Chapter 7

Macrophage phagocytic activity towards adhering staphylococci on cationic and patterned hydrogel coatings versus common biomaterials

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7.1 Abstract

Biomaterial-associated-infection causes failure of biomaterial implants. Many new biomaterials have been evaluated for their ability to inhibit bacterial colonization and stimulate tissue-cell-integration, but neglect the role of immune cells. This paper compares macrophage phagocytosis of adhering *Staphylococcus aureus* on cationic-coatings and patterned poly(ethylene)glycol-hydrogels versus common biomaterials and stainless steel in order to identify surface conditions that promote clearance of adhering bacteria. Staphylococci were allowed to adhere and grow on the materials in a parallel-plate-flow-chamber, after which murine macrophages were introduced. From the decrease in number of adhering staphylococci, phagocytosis-rates were calculated, and total macrophage displacements during an experiment determined. Hydrophilic surfaces had the lowest phagocytosis-rates, while common biomaterials had intermediate phagocytosis-rates. Patterning of poly(ethylene)glycol-hydrogel coatings increased phagocytosis-rates to the level of common biomaterials, while on cationic-coatings phagocytosis-rates remained relatively low. Likely, phagocytosis-rates on cationic coatings are hampered relative to common biomaterials through strong electrostatic binding of negatively-charged macrophages and staphylococci. On polymeric biomaterials and glass, phagocytosis-rates increased with macrophage displacement, while both parameters increased with biomaterial surface hydrophobicity. Thus hydrophobicity is a necessary surface condition for effective phagocytosis. Concluding, next-generation biomaterials should account for surface effects on phagocytosis in order to enhance the ability of these materials to resist biomaterial-associated-infection.
7.2 Introduction

Biomaterials play an important role in human life to support and restore function after wear, trauma or surgical intervention, the most common examples being total hip- or knee prostheses made of a combination of polymeric and metallic biomaterials. Biomaterial implants and devices provide foreign surfaces, alien to the human body, to which bacteria can adhere and start forming biofilms. Accordingly, biomaterial-associated infection (BAI) is the number one cause of failure of biomaterial implants and devices presenting high costs to the healthcare system. Bacterial contamination of a biomaterial surface during surgical implantation has been recognized as an important route of contamination, but whether or not such contamination eventually results in BAI depends on the outcome of the “race for the surface” between tissue integration and biofilm formation.\[1\] If tissue cells win this race, the implant surface will be covered by a cellular layer and is then less vulnerable to biofilm formation and associated infection. Alternatively, in the inverse case, bacteria will colonize the implant surface and tissue cell functions are hampered by bacterial virulence factors and excreted toxins.\[1-3\] BAI is often difficult to treat, as the biofilm mode of growth protects pathogenic microorganisms against both the host defense system and antibiotics.\[4\] In most cases, the final outcome of BAI is the removal of the implant in order to eradicate infection and subsequent replacement. Consequently, an important next challenge in biomaterials development is to preserve or enhance the ability of an implant or device to facilitate tissue integration while simultaneously inhibiting colonization by bacteria.\[1-3\] In an era of an increasing prevalence of antibiotic-resistant strains\[6\] and considering the protection offered to colonizing bacteria by their biofilm mode of growth, these innovative next-generation biomaterial surfaces should have an efficacy that eliminates the need to use post-operative antibiotics.

Many next-generation biomaterials or coatings have been proposed over the past decades (see Campoccia et al.\[7\] and Hasan et al.\[8\] for excellent reviews). Several non-adhesive modifications of biomaterial surfaces have been developed to mitigate bacterial colonization, such as poly(ethylene)glycol (PEG) coatings.\[9-11\] However, while they inhibit bacterial colonization, they simultaneously prevent tissue integration unless appropriately patterned. Recently, it has been observed that bacterial colonization can be confined to small adhesive patches in a PEG-hydrogel coating that at the same time provide sufficient anchoring points for tissue cells to adhere, spread and grow.\[12-14\] Another category of innovative surfaces is constituted by cationic coatings, either of biological\[15\] or synthetic\[16\] origin and possessing the unique quality of killing adhering bacteria upon contact.\[17\] There is a minimal cationic charge density required for bacterial contact-killing, but also a maximal one to ensure survival of tissue cells on such surfaces.\[18\] This leaves a narrow-window of positive charge density available for clinical application.

The pathogenesis of BAI is complex, however, and the outcome of the race for the surface depends not only on how tissue cells and contaminating bacteria interact on a biomaterial surface, but also on how a biomaterial influences the host immune system.\[19\] Following biomaterial implantation, tissue trauma and injury trigger a cascade of events that activate the immune system.\[20\] Macrophages are one of the most predominant immune cells that arrive within minutes to hours after surgery at an implant site and can remain at a biomaterial surface for several weeks to orchestrate the inflammatory process and foreign body reactions.\[20,21\] During infection,
macrophages detect bacteria via cell surface receptors that bind to bacterial ligands and opsonins.\textsuperscript{21-23} Subsequently, macrophages ingest pathogens and activate cellular functions such as proliferation, secretion of proteins and cytokines, and respiratory burst to destroy phagocytized organisms and recruit other cells from the adaptive immune system.\textsuperscript{21,23} Therefore, bacteria-biomaterial-immune cell interactions are important factors in the pathogenesis of BAI. Immune cell interactions with bacteria on a biomaterial surface are extremely hard to study as they require complicated culture conditions in which neither immune cells nor bacteria are put at too big an (dis)advantage with respect to each other. As a consequence, such studies are rarely done.\textsuperscript{24-25} Yet, co-culture studies are urgently needed to advance next-generation biomaterials or coatings to clinical use and possess the potential of reducing the number of animal studies required, since many new biomaterials or coatings can be discarded beforehand on the basis of improved \textit{in vitro} models, such as a co-culture one.\textsuperscript{5} Co-culture studies involving bacteria and tissue cells have been performed under static conditions,\textsuperscript{26} under flow in macroscopic flow perfusion systems\textsuperscript{27} or in microfluidic devices\textsuperscript{28} and importantly have shown results that are consistent with clinical studies.\textsuperscript{25,29} Moreover, biomaterial surface conditions have been revealed on which the presence of low levels of adhering \textit{Staphylococcus epidermidis} enhances tissue integration\textsuperscript{30-31} or completely negates positive effects of cell-adhesive sites on tissue integration indicated in mono-culture studies.\textsuperscript{32} Co-culture studies involving bacteria and macrophages\textsuperscript{33} have revealed differences in clearance of adhering staphylococci from a surface between murine macrophages and human phagocytes, which require differentiation from their monocyte or promyelocytic state during an experiment. In addition, surface thermodynamic analysis indicated that phagocytosis of adhering pathogens is determined by interplay of physical attraction between pathogens, phagocytes and the influence of bacterial chemo-attractants.\textsuperscript{34-36}

Next-generation biomaterials coatings like patterned PEG-hydrogel coatings and cationic coatings, have never been subjected to co-culture studies with macrophages and bacteria. Therefore the aim of this paper is to compare macrophage phagocytosis activity towards adhering staphylococci on cationic coatings and patterned PEG-hydrogels \textit{versus} common biomaterials (polymers with different hydrophobicity and stainless steel) in order to identify surface conditions that promote clearance of adhering bacteria (see Figure 7.1 for a schematics of the different surfaces involved). \textit{Staphylococcus aureus} was chosen as a pathogen as it is frequently found in infections associated with biomaterial implants and devices. The murine macrophage cell line J774 was chosen because this cell line readily phagocytoses \textit{S. aureus},\textsuperscript{37-39} while being activated by lipoteichoic acid and other cell wall antigens of Gram-positive bacteria.\textsuperscript{40-44}
Macrophage phagocytic activity towards adhering staphylococci on various surfaces

Figure 7.1 Schematic drawings of common polymers (silicone rubber, polymethylmethacrylate, tissue culture polystyrene), a cationically coated surface, differently patterned polyethylene glycol coatings and a metal surface, emphasizing the unique features of these different classes of materials involved in this study with respect to their interaction with bacteria and macrophages.

7.3 Materials and methods

7.3.1 Polymeric and metallic biomaterials

Bacterial-macrophage interaction was evaluated on different common polymeric and metallic biomaterial surfaces: silicone rubber (SR, water contact angle 103°), polymethyl methacrylate (PMMA, water contact angle 73°), tissue culture polystyrene (TCPS, 48°), stainless steel (SS, 71°) and glass (37°), though not a common biomaterial. All surfaces were cleaned in 2% RBS 35 detergent solution (Omnilabo International BV, Breda, The Netherlands) under sonication and rinsed abundantly with ultrapure water, submerged in 70% ethanol and washed again with sterile ultrapure water. Finally, surfaces were placed inside a parallel plate flow chamber (175 x 17 x 0.75 mm³) and rinsed with phosphate-buffered saline (PBS, 10 mM potassium phosphate, 150 mM NaCl, pH 6.8).

7.3.2 Cationically coated glass surfaces

Cationic hyperbranched polyethylenimine (PEI) coatings were prepared as described by Asri et al.[45] Briefly, glass slides were activated with Piranha treatment (3:1 of 98% sulfuric acid and 30% H₂O₂), functionalized with 2-oxo-N(3-triethoxysilyl)propyl)azepane-1-carboxamide as a coupling agent, placed in a vacuum oven at 110°C for 2 h, after which unreacted coupling agent was removed by washing with ethanol. A solution of 5 wt% hyperbranched polymer was spin-coated at 2000 rpm for 60 s. After annealing at 145°C for 2 h, non-anchored polymers were removed by extraction, and a 15 wt% solution of PEI in methanol was dropped on the hyperbranched coating and spin-coated. The anchoring reaction was carried out at 125°C for 52 h under nitrogen, followed by extraction of unreacted components in methanol at 65°C for 1 day. Next, the PEI was alkylated by immersion in 150 ml 1-bromohexane and heated under nitrogen at 90°C overnight after which a suspension of 0.6 g potassium hydroxide powder in 50 ml 2-methyl-2-butanol was added for
another 3 h at 90°C. Finally, coatings were three times sonicated in 100 ml methanol for 20 min at room temperature. Next, a second alkylation was done by immersion in a solution of 20 ml iodomethane in 150 ml 2-methyl-2-butanol at 42°C for 18 h. Finally, coatings were sonicated again followed by extraction and another sonication in methanol and dried and stored under nitrogen. Water contact angles on the coatings amounted 51°.\[45\]

7.3.3 Patterned PEG-hydrogel coatings

Adhesive patches in a PEG-matrix on glass were prepared using established procedures involving electron-beam lithography.\[46-47\] Briefly, Piranha treated glass slides were exposed to low-pressure O₂ plasma (300 mTorr, 1.75 W) for 10 min. The slides were then silanized with 2% (v/v) vinyl-methoxy siloxane homopolymer in ethanol for 10 min, rinsed with deionized water, dried and incubated at 110°C for 2 h. After cooling, thin films of PEG (thickness around 100 nm) were spin cast onto these substrata from 2 wt% PEG (6 kDa) in tetrahydrofuran. PEG was locally crosslinked using a focused electron beam.\[48-49\] For patterning, spin-coated PEG thin-films were irradiated using a Zeiss Auriga Scanning Electron Microscope (SEM) with a Schottky field-emission electron source (point dose of 10 fC and incident electron energy of 2 keV). The e-beam position and dwell time were controlled using a NABity Nanometer Pattern Generation System. Subsequently, slides were rinsed in deionized water for 30 min to remove unirradiated PEG and expose the silanized glass in the unirradiated areas. The resulting surface consisted of silanized glass patches with a diameters of 2.5, 5.0 or 10.0 µm separated by surface-bound PEG at inter-patch distances of 5.0 or 10.0 µm. Twelve array patterns were created each with a size of 200 µm x 200 µm on one glass slide. Whereas water contact angles on patterned PEG-hydrogel coatings are fairly meaningless, water contact angles on a fully PEG-hydrogel coated surface amounted 14°.\[50\]

7.3.4 Bacterial culture conditions and harvesting

A strain of \textit{S. aureus} (NCTC 8325-4) was provided by T.J. Foster (Moyne Institute of Preventive Medicine, Dublin, Ireland). Bacteria were inoculated on trypsin soy broth (TSB, Oxoid) agar plates and then incubated overnight at 37°C. One colony was removed and subsequently grown in 10 ml TSB overnight under constant rotation (120 rpm) and later used to inoculate 190 ml TSB. After 2 h of incubation, this culture was centrifuged (6500 g, 5 min, 10°C), bacterial pellet suspended in sterile PBS and washed twice in sterile PBS. Bacterial aggregates were dispersed using mild, intermittent sonication on ice (3x, 10 s, 30 W, Wibra Cell model 375, Sonics and Materials Inc., Danbury, Connecticut, USA). Next, bacteria were re-suspended in sterile PBS to a concentration of 3 × 10⁸ bacteria per ml. The bacterial concentration was later adjusted when required to obtain similar initial numbers of adhering staphylococci on the different materials and coatings (see 7.3.6).

7.3.5 Macrophage culture conditions and harvesting

A murine macrophage cell line (J774A.1; ATCC TIB-67) was used in this study. Macrophages were routinely cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 4.5 g l⁻¹ D-glucose, pyruvate and 10% FBS containing fibronectin (referred as DMEM-HG + FBS), in TCPS. The TCPS flasks were maintained at 37°C in a humidified atmosphere with 5% CO₂ and cells were passaged at 70 – 80% confluence by scraping. Cells were harvested by centrifugation (5
min at 150 g) in DMEM-HG + FBS previous to experiments. The harvested cells were counted using a Bürker-Türk hemocytometer and subsequently diluted to a concentration of 6 x 10⁵ cells ml⁻¹ in DMEM-HG + FBS.

7.3.6 Staphylococcal adhesion and phagocytosis rates

Staphylococcus-macrophage interactions on the different surfaces were followed in a parallel plate flow chamber, equipped with heating elements and kept at 37°C throughout the experiments. Experiments were done as described before⁹⁹, keeping a constant shear rate of 11 s⁻¹ and in situ monitoring by using a CCD camera (Basler AG, Germany) mounted on the phase-contrast microscope (Olympus BH-2; 40× objective). Briefly, the system was filled with sterile PBS to remove air-bubbles from the tubing and flow chamber, and perfused for 30 min with a laminar flow. Then, the bacterial suspension was perfused through the chamber until around 10⁶ bacteria cm⁻² were observed adhering on the polymeric and metallic surfaces and cationic coating (approximately 30 min), as assessed by an automated counting algorithm (software based on the Matlab Image processing Toolkit (The MathWorks, Natick, MA, USA). Next, sterile PBS was perfused through the system to remove non-adhering bacteria. Then, PBS was replaced by TSB, which was perfused for 2 h to allow bacterial growth. By allowing 2 h for growth, we restricted staphylococcal growth to the monolayer level, which facilitated bacterial visualization and quantification of single bacteria by phase-contrast microscopy. DMEM-HG + 10% FBS was then perfused through the system also at a shear rate of 11 s⁻¹ to remove non-adhering bacteria. Finally, the suspension of macrophages was inserted and the flow was arrested for 2 h to prevent removal of macrophages from the surface. Phagocytosis was monitored during this 2 h timespan by taking images of the surface at 1 min intervals. Despite the fact that bacterial concentrations in suspension were adjusted to obtain similar numbers on the different surfaces as much as their different surface chemistries allowed. Yet it could not be avoided that bacterial numbers differed on the different surfaces especially after 2 h growth, which is accounted for in the calculation of phagocytosis rates.

The number of bacteria that had remained adhering after phagocytosis was determined, subtracted from the number of bacteria adhering before insertion of macrophages and divided by the number of macrophages present. By normalizing this value, representing the number of bacteria phagocytized per macrophage, with respect to the number of staphylococci initially adhering per unit area and the time allowed for phagocytosis, phagocytosis rates were obtained, as previously outlined in detail.⁹⁹ The total displacement of macrophages during the 2 h timespan of an experiment was determined as well by Scion image software for 20 macrophages on each surface, as divided over three experiments carried out on each material or coating. All analyses were done on results of triplicate experiments with separately grown bacterial and macrophage cultures.

7.3.7 Statistics

Data are presented as a mean with standard deviation. ANOVA was applied followed by Student-Newman-Keuls Method or a Tukey’s HSD post-hoc test to demonstrate statistically significant differences. P-values smaller than 0.05 were considered significant.
7.4 Results

Phagocytosis of staphylococci by macrophages on different biomaterial surfaces and coatings was quantified by enumerating the number of adhering staphylococci on the substrata, in the different phases of an experiment (Figure 7.2). After 30 min of initial bacterial adhesion approximately $1 \times 10^6$ bacteria cm$^{-2}$ could be found on each surface with minor differences between the different materials and coatings. These differences became larger, however, after the bacterial growth period. At $t = 190$ min, TCPS, PMMA and SR revealed similar numbers of adhering staphylococci ($2.2 \times 10^6$ bacteria cm$^{-2}$) that were higher than on glass ($0.9 \times 10^6$ bacteria cm$^{-2}$) and SS ($1.2 \times 10^6$ bacteria cm$^{-2}$). Interestingly, due to their contact-killing quality,[45] no bacterial growth was visible on cationic coatings and staphylococcal numbers remained at $1.0 \times 10^6$ bacteria cm$^{-2}$. Staphylococcal adhesion was lowest across all surfaces on patterned PEG-hydrogel coatings and here, too, due to bacterial confinement,[51] no bacterial growth was evident and less than $0.5 \times 10^6$ bacteria cm$^{-2}$ were found adhering.

After growth of the adhering staphylococci, macrophages were introduced and phagocytosis pursued for 2 h up to $t = 310$ min during which the number of adhering staphylococci decreased on all surfaces. This reduction in the numbers of adhering bacteria was taken as the number of phagocytized staphylococci. This number was divided by the number of macrophages involved to yield the number of bacteria phagocytized per macrophage. Next, the number of bacteria phagocytized was normalized by the number of adhering bacteria per cm$^2$ after growth and prior to the introduction of macrophages. This number was finally normalized by the time allowed for phagocytosis to yield a phagocytosis rate. Thus calculated phagocytosis rates are summarized in Table 7.1. Phagocytosis rates varied slightly on the different polymeric biomaterial surfaces, but were two-fold lower on hydrophilic glass ($p < 0.05$ for phagocytosis rates on PMMA versus glass). Phagocytosis rates on SS were in between the ones observed on glass and the polymeric materials although not statistically different. A cationically coated glass surface possessed a significantly ($p < 0.05$) smaller phagocytosis rate than the polymeric biomaterials. The smallest phagocytosis rates were observed on a fully PEG-hydrogel coated surface, but once a pattern of adhesive silanized glass patches was applied, phagocytosis rates increased, depending on the patch diameter and interpatch distance considered. On some patterns, phagocytosis rates even tripled but not in a statistically significant way. Increasing the patch diameter from 5 µm to 10 µm at an inter-patch distance of 5 µm, however, had a negative effect on the phagocytosis rate, although still remaining higher than of a fully PEGylated hydrogel coating.
Figure 7.2 Number of adhering *S. aureus* NCTC 8325-4 on different biomaterials and coatings as a function of time after initial adhesion (t = 0-30 min), PBS washing (t = 30-60 min), growth (t = 60-180 min), DMEM-HG + FBS washing (t = 180-190 min), insertion of macrophages (t = 190 min) and interaction with macrophages (t = 190-310 min).

A: Numbers of adhering staphylococci on glass and polymeric materials,
B: Numbers of adhering staphylococci on stainless steel,
C: Numbers of adhering staphylococci on cationically coated glass (QAC),
D: Numbers of adhering staphylococci on patterned PEG-hydrogel coatings with different patch diameters α and inter-patch distances β, including fully PEG-hydrogel coated glass.

All data are represented as average and standard deviation over three different experiments with separately cultured bacteria and macrophages.
Table 7.1 also summarizes the total distances a macrophage could move on the different biomaterial surfaces within the 2 h timespan allowed for phagocytosis (note that in this stage of the experiment, flow was arrested and macrophage displacement is solely due to intrinsic mechanisms). Macrophages had the smallest displacement on glass and the cationic coatings. Interestingly, despite both being hydrophilic in nature, macrophage displacement on fully PEG-hydrogel coated glass surface was twofold higher than on glass. Patterning did not affect macrophage displacement, which remained similar as on a fully PEG-hydrogel coated glass surface. Macrophage displacement was significantly larger on SS than on most other surfaces, indicating a completely different adhesion mechanism of macrophages on SS than on polymers or glass.

Accounting for the fact that glass and polymers, SS, cationic coatings and PEG-hydrogel coatings possess adhesion mechanisms with bacteria and macrophages that differ widely (charge transfer and mirror charges on SS, electrostatic attraction on cationic coatings, and the hydrated nature of PEG-hydrogels), relations between phagocytosis parameters with hydrophobicity of the materials were only sought for glass and polymeric biomaterials. Figure 7.3 relates phagocytosis rates with macrophage displacement, indicating an increase in phagocytosis rate with displacement. However, phagocytosis rates appear limited to about 15 x 10^6 cm^2 min^-1 under the current experimental conditions. Both phagocytosis rates as well as displacement increase with increasing water contact angles of the substrata. This suggests that substratum hydrophobicity stimulates effective phagocytosis of adhering bacteria (Figure 7.3).

Table 7.1 Phagocytosis rates and total macrophage displacement during the 2 h timespan allowed for phagocytosis for various different classes of common biomaterials and innovative cationic and PEG-hydrogel coatings. All data are represented as averages and standard deviations over three different experiments with separately cultured bacteria and macrophages.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Phagocytosis rate (10^-8 cm^2 min^-1)</th>
<th>Macrophage displacement (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass and polymeric biomaterials</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glass</td>
<td>6.9 ± 2.5</td>
<td>36 ± 18</td>
</tr>
<tr>
<td>Tissue Culture Polystyrene</td>
<td>11.8 ± 1.7</td>
<td>65 ± 20</td>
</tr>
<tr>
<td>Polymethylmethacrylate</td>
<td>14.2 ± 3.9</td>
<td>106 ± 56</td>
</tr>
<tr>
<td>Silicone rubber</td>
<td>13.4 ± 2.8</td>
<td>186 ± 68</td>
</tr>
<tr>
<td>Metallic biomaterials</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stainless steel</td>
<td>10.9 ± 1.4</td>
<td>239 ± 90</td>
</tr>
<tr>
<td>Cationic coating</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEI-coating</td>
<td>7.6 ± 1.7</td>
<td>39 ± 26</td>
</tr>
<tr>
<td>Patterned PEG-hydrogel coatings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fully PEG coated</td>
<td>4.6 ± 6.0</td>
<td>75 ± 38</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patch diameter (µm)</th>
<th>Interpatch distance (µm)</th>
<th>Phagocytosis rate (10^-8 cm^2 min^-1)</th>
<th>Macrophage displacement (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>5.0</td>
<td>15.7 ± 5.5</td>
<td>89 ± 41</td>
</tr>
<tr>
<td>5.0</td>
<td>2.5</td>
<td>14.3 ± 4.7</td>
<td>70 ± 60</td>
</tr>
<tr>
<td>5.0</td>
<td>5.0</td>
<td>16.1 ± 8.2</td>
<td>68 ± 24</td>
</tr>
<tr>
<td>5.0</td>
<td>10.0</td>
<td>15.1 ± 2.1</td>
<td>98 ± 56</td>
</tr>
<tr>
<td>10.0</td>
<td>5.0</td>
<td>8.2 ± 5.5</td>
<td>87 ± 61</td>
</tr>
</tbody>
</table>
Figure 7.3 Rate at which adhering *S. aureus* NCTC 8325-4 are removed by macrophages as a function of macrophage displacement on glass and different polymeric materials. All data are represented as averages and standard deviations over three different experiments with separately cultured bacteria and macrophages.

7.5 Discussion

The interaction of immune cells with bacteria adhering on the surfaces of biomaterial implant and devices has rarely been taken into account in the design of infection-resistant materials and coatings. In this paper, we fill this gap with respect to cationic and patterned PEG-hydrogel coating by carrying out co-culture studies with murine macrophages and an *S. aureus* strain adhering to different common biomaterials and the two type of coatings.

Phagocytosis studies are mostly carried out with bacteria in suspensions, but here we applied a recently developed method to quantify phagocytosis of bacteria adhering to solid surfaces. The number of bacteria phagocytized was inferred from the decrease of the number of bacteria adhering on a substratum surface and for that reason we called this approach “indirect”. Direct quantification of the number of green-fluorescent bacteria internalized by macrophages however, yielded identical results confirming the validity of the method applied. A major advantage of the indirect method as applied here is that it not only includes live but also dead bacteria, as expected on cationically-coated surfaces. Note that the results of this study (Figure 7.1, initial adhesion phase) may not be interpreted in terms of differences in their adhesiveness to *S. aureus* as the study was set up to obtain numbers of initially adhering bacteria within an as narrowly possible, limited range of values. Though outside the scope of this paper and discussed and explained in several other papers, it is of importance to mention that bacterial growth was virtually absent on patterned PEG-hydrogel coatings and cationically coated surfaces.

There is quite a bit of concern on the applicability of cationic coatings to control bacterial colonization and a frequently heard argument is that once adhering bacteria are killed upon contact, they form a layer of dead bacteria to which new bacteria can adhere and form a biofilm. This criticism is persistent, despite the fact that animal studies in both rats and sheep have demonstrated efficacy of cationic coatings in infection control. Schaer et al argued that “ensuing bacterial debris is available to the host’s immune surveillance”, but experimental evidence that
immune cells function on a cationic surface has never been provided. Our study shows that macrophages indeed have difficulty clearing adhering bacteria from cationically charged coatings. Both phagocytosis rates as well as the displacement of adhering macrophages over the cationic coating are reduced relative to those on common biomaterials (see Table 7.1). The reason for this may be twofold. First, bacterial cell surfaces are generally negatively-charged\cite{61} and accordingly\cite{62} adhere strongly to a positively-charged surface, which is in fact the basis for bacterial contact-killing.\cite{63} Thus, bacteria adhering to cationic coatings will be difficult to engulf by macrophages, a process which requires bacterial detachment from the surface. Second, macrophage cell surfaces are negatively charged, and consequently macrophages will have difficulty in displacing themselves over positively charged surfaces. As the results of this study show (Figure 7.3), displacement is a prerequisite for effective phagocytosis. Yet, we observed sizeable phagocytosis of adhering staphylococci by murine macrophages (Table 7.1), explaining the anti-infection resistance of cationic coatings in animal studies and supporting the suggestion\cite{60} that dead bacteria and ensuing debris can be removed by immune cells.

Fully PEG-hydrogel coated surfaces constitute another class of surfaces on which phagocytosis rates are reduced as compared to common biomaterials (Table 7.1). The mechanism behind this reduction is likely opposite to the one offered above for cationic surfaces. It has been demonstrated that due to the highly hydrated nature of PEG-hydrogels, cells, bacteria and proteins have great difficulty interacting with such surfaces.\cite{64} Consequently, macrophages are unable to establish the attachment points needed to displace themselves and subsequently engulf adhering bacteria, despite the fact that bacteria adhere weakly.\cite{48}

Attachment points to facilitate macrophage displacement on a fully PEGylated surface can be provided by introducing adhesive patches in an otherwise fully PEG-hydrogel coated surface, restoring macrophage displacement to the level observed on common, polymeric biomaterials irrespective of patch diameter and interpatch distance (see also Table 7.1). The adhesive patches are formed by vinyl-methoxy siloxane groups and are thus hydrophobic. In general, hydrophobic surfaces are not known to enhance macrophage adhesion,\cite{24,65} but relative to their highly hydrated surrounding of the PEG-hydrogel, hydrophobic patches may provide sufficient opportunity for macrophages to adhere and phagocytize adhering staphylococci to the same level as on common biomaterials. Macrophages use small cellular projections (“filopodia”) to search for bacteria.\cite{27} When the adhesion of bacteria is confined into small patches, this search will evidently be more successful than when adhering bacteria adhere scattered over a surface, which explains why macrophages were more effective in eradicating bacteria from patterned surfaces. However, when patches become too large, the possibility of a more scattered adhesion within the patch increases which in turn reduces the phagocytosis rate (Table 7.1). It is unfortunate that the few studies pertaining to immune cell adhesion to biomaterials have been done in mono-culture studies\cite{66} and therewith yield no confirmative clues as to the implications of these interactions for phagocytosis of adhering bacteria. However, Figure 7.4 indicates that a certain degree of substratum hydrophobicity is needed for effective phagocytosis despite the fact that monoculture studies conclude that substratum hydrophobicity may reduce macrophage adhesion.\cite{24}
Explaining the phagocytosis rate and macrophage displacement on SS is most difficult, as metallic biomaterials have yet a very different mechanism of interaction than polymeric ones. Charge transfer upon bacterial adhesion is a demonstrated mechanism\[67\] that will occur as well with macrophages. Then there is an additional attraction between charged particles adhering to metal surfaces through the development of so-called mirror charges in the metal. The net effect of all these is that phagocytosis rates on SS are very comparable to those on polymeric biomaterials, while macrophages appear to displace themselves quite easily on SS. This may point to a relatively strong adhesion of micron-sized bacteria to SS as compared to macrophages. Strong adhesion forces for oral streptococci have indeed been found on SS.\[68\]

**Figure 7.4** Rate at which adhering *S. aureus* NCTC 8325-4 are removed by macrophages and macrophage displacement on glass and different polymeric materials as a function of water contact angles on the materials.

Top: Phagocytosis rates versus water contact angles.

Bottom: Displacement versus water contact angles.

All data are represented as averages and standard deviations over three different experiments with separately cultured bacteria and macrophages.
7.6 Conclusions

In order to prepare advanced health-care materials that resist bacterial colonization in an effective way, the interaction of immune cells with adhering bacteria has to be accounted for in co-culture studies. When doing so, it becomes apparent that both phagocytosis rate as well as macrophage displacement increase with increasing hydrophobicity of the substrata. This suggests that hydrophobicity is a necessary surface condition for effective phagocytosis to occur, which explains why the introduction of silanized adhesive patches in hydrophilic PEG-hydrogel coatings stimulates phagocytosis. Phagocytosis is hampered with respect to common biomaterials on cationic coatings, possibly through strong electrostatic binding of negatively-charged adhering staphylococci but not to the extent that it impedes clinical application.

7.7 Acknowledgements

This project was funded by the UMCG and partially supported by the U.S. Army Research Office via grant #W911NF-12-1-0331.

7.8 Disclosures

H. J. Busscher is also director of a consulting company, SASA BV (GN Schutterlaan 4, 9797 PC Thesinge, The Netherlands). The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article. Opinions and assertions contained herein are those of the authors and are not construed as necessarily representing views of the funding organizations or their respective employers.
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