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Adsorption-associated orientational changes of immunoglobulin G and regulated phagocytosis of *Staphylococcus epidermidis*

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Abstract: Understanding the adsorption of immunoglobulin G (IgG) on biomaterials surfaces is crucial for design and modification of the surfaces to alleviate inflammatory responses after implantation. Here, we report direct visualization and two-dimensional (2D) image interpretation of the IgG molecule adsorbed on simplified surfaces by single particle electron microscopy and atomic force microscopy. Influence of the orientational changes in adsorbed IgG on phagocytosis of macrophages against *Staphylococcus epidermidis* is further examined. Untreated amorphous carbon film and –COOH and $–NH_2$ grafted carbon films are employed as the model surfaces for the adsorption testing. Results show that IgG displays flat orientation lying on the untreated surface, while forms vertical orientations standing on the functionalized surfaces. These specific spatial alignments are associated with altered unfolding extent and structure rearrangement of IgG domains, which are influenced synergistically by surface charge and wettability of the substrata. The changes in interdomain distance of IgG molecules subsequently regulate immune behaviors of macrophages and phagocytosis of *S. epidermidis*. The results would give insight into appropriate design of biomaterial surfaces in nanoscales for desired inflammatory responses. © 2018 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A: 1–12, 2018.

Key Words: immunoglobulin G, orientational changes, protein adsorption, single particle electron microscopy, phagocytosis

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INTRODUCTION

Immunoglobulin G, ~150 kDa glycoprotein with a typical concentration of ~11 mg/mL in serum, is a main type of antibody primarily responsible for infection of body tissue.[1] This molecule comprises four peptide chains that are connected by disulfide bonds and non-covalent forces. All the four chains contain distinctive domains, two Fab segments and one Fc segment, together forming a Y-shaped conformation.[2] The $F(ab)_2$ serves as an antigen binding site, binding many kinds of pathogens, such as bacteria, viruses and fungi.[3] Fc fragment can recognize Fc_γ sites of many immune cells, and the whole IgG molecule allows recognition and ingestion by immune cells, resulting in the elimination of pathogens.[4] This process has significant influence on biomaterial-associated infection, which is considered to be the number one cause of implant failure.[5,6] In addition, it has been well established that the interaction of biomaterial surface with blood and fluids results in protein adsorption, in turn transforming the surface characters into subsequent cellular responses, among which a significant component is the inflammatory response that is related to IgG.[6] To function properly, IgG must remain bioactive upon adsorption, and its biological activities are directly decided by its conformation. Clarifying the structural changes of IgG upon adsorption is essential for understanding the interactions of IgG with biomaterials for designing and constructing appropriate surfaces.

Extensive studies have been carried out to elucidate the adsorption behaviors of IgG molecules on many surfaces such as silica,[7] porous calcium phosphate (CaP),[8] polymer,[9] and metals.[10] Alternative techniques have been employed for assessing the adsorption, for example time of flight secondary ion mass spectrometry (ToF-SIMS),[11] computer simulation,[12–14] surface plasmon resonance (SPR),[15] infrared reflection spectroscopy,[16] quartz crystal microbalance (QCM),[17,18] and radio-labeling.[19] A study by molecular dynamic simulation and atomic force microscopy (AFM) characterization proposed that upon adsorption, IgG retained its secondary and tertiary structures and exposed at least one Fab binding site for recognition events upon deposition

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on hydrophobic graphene slab surface.[20] Electrokinetic measurements also showed irreversible adsorption of IgG as a monolayer on mica.[21] In addition, previous study on the functional orientation of adsorbed IgG as examined by molecular probes suggested fully exposed Fc portion of IgG, and the sites of attachment to crystal surface remained on the Fab moiety of IgG.[22] It has been widely accepted that the molecule structure has a great impact on its function. Fc fragment is a homodimer of N-linked glycopeptide, and the removal of sugar residues could transform its conformation from "open" to "closed" state, thus negatively affects the binding of Fc_γ receptors.[23] Fab fragments could also facilitate adhesion of some immune cells like macrophages after long-term culturing.[1] Study on conformation of IgG molecule adsorbed on biomaterials surfaces is therefore of crucial importance for controlling inflammatory response and foreign body reaction after implantation.

To comprehensively understand the adsorption of the molecule on biomaterials surfaces and its influence on subsequent phenomena, it is critical to acquire the conformational information of the adsorbed antibody. It has been clear that protein adsorption is a complex phenomenon involving a variety of key materials properties like surface chemistry, wettability, charge and various physicochemical properties of physiological media.[9,24,25] Appropriate design of simplified model surfaces for the adsorption testing of IgG and selection of appropriate characterization techniques are critical for the research.

Single particle electron microscopy (EM) is a developing high-resolution approach for determining the structure of biomolecules in solution, for the pioneering work of which Jacques Dubochet, Joachim Frank and Richard Henderson were conferred the Nobel Prize in chemistry. By using this method, 3D structures of molecules are reconstructed from acquired 2D EM images that show all views of the molecules. This however requires random orientation of the proteins for EM observation, and the approximate orientation of each particle is determined by comparing the particles with a set of trial projections.[26] For the proteins with specific binding to biomaterial surface, it is possible to clarify their projections into reference-free 2D classes, which would provide valuable structural information about the adsorption orientation of the proteins. This method has been employed to visualize the conformational changes of adsorbed vitronectin in our previous work.[27] Complementary AFM visualization could further give height information of the adsorbed proteins, since AFM is capable of generating height maps with sub-nanometer resolution.[20,28] In this work, adsorption of IgG on typically designed model surfaces, amorphous carbon film, −COOH and $-NH_2$ grafted carbon films, was characterized by single particle EM and AFM. Macrophage, one of the major immune cells playing vital roles in regulating inflammatory response to biomaterials, was used to assess the adhesion and spreading behaviors on IgG-adsorbed surfaces. Furthermore, effect of the IgG adsorption on phagocytosis of *Staphylococcus epidermidis* (*S. epidermidis*) was also examined and elucidated. The findings gained from the high resolution EM visualization of IgG after adsorption on the surfaces could provide helpful clues to understanding protein-biomaterial interaction and shed light on design of nanostructured biomaterials surfaces for desired anti-inflammatory effects.

MATERIALS AND METHODS

Preparation and characterization of functionalized surfaces

Human IgG was purchased from Sigma-Aldrich (I4506). It was purified from human serum, containing four subtypes, namely IgG1, IgG2, IgG3 and IgG4. Carboxylic acid (−COOH) and amine ($-NH_2$) grafted surfaces were prepared by plasma grafting acrylic acid and allylamine on carbon films according to previously established protocols.[29,30] Prior to the grafting, amorphous graphitic carbon films were fabricated by evaporating carbon layers under high vacuum (Cressington 208 C Turbo Carbon Coater). The deposition was carried out at 4.3 V for 20 s. The plasma grafting was made using an anode layer ion beam source. Surface chemistry was investigated by X-ray photoelectron spectroscopy (XPS, ESCALAB 250, Thermo) using Al (mono) K_α irradiation, and C1s, O1s, and N1s core level signals were acquired. Surface roughness of the films was analyzed by AFM (Bruker FastScan, Germany). Wettability of the films was assessed by measuring the contact angle of 3 μL water droplets standing on the samples using a video-based optical contact angle measurement instrument (Dataphysics OCA20, Germany).

IgG adsorption testing

For the testing, IgG molecules were diluted in PBS solution, and a drop of 5 μL IgG solution (15 μg/mL) was added onto the thin film samples standing on 300-mesh copper grids (TED Pella Inc.) and incubated for 2 min. The sample grids were stained by two 5 μL drops of 2% (w/v) uranyl acetate solution. After removal of the excess stain by blotting with filter article, the grids were quickly dried by flowing air. Images were recorded under low-dose conditions (10 $e/Å^2$) on field emission transmission EM (FETEM, FEI Tecnai 20) operated at 200 kV. Micrographs were acquired with a Gatan Ultrascan 4 k × 4 k charge coupled device camera at a magnification of 71,000×, corresponding to a pixel size of 1.97 Å at the sample level. Picking and image processing of individual particle images were carried out using the software package EMAN.[26] Reference-free 2D classifications were made and interpreted. The software ImageJ was used to measure the longest dimension of the reference-free 2D classification images. The adsorbed IgG was further characterized by AFM imaging operated in air using peak force mode. 100 μL IgG solution (15 μg/mL) was applied to the surfaces of the films coated on silicon wafer. Sharp AFM probes (SCANASYST-AIR) were used for acquiring the images, which were further analyzed by the software Nanoscope Analysis. For quantitative assessment of IgG adsorption, further micro BCA protein assay was conducted. For the testing, 200 μL IgG solution (20 μg/mL) was plated on each of the samples for 2 min at 37°C, and then each well was washed for three times. Subsequently, the samples were incubated in 300 μL 2% sodium dodecyl sulfate (SDS) for 24 h at 37°C.

Concentration of IgG in the SDS solution was measured using a micro BCA protein assay regent kit (Sangon Biotech, China).

Culturing of bacteria and macrophages

The bacteria *S. epidermidis* (CMCC(B)26069) were cultured on tryptone soy agar (TSA) plates and grew overnight at 37°C. The plates were then kept at 4°C for further use. For each testing, a single colony was inoculated into 50 mL tryptone soy broth (TSB) and grew for 24 h at 37°C. The bacteria were harvested by centrifugation at 1200 rpm for 10 min and washed twice with sterile PBS. Concentration of the bacteria was measured by a spectrophotometer operated at 600 nm wavelength, and a final concentration of 1×10^8 cells/mL was used.

The macrophage cell line (RAW 264.7, the Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) was employed for the cell culturing. Macrophages were cultured in Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 2% penicillin-streptomycin (Gibco) in humidified atmosphere at 37°C and 5% CO_2. The cells were harvested with trypsin/EDTA that could be inactivated by addition of FBS. Trypsinized cells were washed twice with serum-free medium to remove trace of serum proteins. The harvested cells were counted and diluted to a concentration of 6×10^5 cells/mL for further use. After culturing in macrophages-containing media of the samples with/without preadsorption of IgG on their surfaces, the adherent cells were fixed with 4% paraformaldehyde for 30 min. Morphology of macrophages was examined by FESEM (FEI Quanta FEG 250). Cell polarity was calculated after the size measurement using a digital image processing software.

Staphylococcus epidermidis adhesion and phagocytosis testing

Staphylococcus epidermidis-macrophage interactions on the different surfaces were examined in standard 24-well culture plates under static condition. Before bacterial adhesion, the samples were immersed in IgG solution (15 μg/mL) for preadsorption of IgG on their surfaces, or in ultrapure water as control. The *S. epidermidis*-containing PBS suspension was then added and cultured for 2 h. The samples were then rinsed with sterile PBS twice to remove non-adsorbed bacteria, and macrophages were then seeded. After incubated for 2 h, the cells were rinsed with PBS for 3 times, and fixed with 4% paraformaldehyde for 30 min. Morphology of *S. epidermidis* and macrophages were characterized by FESEM. Each testing was performed in triplicate.

RESULTS AND DISCUSSION

XPS measurements evidenced successful grafting of the functional groups on the surfaces (Fig. 1). The peak at 284.6 eV is attributed to hydrocarbon (CH_x), and the peaks of C-O

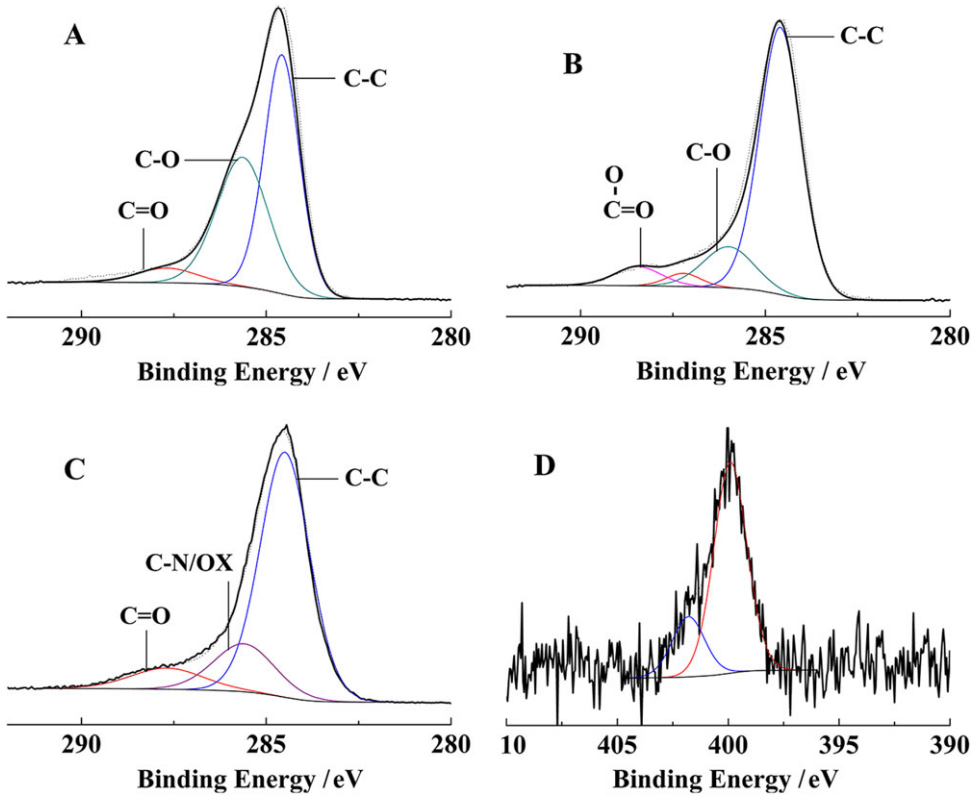

FIGURE 1. XPS spectra of the functionalized carbon films: (A) the C 1s peak of the untreated sample, (B) the C 1s peak of the –COOH grafted sample, (C) the C 1s peak of the $–NH_2$ grafted sample, and (D) the N 1s peak of the $–NH_2$ grafted sample. All the spectra were charge-corrected to C-C 1s core level at 284.6 eV.

TABLE I. Water Contact Angle and Surface Roughness of the Three Films Used for the IgG Adsorption Testing

Surface	Charging State	Water Contact Angle	Roughness (R_q, nm)
Untreated film	Neutral	65.3 ± 1.5°	0.91
–COOH grafted film	Negative	27.5 ± 1.6°	0.89
$–NH_2$ grafted film	Positive	43.5 ± 1.3°	1.26

(alcohol or ether) and C=O (carbonyl) are seen at 286.2 and 287.7 eV, respectively. For the acrylic acid grafted surfaces, the peak at 288.2 eV is assigned to C(=O)O, indicating successful grafting of –COOH groups on carbon film. For the film with plasma-grafted allylamine, the peak at 285.3 eV is assigned to C-N bonds, demonstrating the existence of primary, secondary, tertiary, or quaternary amines.[31] The N 1s signal suggests the presence of two components at 399.8 eV and 402.4 eV, which refer to amine and protonated amine environments. In addition, the wettability and roughness values of the samples are assessed (Table I). The water contact angle is decreased from 65.3° to 27.5° for the acrylic acid treated sample, and from 65.3° to 43.5° for the allylamine grafted surface. However, the roughness has not been altered remarkably by the plasma grafting treatment. The results show clearly that the –COOH and $–NH_2$ functional groups were successfully grafted on the surfaces.

EM visualization suggests quick adsorption of IgG on the three surfaces [Fig. 2 (A–C)]. It is noted that the untreated amorphous carbon film recruited the least IgG molecules, and both the –COOH and the $–NH_2$ modified surfaces supported more pronounced adsorption. This is consistent with the quantitative testing result. The micro BCA protein assay showed the adsorbed IgG amount of 1.18 ± 0.09 μg/cm^2, 2.39 ± 0.42 μg/cm^2, and 1.47 ± 0.71 μg/cm^2 for the untreated surface, the –COOH treated surface, and the $–NH_2$ treated surface, respectively. These indicate remarkable

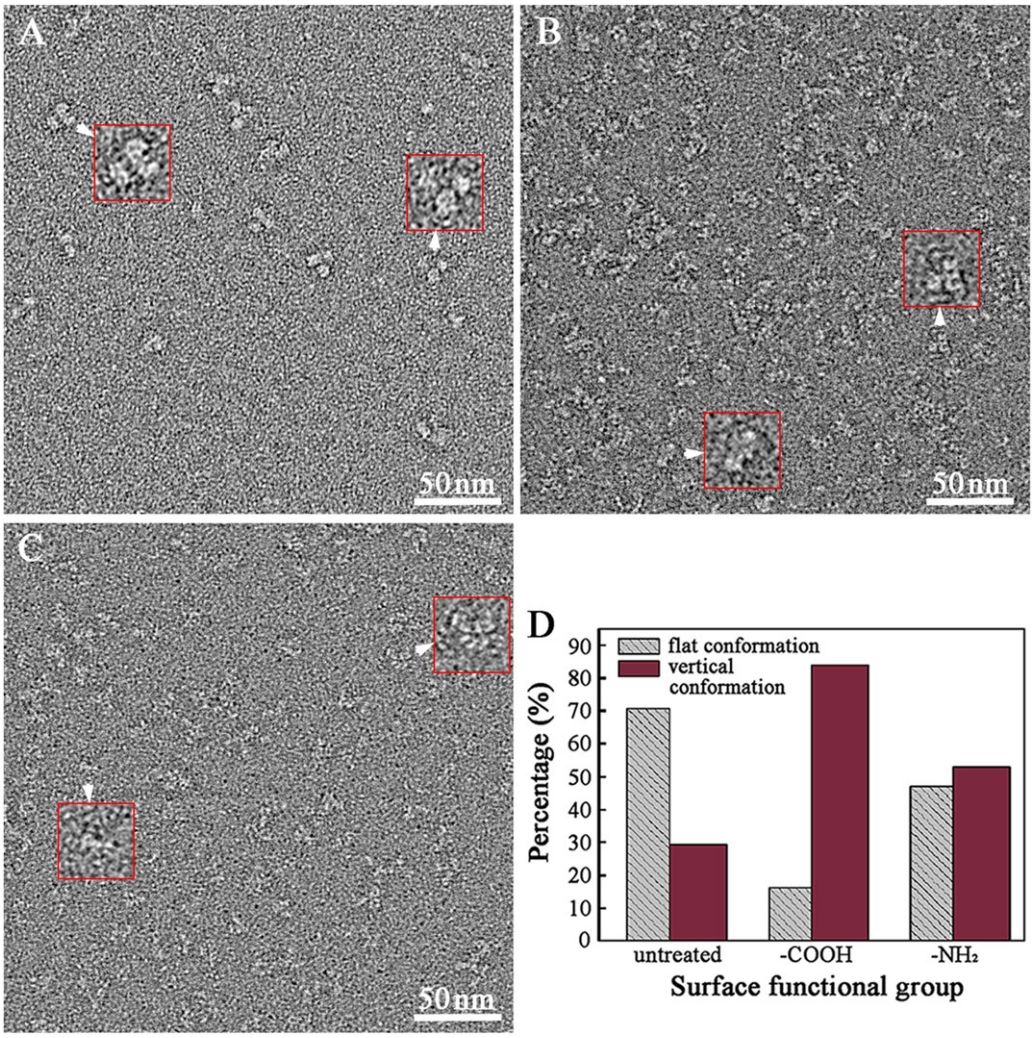

FIGURE 2. Negative-staining EM images of IgG adsorbed on the surfaces of the untreated amorphous carbon film (A), the –COOH grafted surface (B), and the $–NH_2$ grafted surface (C); and (D) statistical analysis result of the contour of the adsorbed IgG in terms of flat and vertical orientations.

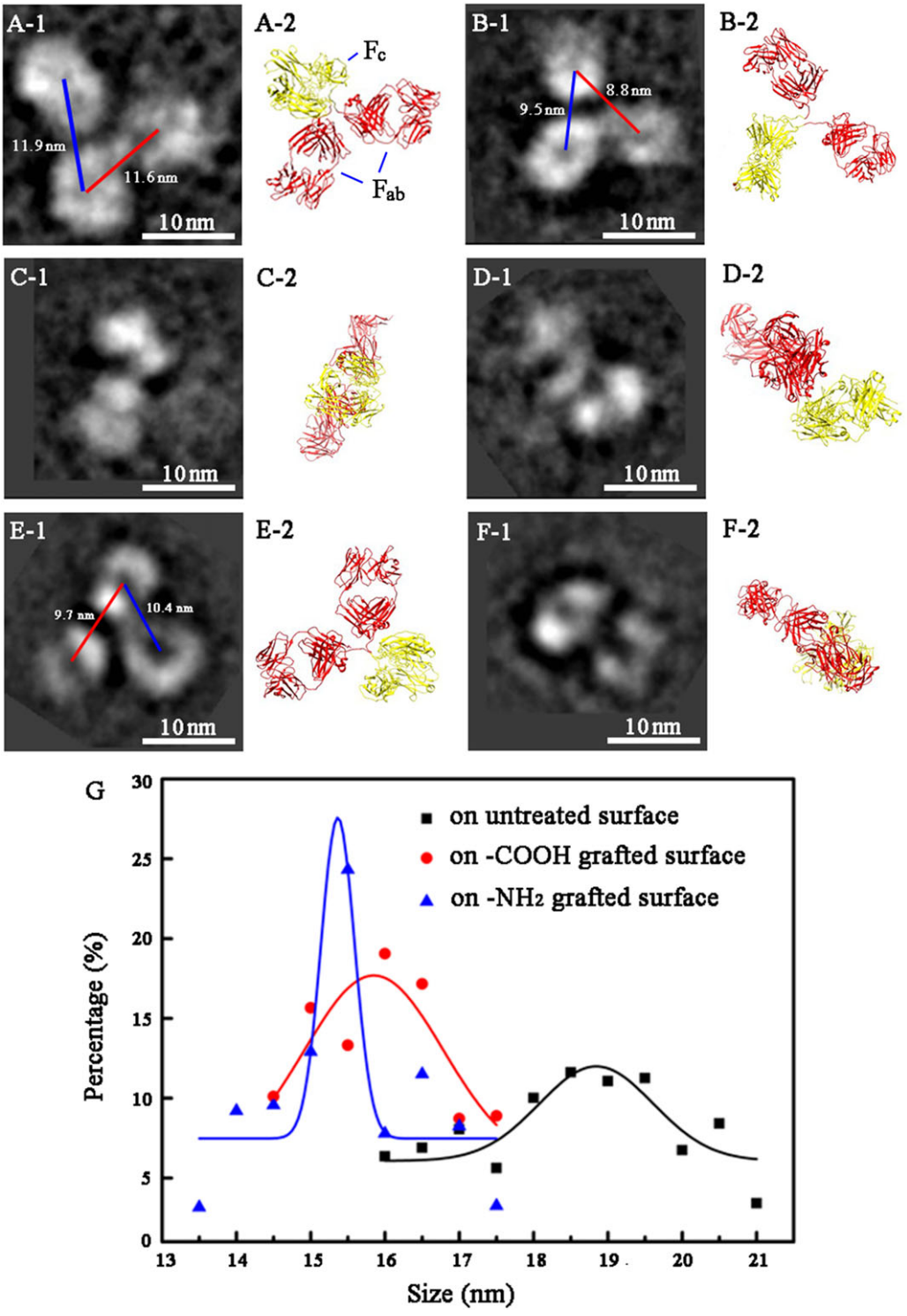

FIGURE 3. Domain organization and negative-staining EM image analysis of IgG adsorbed on the surfaces of the samples. A-1: the selected 2D class average of IgG adsorbed on the untreated amorphous carbon film in comparison with the side view of IgG atomic structure (A-2) (PDB ID 1IGY); B-1, C-1, and D-1: the three selected 2D class averages of IgG adsorbed on the –COOH grafted surface in comparison, respectively, with the side view of the IgG atomic structure (B-2, C-2, and D-2); E-1 and F-1: the two selected 2D class averages of IgG adsorbed on the $–NH_2$ grafted surface in comparison, respectively, with the side view of the IgG atomic structure (E-2 and F-2); G: particle size distribution of the adsorbed protein on the surfaces.

influence of the functionalized groups on IgG adsorption. Enlarged views of the typically selected IgG particles show distinctive conformations depending on the substrata. The adsorbed IgG can be easily classified into two predominate groups by their two preferential conformational orientations along flat and vertical directions, which are consistent with

previously reported findings.[20] The flat orientation with a typical Y-shape illustrate three lobes (subunits) for the particles. The vertical orientation can be recognized by the presence of two lobes or one plus a protrusion located nearby. To recap the conformations of the adsorbed IgG on the specific surfaces, statistical analyses was conducted [Fig. 2(D)]. Result shows that the adsorbed molecules favor a flat orientation on the untreated carbon film, a vertical orientation on the –COOH grafted surface, and a flat/vertical hybrid orientation on the $-NH_2$ modified surface.

Further reference-free 2D class-averaging was made using over 5,000 IgG particles adsorbed on each sample surface (Fig. 3). The flat and vertical conformations are clearly recognized. Alignment of the atomic structure of human IgG1 (1IGY, PDB databank) to the EM densities reveals the adsorption orientation of the molecule (Fig. 3 A-2, B-2, C-2, D-2, E-2, and F-2). It seems clear that adsorbed IgG with the vertical orientation can be further grouped into head-on adsorption (adsorption through both Fab segments, Fig. 3 C-1, C-2) and sideway adsorption (adsorption through one Fab domain and Fc domain, Fig. 3 D-1, D-2) on the –COOH grafted surface. Whereas the adsorbed IgG on the $-NH_2$ modified surface shows the sideway orientation (Fig. 3 F-1, F-2). A simulation study by Jiang et al.[13] also reported that IgG favors head-on orientation on negatively charged surfaces, and end-on (adsorption through Fc fragment) and sideway orientations on positively charged surfaces. Our direct EM visualization shows clearly the experimental proof that the adsorption orientation of IgG molecules is predominately determined by physicochemical features of the surfaces.

In addition, the EM characterization further provides statistic size information of IgG adsorbed on the three surfaces [Fig. 3(G)]. The antibody shows large lateral size of 18–20 nm on the untreated amorphous carbon film. Its size drops to 15–16 nm on the $-NH_2$ and the –COOH grafted surfaces. The inter-domain distance for each conformation of the adsorbed molecules with the flat conformation is also measured (Fig. 3 A-1, B-1, E-1). The molecules adsorbed on the untreated film show the longest inter-domain distance. X-ray crystallographic examination already revealed the dimension of IgG molecule of $3.8 \times 8.5 \times 14.2$ nm.[2,32] Taking into account the possible broadening effect observed in our study, it is speculated that there exists different degree of unfolding and structure rearrangement upon adsorption on the three surfaces, which was also claimed by AFM examination of the adsorbed molecules.[33]

To gain height information of the adsorbed IgG, AFM measurement was further conducted (Fig. 4). The predominantly isolated particles are in globular shapes with a mean height ranging from 2.0 to 3.5 nm [Fig. 4(D)]. It is lower than the nominal height, which is likely attributed to deformation of the antibody in vertical direction by the force applied by the AFM probe during imaging. It was reported that a deformation

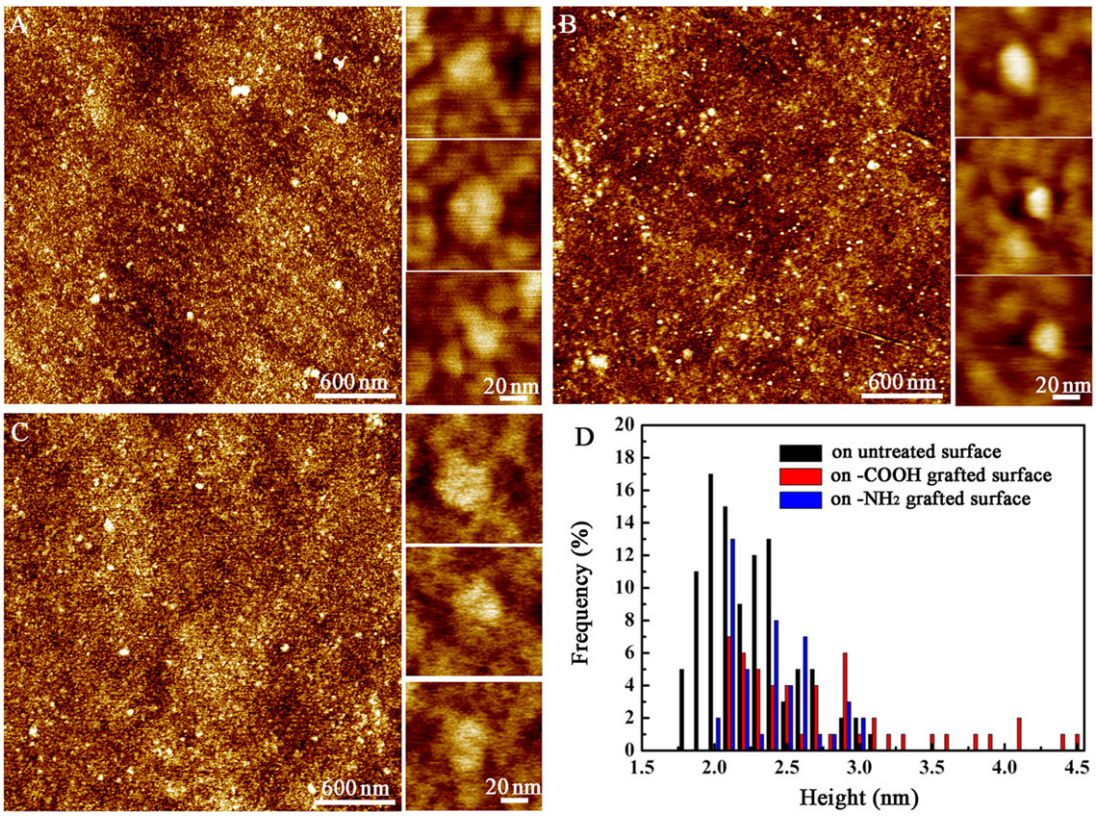

FIGURE 4. AFM images showing the IgG molecules adsorbed on the untreated amorphous carbon film (A), the –COOH modified surface (B), and the $-NH_2$ grafted surface (C); the right panel of the AFM images shows enlarged views of typically selected IgG molecule; and D: statistical height analysis of the adsorbed protein.

of ~2 nm is anticipated when the peak force reaches 400 pN.[34] The AFM images also show clearly Y-shaped orientation of the molecule [Fig. 4(A)]. The molecule shows a flat orientation with a mean height of ~2.26 nm standing on the untreated amorphous carbon film. A similar structure is also observed for the antibody adsorbed on the $-NH_2$ grafted surface, but with an enlarged height of ~2.61 nm [Fig. 4(C)]. For the IgG adsorbed on the −COOH modified surface, they are more like an oval-shaped particles with the mean height of ~3.21 nm [Fig. 4(B)]. These results are consistent with the lateral dimensions obtained from the EM analyses [Fig. 3(G)]. This is not surprising since orientations of adsorbed molecules are directly related to their 3D dimensions. In addition, the anticipated denaturing of the molecules triggered by specific surface characteristics also influences their sizes.[33]

The orientation of adsorbed IgG molecules is strongly dependent on the physicochemical characteristics of the surfaces, such as chemistry, wettability, roughness and charging state.[10,35,36] The isoelectric point (IEPs) of IgG1 and its Fab and Fc fragments is 6.8, 8.3 and 6.0, respectively, thus

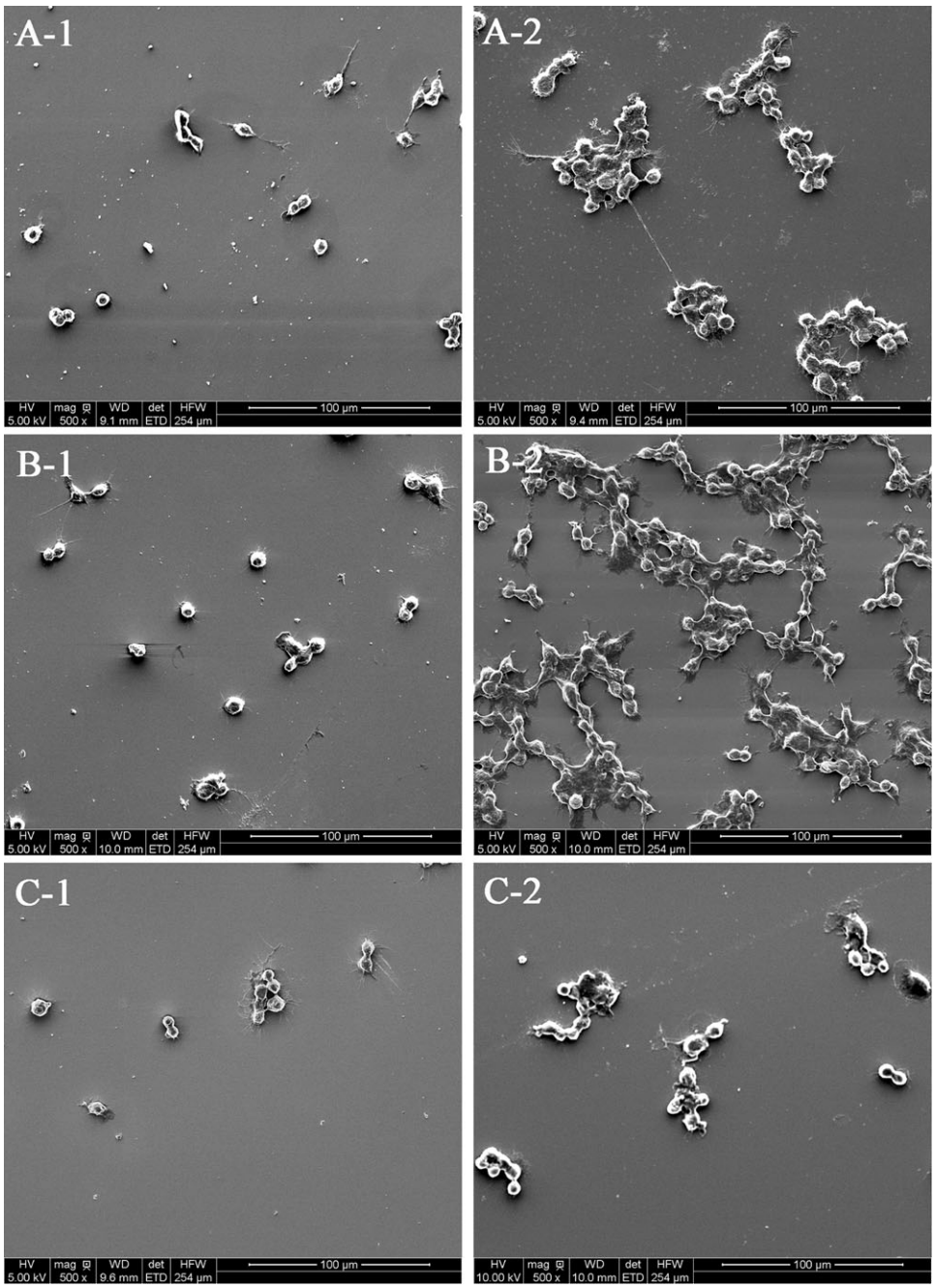

FIGURE 5. FESEM images showing the macrophages adhered on the sample surfaces without (A-1, B-1, and C-1) and with (A-2, B-2, and C-2) the preadsorption of IgG. A-1 and A-2: the untreated amorphous carbon film, B-1 and B-2: the –COOH grafted carbon film, and C-1 and C-2: the $–NH_2$ grafted carbon film.

forming a dipole pointing from the Fc to $(Fab)_2$ fragment. When the molecule is adsorbed onto a charged surface, electrostatic interaction would play a dominant role in regulating the adsorption.[13] This well explains why they prefer vertical orientation upon adsorbing on the −COOH and $-NH_2$ grafted surfaces. IgG opts to show head-on orientation on negatively charged surfaces, whereas end-on orientation on positively charged surfaces.[15] For neutral surfaces where van der Waals interaction dominates, IgG favors lying-on (adsorption through the whole molecule, showing a Y shape) orientation, which is in agreement with our observation of the Y-shaped molecules adsorbed on the untreated surface [Fig. 2(A)].

Other factors like wettability and surface roughness of the substrata would also influence adsorption behaviors of IgG. It has been reported that the hydrophobic/hydrophilic interactions on material surface have profound influence on protein adsorption.[8,36] In aqueous solution, the hydrophilic part is in outer surface with a hydrophobic core. However, when a solid substrate replaces aqueous solution, the tertiary structure elements, which result from the participation of hydrophobic groups, would be destabilized, in turn resulting in a denatured conformation.[33] In addition, according to X-ray crystallography results, the predominant secondary structure elements in IgG are anti-parallel β-sheets and random coils.[2] These structure elements are partially stabilized by intermolecular hydrogen bonding. When IgG molecules got adsorbed onto the surfaces, the amount of β-sheets was strongly reduced, whereas significant increase in random coils was seen.[37] It is believed that amino acids from unfolded parts of a protein would spread on substratum, and thus, peptide units would also be released, occupying a large protein-surface contact region. These speculated behaviors would in turn reduce ordered structures in the adsorbed protein, which could explain the larger lateral size yet smaller vertical size of the IgG particles observed from our EM and AFM characterization. This phenomenon was also reported for several other proteins.[38,39] Variations in the lateral and the vertical dimensions may reflect different unfolding degree of the protein. On the untreated amorphous carbon film, the least hydrophilic surface among the films, the adsorbed molecules show remarkably reduced height and the adsorbed layer would occupy the largest surface area, representing the maximum extent of unfolded protein structure. Whereas on the most hydrophilic surface, the adsorbed IgG shows constrained internal structural rearrangement. This result is consistent with a previous study stating that adsorption of IgG onto a hydrophobic surface induces significant structural changes.[39] Taken together, the adsorption of IgG molecules is a complex process, and electrostatic, van der Waals and hydrophobic interaction play synergistic roles in deciding its conformation. The electrostatic forces determine the predominant orientation of the adsorbed molecules, and the hydrophobic interactions affect the unfolding degree of the proteins, which would ultimately affect following cellular behaviors.

Impact of the orientational changes of IgG upon adsorption on immune responses was further assessed. It is known that IgG plays crucial roles in regulating inflammatory response to biomaterials, and many variables like electrostatic and hydrophobic interactions would influence the adsorption/desorption of IgG.[40] Fc fragment could specifically bind with Fc_γ sites on macrophage membranes to release lysosomal enzymes, such as TNF-α, IL-1, IL-6, and initiates phagocytosis of pathogens.[41] In this case, the immune behavior of macrophages was examined by cell culturing testing. Since the initial stage of inflammatory reaction is crucial, relatively short-term culturing of macrophages was performed. After 6 h incubation, attachment and spreading of macrophages are clearly seen on the surfaces of the films with/without preadsorption of IgG (Fig. 5). Further examination of the adhered macrophages in terms of their aspect ratio and percentage of polarized cells that have the aspect ratio of higher than 1.5.[42] Enhanced adhesion and polarization of macrophages are suggested on the surfaces with the IgG adsorption. The most abundant macrophages are seen on the −COOH grafted surface, and they are the mostly polarized, exhibiting a spindle shape with numerous filopodia in an extensive spreading state (Figs. 5 B-2 and Fig. 6). This presumably indicates an activated status of the cells. In contrast, lower densities of cells are

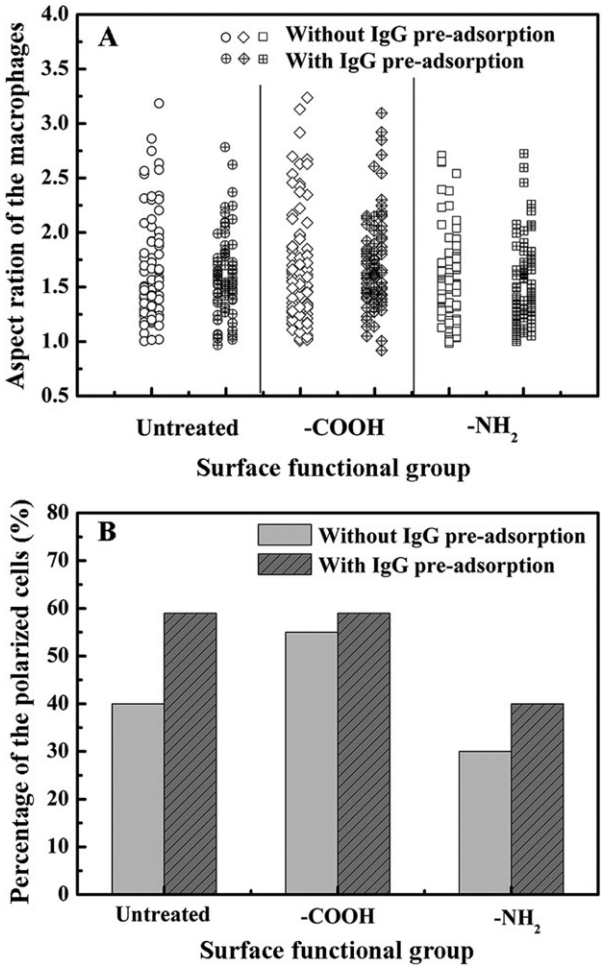

FIGURE 6. Aspect ratio of the adhered macrophages and percentage of the polarized cells showing the influence of preadsorption of IgG on the surfaces of the samples. Cells with the aspect ratio of <1.5 were considered equiaxial, while those with the value of higher than 1.5 were considered polarized.

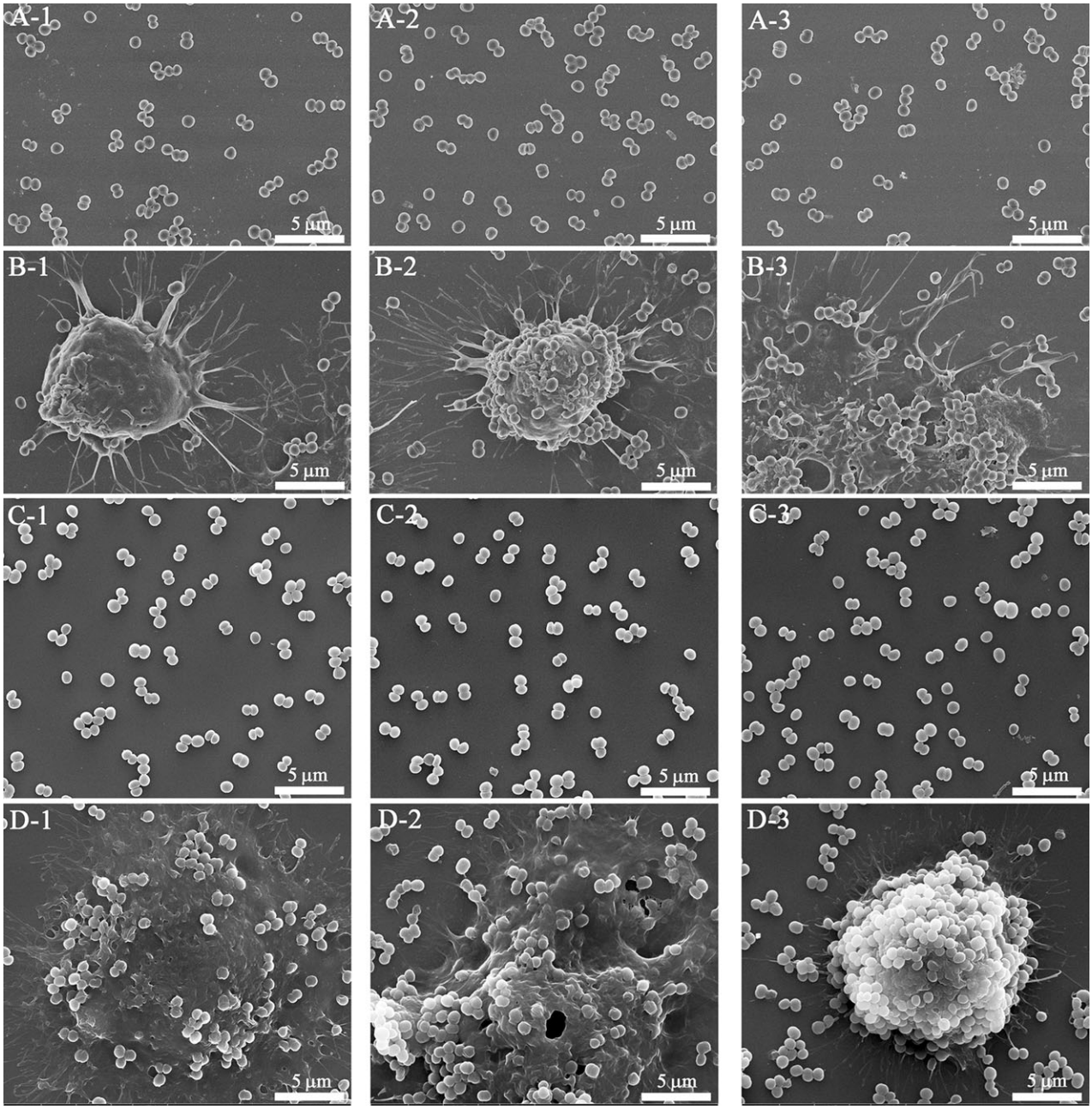

FIGURE 7. FESEM images showing the influence of preadsorption of IgG molecules on adhesion and colonization of *Staphylococcus epidermidis* and subsequent phagocytosis of the bacteria as achieved by participation of macrophages. A-1, A-2, and A-3: morphology of the adhered bacteria without IgG preadsorption on the untreated surface (A-1), the –COOH grafted surface (A-2), and the $–NH_2$ grafted surface (A-3); B-1, B-2, and B-3: typical phagocytosis of the bacteria by macrophages without IgG preadsorption for the untreated surface (B-1), the –COOH grafted surface (B-2), and the $–NH_2$ grafted surface (B-3); C-1, C-2, and C-3: morphology of the bacteria adhered on the surfaces with preadsorption of IgG molecules, (C-1) the untreated surface, (C-2) –COOH grafted surface, and (C-3) the $–NH_2$ grafted surface; D-1, D-2, and D-3: typical phagocytosis of the bacteria by macrophages for the surfaces with preadsorption of IgG molecules, (D-1) the untreated surface, (D-2) –COOH grafted surface, and (D-3) the $–NH_2$ grafted surface.

observed on the untreated and the $–NH_2$ grafted surfaces, and in the presence of IgG preadsorption, the polarization was promoted significantly [Figs. 5(A, C) and 6]. These results are consistent with a previous study reporting that preadsorption of IgG could dramatically maximize macrophage adhesion.[1] It was proposed that polarization of macrophages could be used as an index of cell activation.[43] Activated macrophages display strengthened ruffling of their plasma membrane, giving rise to promoted cell adhesion and formation of pseudopods. It seems that the –COOH grafted surface with preadsorption of IgG remarkably stimulates the activation of macrophages.

To further clarify the influence of preadsorbed IgG on immune responses, macrophage phagocytosis of adherent *S. epidermidis* was investigated. In inflammatory environment,

it has been established that macrophages and IgG interact with each other during the clearance of opsonized pathogens, such as *S. epidermidis*, one of the major bacteria in BAI.[44] In this study, the bacteria were allowed to adhere and grow on the surfaces of the films, and the murine macrophages were introduced after colonization of the bacteria. The phagocytosis of the bacteria by macrophages was quantified by enumerating the number of adhered bacteria in different phases, which was proven to be a reliable approach.[45] Preadsorption of IgG promotes the adhesion of *S. epidermidis* on all the three surfaces after 2 h incubation [Fig. 5(C) vs. A]. Further quantification of the attached bacteria is also conducted. The −COOH functionalized surface without preadsorption of IgG shows the most abundant adhered bacteria [Fig. 8(A)]. After the preadsorption of IgG on their surfaces (formation of conditioning layer), all films show close adhesion ratios of the bacteria [Figs. 7(C) and 8(B)]. This nevertheless indicates that the presence of IgG layer on the films already alleviates the remarkable impact of the special functionalization of the surface (surface grafting). In fact, the enhanced bacteria adhesion as a result of the preadsorption of IgG is not surprising, since conditioning layer (IgG layer in this case) usually promotes formation of bacterial biofilm.[46–48] Many other serum protein layers, such as collagen,[49] fibronectin[50] and vitronectin,[48] were reported to have similar effect. It was found that *S. epidermidis* could specifically bind Fbe sites to fibrinogen, SdrG to collagen, autolysins AtlE and Aae to vitronectin, in turn accelerating the formation of bacterial biofilm.[51–53]

For all the samples, macrophages in the culturing trigger obvious phagocytosis (Fig. 7 B-1, B-2, B-3, D-1, D-2, D-3 and Fig. 8). Phagocytized staphylococci is clearly seen, and the macrophages adhered on the surfaces with preadsorption of IgG exhibit larger sizes with extensively stretched filopodia than those without preadsorption of IgG [Fig. 7(D) vs. B]. It was already shown that macrophages prefer to adhere on the IgG-adsorbed surfaces (Fig. 5), which can well explain the best phagocytosis observed for the −COOH-grafted surface (Figs. 7 and 8). The functional group −COOH alone does not facilitate the adhesion of macrophages (Fig. 5 B-1 vs. A-1), instead, it is the preadsorption of IgG that promotes the adhesion (Fig. 5 B-2 vs. A-2 and C-2). Some other serum proteins, such as fibronectin, fibrinogen, and vitronectin, have been realized to enhance macrophage adhesion.[42,54,55] The composition and orientation of this adsorbed protein layer produce a unique surface of exposed protein sequences, functioning as ligands for cellular responses. Besides, enhanced adhesion of macrophages gives rise to significantly accelerated phagocytosis of bacteria (Fig. 8 B vs. A). It therefore suggests that the surfaces that can promote adsorption of IgG would ultimately benefit phagocytosis of bacteria.

The promoted phagocytosis is associated with enhanced adhesion of macrophages on the functionalized surfaces, further suggesting stimulated activation of macrophages by IgG adsorption. It was already found that receptor Fc_γ from the plasma membrane of macrophage could recognize adsorbed IgG for increased adhesion and activated phagocytosis.[56] The surfaces with preadsorption of IgG could strongly activate the complement cascade and subsequently stimulate

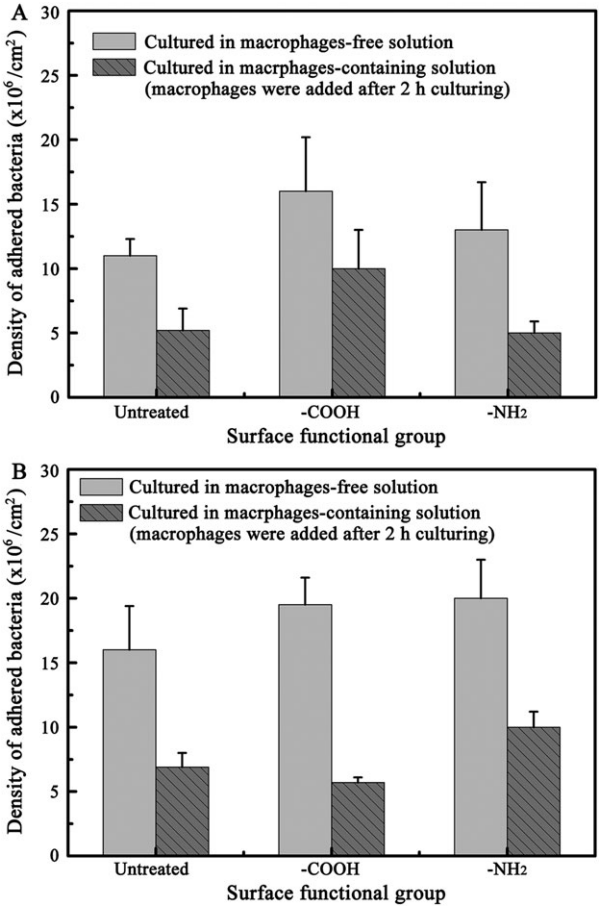

FIGURE 8. Statistical analyses of the adhered *Staphylococcus epidermidis* on the surfaces without (A) and with (B) preadsorption of IgG molecules. The phagocytosis was initiated by adding the macrophages into the solutions after 2 h culturing of the bacteria.

macrophages.[57] Surprisingly, it is noted in this study that IgG pre-adsorption might play a minor role in affecting the activity of macrophages on the $-NH_2$ grafted surface, but the preadsorption enhances the activity on the −COOH grafted surface. This on the other hand suggests tailored activity of macrophages by changing surface chemistry of biomaterials, which is accomplished by regulating the orientations of adsorbed IgG. Our results already showed that the adsorbed IgG molecules favor a flat orientation on the untreated surface, a vertical conformation on the −COOH grafted surface, and a flat/vertical hybrid orientation on the $-NH_2$ modified surface. It has been reported that the head-on orientation is favorable on negatively charged surfaces, and the end-on and the sideway orientations on positively charged surfaces.[13] The orientational information of IgG gained from the EM analyses could explain the altered macrophage behaviors on the different surfaces. On the −COOH terminated surface, IgG prefers to have the head-on orientation, likely providing maximum access to the ligand Fc for strongly enhanced macrophage adhesion. In contrast, however, on the untreated and the $-NH_2$ grafted surfaces, the molecule favors flat and end-on orientation, obscuring the ligand Fc to minimize macrophage interaction. These results further indicate the

impact of orientational changes of adsorbed IgG on immune cell behaviors.

CONCLUSIONS

Orientation changes of IgG after adsorption on typically designed surfaces were characterized by single particle EM and AFM measurements. Charging state of the simplified model surfaces significantly affects adsorption orientation of the molecule. The adsorbed IgG on neutral surface displays a Y-shaped contour, whereas shows vertical orientation on charged surfaces. Unfolding and structural rearrangement of the molecule is accompanied by altered conformation, which further affects adhesion of microphages for tailored phagocytosis of *S. epidermidis.* The direct visualization by single particle EM of IgG upon its adsorption and interpretation of its orientational changes would give insight into characterizing at molecular level protein-biomaterial interactions for appropriate design and construction of biomaterials surfaces.

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