Early Polymicrobial Biofilm Adhesive Events. *BMC Microbiol.* 2014, 14, 11.
- (28) Zhou, Z. N.; Li, W. P.; He, T. R.; Yu, P.; Tan, G. X.; Ning, C. Y. Controllable Protein Adsorption and Bacterial Adhesion on Polypyrrole Nanocone Arrays. *J. Mater. Sci. Technol.* 2016, 32 (9), 950-955.
- (29) Kovacevic, D.; Pratnekar, R.; Torkar, K. G.; Salopek, J.; Drazic, G.; Abram, A.; Bohinc, K. Influence of Polyelectrolyte Multilayer Properties on Bacterial Adhesion Capacity. *Polymers* 2016, 8 (10).
- (30) Hou, W.; Liu, Y.; Zhang, B.; He, X.; Li, H. Adsorption-Associated Orientational Changes of Immunoglobulin G and Regulated Phagocytosis of *Staphylococcus epidermidis*. *J. Biomed. Mater. Res. A* 2018, 106, 11.
- (31) Lehnert, M.; Gorbahn, M.; Rosin, C.; Klein, M.; Koeper, I.; Al-Nawas, B.; Knoll, W.; Veith, M. Adsorption and Conformation Behavior of Biotinylated Fibronectin on Streptavidin-Modified TiO_x Surfaces Studied by SPR and AFM. *Langmuir* 2011, 27 (12), 7743-7751.
- (32) Vadiello-Rodriguez, V.; Bruque, J. M.; Gallardo-Moreno, A. M.; Luisa Gonzalez-Martin, M. Surface-Dependent Mechanical Stability of Adsorbed Human Plasma Fibronectin on Ti6Al4V: Domain Unfolding and Stepwise Unraveling of Single Compact Molecules. *Langmuir* 2013, 29 (27), 8554-8560.
- (33) Szymanski, J. M.; Sevcik, E. N.; Zhang, K.; Feinberg, A. W. Stretch-Dependent Changes in Molecular Conformation in Fibronectin Nanofibers. *Biomater. Sci-UK* 2017, 5 (8), 1629-1639.
- (34) Aderem, A.; Underhill, D. M. Mechanisms of Phagocytosis in Macrophages. *Annu. Rev. Immunol.* 1999, 17, 593-623.
- (35) Anderson, J. M.; Rodriguez, A.; Chang, D. T. Foreign Body Reaction to Biomaterials. *Semin. Immunol.* 2008, 20 (2), 86-100.
- (36) Feng, X. Y.; Deng, T. T.; Zhang, Y.; Su, S. B.; Wei, C. J.; Han, D. S. Lipopolysaccharide Inhibits Macrophage Phagocytosis of Apoptotic Neutrophils by Regulating the Production of Tumour Necrosis Factor Alpha and Growth Arrest-Specific Gene 6. *Immunology* 2011, 132 (2), 287-295.

TOC Graphic



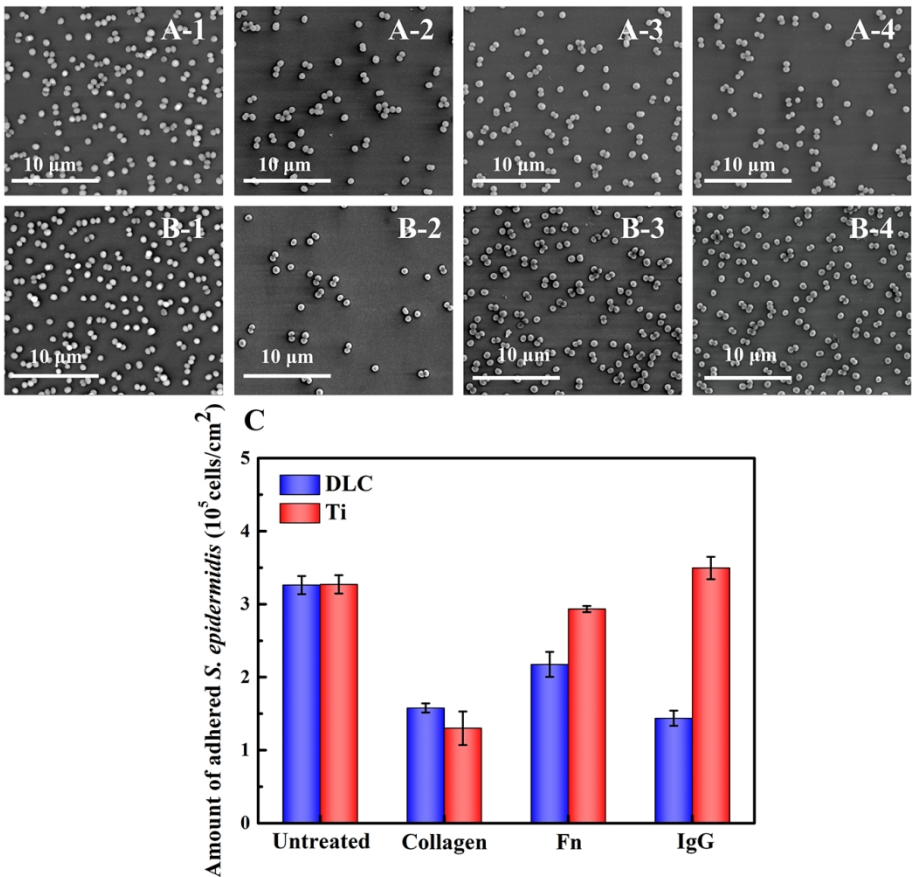


Fig. 1 A, B: FESEM images showing the initial adhesion of *S. epidermidis* on DLC (A) and Ti (B) films without (A-1, B-1) or with the pre-adsorption of type I collagen (A-2, B-2), Fn (A-3, B-3), and IgG (A-4, B-4). C: The density of adherent *S. epidermidis* on the DLC and Ti surfaces with/without protein adsorption.

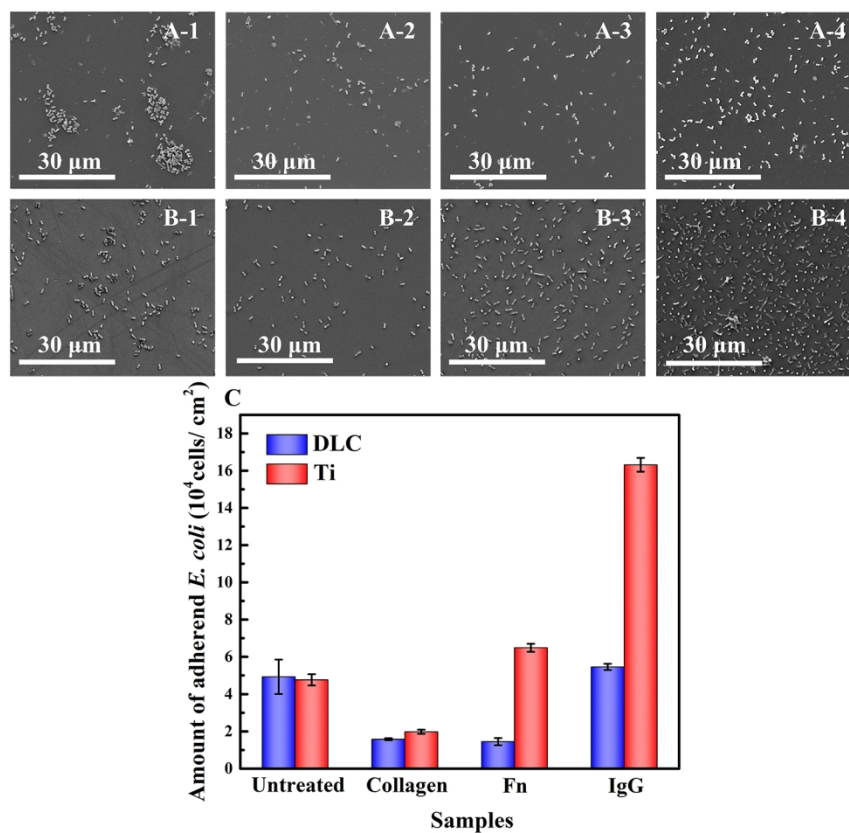


Fig. 2 A, B: FESEM images showing the initial adhesion of *E. coli* on DLC (A) and Ti (B) films without (A-1, B-1) or with the pre-adsorption of type I collagen (A-2, B-2), Fn (A-3, B-3), and IgG (A-4, B-4). C: The density of adherent *E. coli* on the DLC and Ti surfaces with/without protein adsorption.

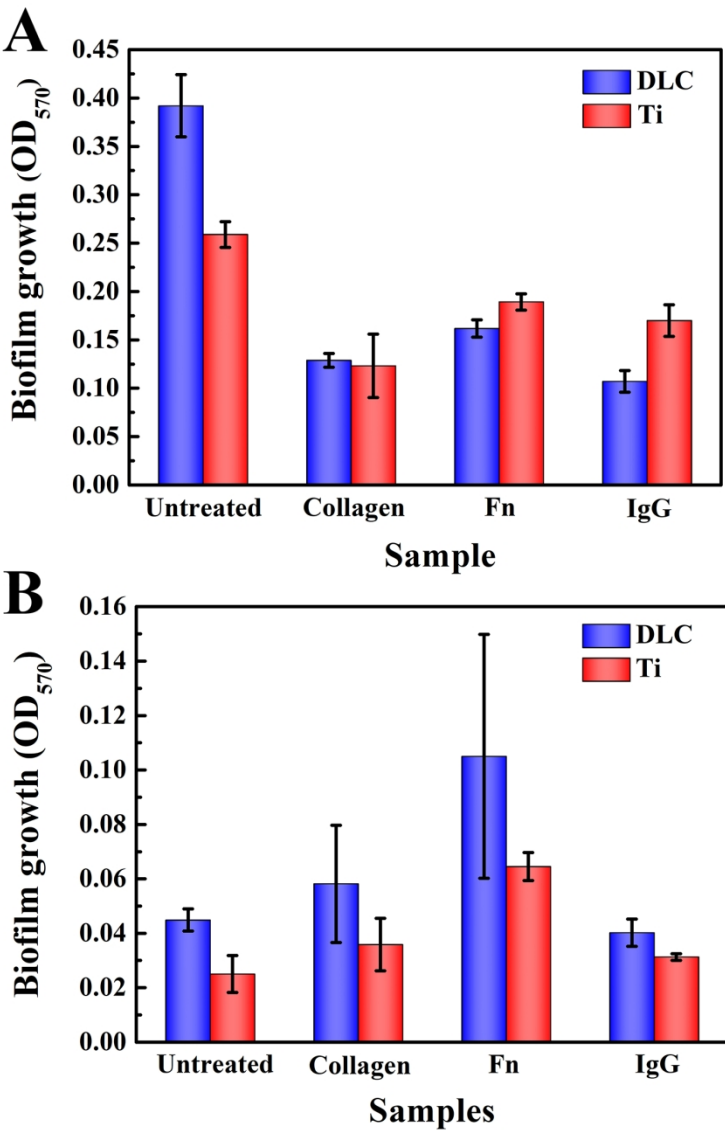


Fig. 3 Biofilm formation of the *S. epidermidis* (A) and *E. coli* (B) on the DLC and Ti surfaces with/without protein adsorption.

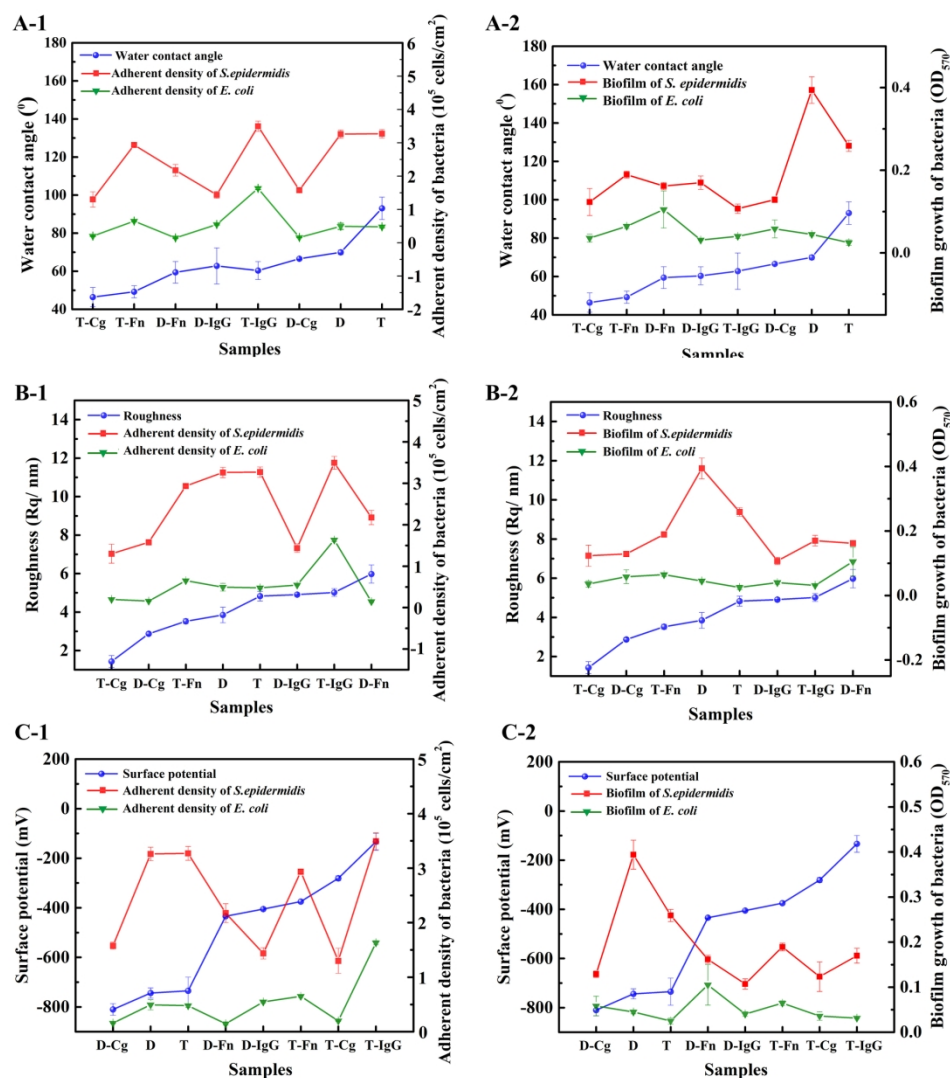


Fig. 4 The variation of the density of adherent bacteria (-1) and biofilm formation (-2) with surface wettability (A), surface roughness (B) and surface potential (C) on DLC and Ti surfaces with/without protein adsorption.

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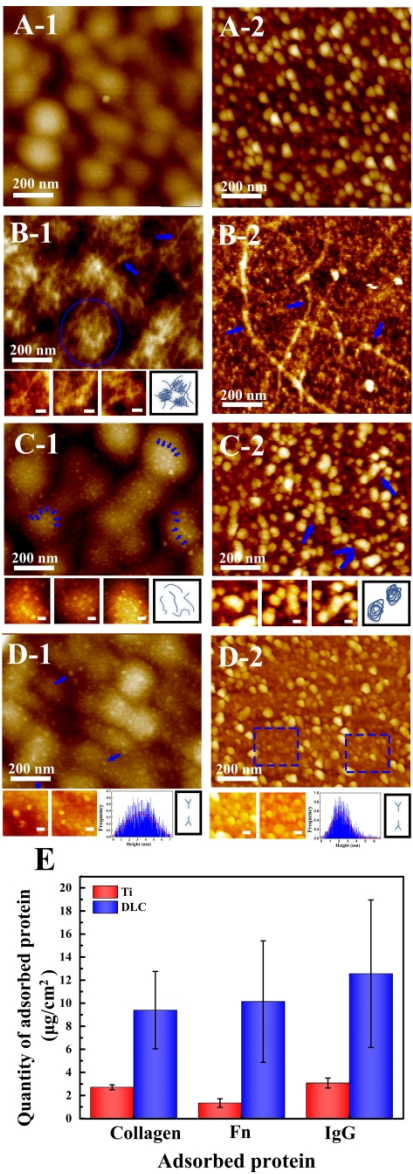


Fig. 5 A, B, C, D: AFM images showing the surface morphologies of the DLC (-1) and Ti (-2) films with/without protein pre-adsorption. The bare films (A) and the films pre-adsorbed with type I collagen (B), Fn (C) and IgG (D). The typical molecules adsorbed on the films are highlighted in the framed and arrow-pointed areas. The magnified images of adsorbed proteins are on the bottom of B, C and D (the scale bar is 50 nm). Graphical depictions of the typical protein molecules are shown in the right-bottom corners of B, C and D. The height distributions of the adsorbed IgG particles are shown on the bottom of D-1 and D-2. E: The quantity of adsorbed proteins on DLC and Ti surfaces.

151x399mm (300 x 300 DPI)

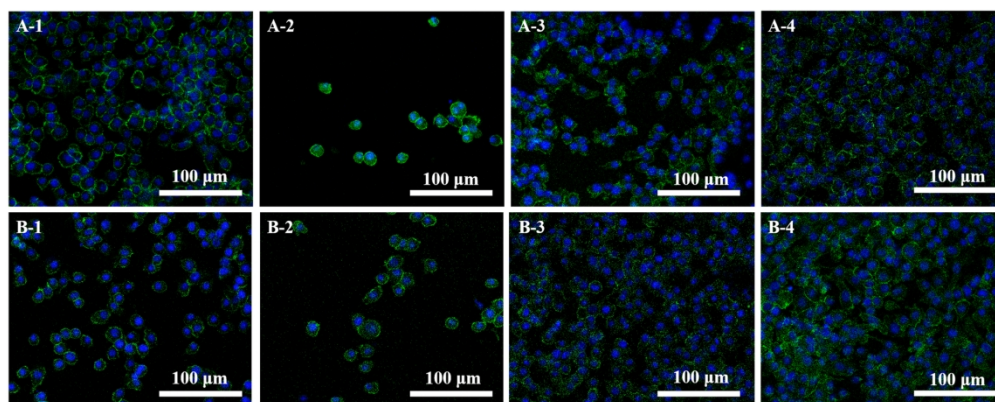


Fig. 6 CLSM images showing the macrophages adhered on the DLC (A) and Ti (B) films. The bare films (-1) and the films pre-adsorbed with collagen (-2), Fn (-3) and IgG (-4).

175x70mm (300 x 300 DPI)

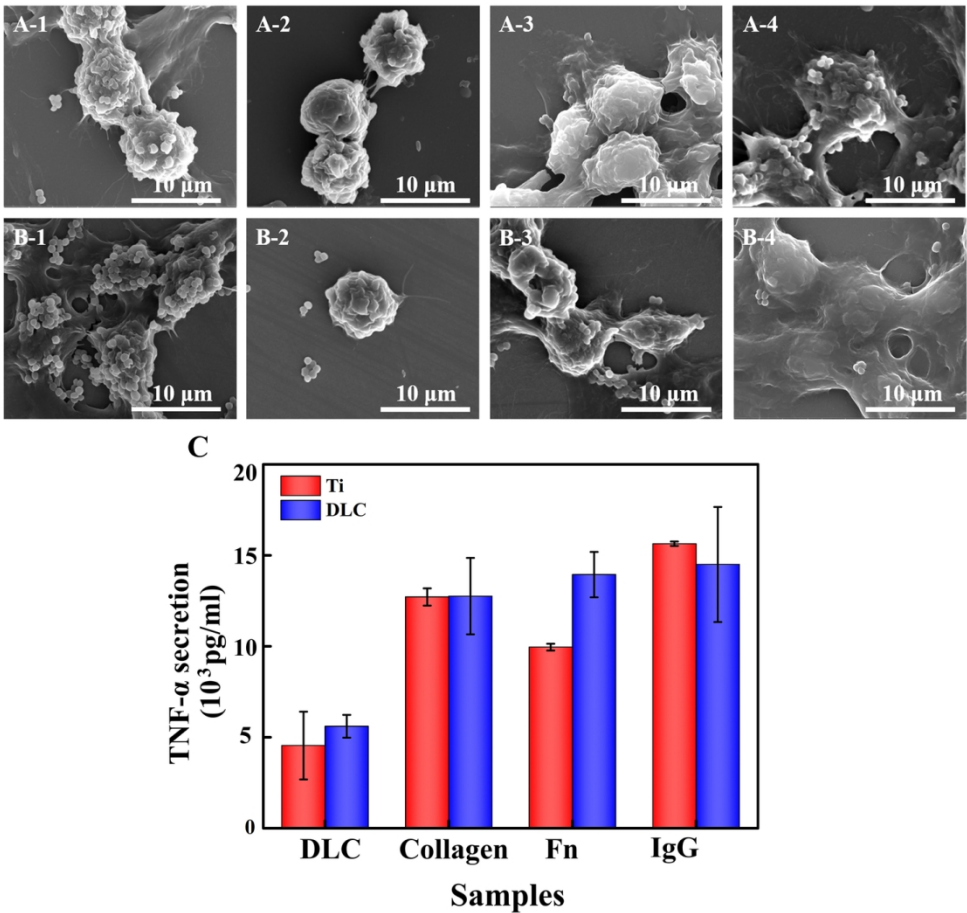


Fig. 7 A, B: FESEM images of macrophage phagocytosis against *S. epidermidis* on the DLC (A) and Ti (B) films. The bare films (-1) and the films pre-adsorbed with collagen (-2), Fn (-3) and IgG (-4). C: TNF-α secretion of macrophages with and without protein pre-adsorption.

129x120mm (300 x 300 DPI)

