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The Importance of Not Being Too Attached: Pharmaceutical Equipment Characteristics and Bacterial Attachment

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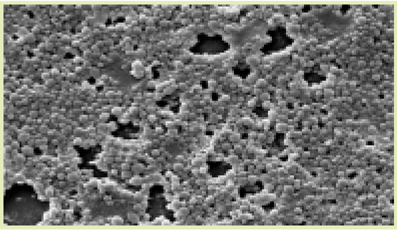

INTRODUCTION

The ability to attach to and subsequently detach from biotic and abiotic surfaces is a characteristic shared by all bacterial cells. This is because the mechanisms of attachment are advantageous for survival in the natural environment. Moreover, attachment enables microorganisms to exert some control over their nutritional environment. The ability to, and likelihood of, attachment is as likely within a pharmaceutical or healthcare environment compared with any other environment, albeit with the expectation that populations are lower. It is important that as part of hygienic equipment design that surface characteristics are specified, especially with materials like stainless steel (1).

Attachment can lead to adhesion, and the two are different but related concepts. Attachment is a physical activity; as bacteria approach a surface, cell appendages (such as fimbriae, pili, and flagella) may stick to it. Adhesion can occur when adhesive molecules expressed on the bacterial surface bind to host surface receptors; this is associated with biofilm formation (2). In both cases, bacteria can sense that they are in contact with a surface and provide the initial cellular responses to surface contact and subsequent adhesion. Factors like temperature and pH influence the bacterial adhesion (processes that are explored in an earlier article by the author for the *Journal of Validation Technology*) (3). It is important to note that surface roughness and cleanability have a relationship, but the relationship is complex. A rougher surface may increase the potential for microbial attachment although to a degree cleanability is subject to variations in terms of the presence of other substrates and time as microorganisms move from the reversible to the irreversible state.

An important factor related to this consideration, especially for when cleanroom equipment is selected, is the surface finish and surface roughness. This focus on topography is particularly useful for stainless steel given the commonality of this material, along with anodized aluminum, for the production of pharmaceutical processing equipment. Hence, specifying appropriate finishes and limits of roughness should form part of equipment and facility specifications, as informed by the principles of quality by design. Durability and resistance to corrosion represent important reasons as to why material grade, finish, and roughness matter. In addition, these specifications are also determinants of the likelihood of microbial attachment and hence they are essential considerations when developing a contamination control strategy. This review paper assesses the factors affecting finish and roughness, primarily in relation to microbial attachment to stainless steel. The objective is to aid with developing equipment design specifications and User Requirement Specifications. This paper is a follow-up to “Good Hygienic Design Principles for Pharmaceutical Manufacturing”, published in the*Journal of Validation Technology* (4).

SURFACE SPECIFICATIONS

Different types of austenitic stainless steel are widely used around the world (and less commonly ferritic stainless steels are used, along with other stainless steels like martensitic, duplex and precipitation hardened steels, which make up the metallurgical families of stainless steel). These types are different in terms of composition, resistance to corrosion, versatility, and price. Key factors to be assessed when purchasing stainless steel include:

- Toughness
- Ductility
- Weldability
- Thermal expansion
- Stress corrosion cracking resistance
- Magnetic properties

Generally, in pharmaceuticals, stainless teel composed of chromium-nickel alloys is used; commonly these are the 304 and 316 grades, as per the AISI/SAE standard, with the addition of the electropolishing of the surfaces being a common requirement. Stainless 304 usually consists of 18% chromium and 8% nickel, whereas, stainless 316 is made up of 16% chromium, 10% nickel and 2% molybdenum. Stainless steel grade 316 is far more expensive than 304 and is preferred for many pharmaceutical applications since it provides a higher corrosion resistance, particularly against chlorides and chlorinated solutions. The grade is commonly followed by one of around twelve application codes. For example, 'S' to denote structural steel; 'P' to denote steel for pressure lines and vessels; and 'L' to denote steel for pipes and tubes.

Electropolishing helps to ensure friction is reduced (with 316 grade being more resistant to corrosion than 304 grade). Practical demonstrations indicate that electropolished and grit 4000-polished steel be relatively corrosion resistant as opposed to grit 80- and 120- polished surfaces (higher grit scores correlate with lower surface roughness) (5). Finish and grade are not, however, commensurate with surface roughness (6). It remains that too few facilities specify for both surface finish and surface roughness, and on some occasions, 'finish' is erroneously taken to be the equivalent of roughness. This can have implications for the likelihood of reversible and irreversible microbial attachment and adhesion.

Scientific literature provides evidence that surface roughness is an important factor with microbial attachment and that increased surface roughness is associated with increased bacterial attachment (7, 8) (albeit there are the variables of microbial species specificity and the nature of the ecological community that develops as discussed below). Further supporting experimental data, for stainless steel specifically, has demonstrated that the possibility of bacterial contamination and hence the potential for biofilm communities forming on stainless steel surfaces is reduced where there are appropriate mechanical and electrochemical treatments have been performed so that the resultant surface morphology and chemical reactions are rendered optimal (9). As to what optimal is, there are different measures that can be deployed (notably the Ra value) and these are outlined below. Achieving this is not 'exact' for with stainless steel, there will always be a slight variation due to the random nature of the surface structure.

It follows that specifying a requirement for the hygienic design of equipment for pharmaceutical manufacturing, such as stipulating the grade of materials for product contact surfaces, is important in order to prevent long-term bacterial attachment and thus for biofilm formation (10).

Ra: The Concept And Its Limitations

The most commonly reported surface roughness parameters are average and root mean square (RMS) roughness (referred to as*Ra* and*Rq* respectively). These are both indicators of the typical height variation of the surface.*Rz* is also sometimes used, representing the difference in height between the average of five highest peaks and five lowest valleys. The outcomes of*Ra, Rq, Rz* and so on often vary meaning that the "roughness" of the same surface can vary depending upon which parameter is used (11). Of these, Ra is the most common measure, expressed in micrometers (µm). There are different methods for assessing this surface roughness (*Ra*) value as set out by bodies such as ASME (12), such as using a stylus method. Here, a stylus (or a profilometer) travels across the surface assessing microscopic peaks and valleys and the differences between the high and low points. The movement of the stylus is amplified, and the signal recorded as an average (mean) value. The resulting*Ra* value (or "microns Ra") is the arithmetic average value of the deviation of the trace above and below the centerline (13).

The Ra value is not ideal, but it is generally taken to be the best measure available (at least as notified to pharmaceutical companies by equipment suppliers). Criticism of*Ra* in terms of providing a full indicator of roughness include the fact that the measure offers no insights into the spatial distribution or the shape of the surface features, such as the undulation of peaks or valleys (which comes under the concept of rugosity, which is a measure of small-scale variations of amplitude in the height of a surface) (14). Nevertheless, as a readily available parameter, it remains the accepted industry marker for surface roughness (15); and hence this is the concept that this paper focuses upon in terms of being of practical value.

Characteristics for Surface Attachment

The presence of microorganisms in pharmaceutical manufacturing presents a higher risk when there is the possibility of transfer to a more critical surface. The main mechanisms are by particles carried in the air (and where the fluid path leads to a critical area through the airflow dynamics) and via surface transfer. With surfaces, where microorganisms are deposited onto critical surfaces, the control mechanism is based on cleaning and disinfection using manual or automated steps. Cleaning validation studies do not always include microbial criteria and the inclusion of microbial testing (surface sampling and final rinse is important) (16).

For any given surface, microorganisms deposited onto a surface will remain present on the surface by a combination of mechanisms such as adhesion, adherence, and entrapment. Adhesion is rendered easier of difficult based on the material type and prevailing conditions. Variations between different surfaces is also demonstrated through different rates of microbial recovery (17). While the roughness of the surface is also a contributing factor in terms of the adhesion mechanism, entrapment is perhaps the variable of greatest concern when considering surface roughness. When expressing this using the Ra value, studies demonstrate a stronger possibility of microbial entrapment at an*Ra* of 0.9 µm compared with an*Ra* of 0.8 µm.

For example, one study looked at the difference between surface finish and surface roughness for 304 grade stainless steel across nine surface finishes. The focus was on the 'cleanability' of the surface. The finishes included: hot rolled and pickled, 2B mechanical polished, and electropolished stainless steel. Cleanability was assessed by using two microorganisms:*Geobacillus stearothermophilus* (in the endospore state) and *Pseudomonas* sp. as a community biofilm. A milk powder was used to represent a soiling substance. The sanitization agent was 100 ppm hypochlorite, applied by depositing the disinfectant onto a surface and with manual (mechanical) wiping. The analysis indicated that surface defects were the optimal determinant in terms of making microorganisms more difficult to remove rather than the finish type. The point at which surface roughness was significant was determined to be*Ra* = 0.82 µm. Hence an*Ra* of ?0.8 µm represents an appropriate specification value to remove microorganisms through a standard cleaning and disinfection regime. It is on the basis of such studies that an*Ra* value of below 0.8 µm has evolved to become an important criterion for hygienic design considerations (18). There are advantages of targeting an*Ra* of below 0.8 µm as the data patterns follow that the risk of surface attachment becomes lower in proportion to the surface becoming smoother (in terms of descending 0.1 µm increments). This happens to a point where anything smoother ceases to make a difference (19). Research places this marginal point as 0.16 µm (20), below which there is no further advantage to be gained. Moving in the other direction, roughened stainless steel (*Ra* 5.38 µm and higher) has been shown to be completely unacceptable for effective cleaning (21) and cleanability becomes easier (although problematic) the further the surface gravitates down the scale (again, this can be assessed in 0.1 µm increments although from a practical value for the pharmaceuticals and healthcare context there is little value in specifying a finish above 0.8 µm).

The margin between 0.8 µm and higher values is set out in the international standard for surface roughness: ISO 4287 (22). Under this standard, an N6 notation corresponds to an Ra of 0.8 µm, whereas the next lowest grade, N7, corresponds to an*Ra* of 1.6 µm. Therefore, for product contact and other critical surfaces within a controlled environment, specifying N6 as a surface roughness value is required. Such data is also of interest in the healthcare context in areas where nosocomial infections are of a concern.

Surface Finish and Material Grade

Surface finish is a more subjective concept than surface roughness (the latter can be assessed quantitatively, as discussed in the section below). Surface finishes are assessed in terms of grades, relating to both the method of creating the surface finish and the subsequent appearance assessment as part of quality control (standards include EN 10088-2 (23) and ASTM A480 / A480M - 20a (24)). There is, nevertheless, an importance in having a high-quality finish in relation to the method of finishing. Mechanically polished surfaces tend to be less well-finished than electropolished surfaces. This is because microscopic crevices resulting from smeared metal from the polishing operation tend to occur with the mechanical polishing process. Electropolishing, in contrast, removes these microscopic crevices and this electrochemical process produces a passive layer with a higher chromium to iron ratio. This not only adds to the appearance, but it also assists with cleanability. It is slightly confusing that a form of electropolishing is also a means to reduce the roughness of a surface (as well as to improve the finish).

However, studies relating to microbial attachment to stainless steel indicate that surface finish is not the primary factor in contrast to the level of roughness or damage occurring through misuse or ageing (25, 26). This is provided that the surface finish is uniform. Furthermore, the influence of surface roughness appears to be influential than other physicochemical properties of stainless steel, such as the surface free energy (which is the excess energy that the surface has compared to the bulk of the material) (27). Passivation is a separate process to 'surface finish', although the two are sometimes confused. Passivation does not affect the surface profile. Passivation is the process of rendering stainless chemically inert by removing iron ions and is undertaken for enhance corrosion resistance.

The grade of stainless is influential. Chemical analysis has found that the difference in the compositions of the passive surface layers on grades 304 and 316 stainless steel is due to the presence of molybdenum (which is added to increase the corrosion resistance of austenitic and duplex stainless steels and is found in 316 stainless steel but not the 304 grade). In experiments, 316 grade stainless steel has recorded lower levels of microbial attachment when both 316 and 304 grades of stainless steel are subject to equivalent microbial challenges and tested under the same conditions (28).

Surface Roughness

It is perhaps easier to discuss the roughness of a surface than the smoothness since all surfaces with contain a level of roughness when assessed on the micron scale. Roughness of a surface either relates to the design of the process or rendering the surface, with some milling processes designed to produce smoother crevices than others; or the degree of roughness occurs in relation to quality variables with machining (29). A general reason why surface roughness is important for pharmaceuticals and healthcare is because rough surfaces usually wear more quickly and may have higher friction coefficients than smooth surfaces. From the contamination control perspective, an increased degree of roughness generally leads to an increase in the contact area for a bacterial cell since the larger the contact area is, then the larger the attachment force will be, and hence microbial attachment becomes easier (30). This is, of course, a generalization because microbial cells exhibit flexibility in terms of shape and there are variations in physiological shape which also affect attachment, and hence attachment is species specific and affected by the condition of the cell. Thus, bacterial attachment in relation to surface roughness can only be considered as a probabilistic concept. This concept is centered on the Ra 0.8 µm value (or, as a non-metric value - 32µin), below which it becomes harder for bacterial cells to attach and above which it becomes easier for cells to attach.

There are limitations with the use of*Ra* based on the fact that*Ra* is an average value and consequently there will be variations with the substratum surface roughness in different locations across the surface (31). With the determination of*Ra*, up to 16% of the measurements can be above the required value and the surface may still pass unless the maximum value is specified. When a maximum value is stated,*Ra* will appear as*'Ra max'* on the material certification and this means that no value has exceeded the required maximum value (32). It follows that very different profiles can have the same*Ra* value and "*Ra* ? 0.8 ?m" does not carry the same meaning as "*Ra max* 0.8 ?m". A further consideration is with the slope of a surface, which can be examined through the contact angle measurement test, where the angle can be a parameter influencing the adhesion rate (33). Generally, bacteria prefer to attach to horizontal surfaces.

In terms of lowering the*Ra* value, this is undertaken by utilizing a combination of chemicals and electricity to carefully dissolve the surface of the steel (a form of electropolishing). This process works on surface peaks, rather than on surface valleys, on the basis that reducing the height of the peaks brings them closer to the depths of the valleys. The quality of the starting material is important since such processes can generally only lower the*Ra* value by up to 10 µm.

It also stands that within a busy controlled environment, roughness will not be a static concept. Surfaces will be subject to periodic cleaning and disinfection, a process that can disassociate microorganisms especially where wiping is involved. This is technique and method dependent and introduces variations with chemicals, application methods, and personnel competency (ineffective cleaning is likely to be influential, especially when soil remains (34)). The ability to clean and disinfect consistently is also affected by surface roughness (in that 'roughened' surfaces are more difficult to clean and microscopic observations demonstrate how the shape and size of surface irregularities further influence on the level of residual soil remaining after standard cleaning(35)). According to one study, the*Ra* range that best facilitates the ease of cleaning was found to be 0.4 to 1.5 µm (36) (although cleanability falls away with higher*Ra* values where long-term microbial attachment occurs, and this again returns the optimum to the 0.8 µm cut-off being preferrable since a more stable microbial community attachment becomes more difficult to maintain).

Another important surface manufacturing factor that can influence microbial attachment arises from the design and construction process. With this, bacteria have been observed to colonize preferentially near welds as a result of surface roughness arising from imperfect fabrication. In particular, as bacteria begin colonizing, attachment will occur most greatly on the grain boundaries of the base metal (between the weld and the base) (37). Various methods, as described in standard ISO 15609: 2019 (38), can be deployed to assess the quality of a weld, including visual inspection, radiography, ultrasonic testing, phased-array ultrasonics, dye penetrant inspection, magnetic particle inspection, or industrial computed tomography. Consideration must be given to any burr from grinding, drilling, milling, engraving, or turning. A 'burr' is when a raised edge remains following the fabrication process. The manufacturer of the material should have a system in place to remove any burr by the process off 'deburring,' for which there are multiple methods including mass-finishing, abrasive flow machining, electrochemical deburring, and the thermal energy method (39).

The process of attachment is also aided by surface irregularities, such as with water systems where a bend in a pipe can protect colonizing bacteria from shear forces and hence the organisms can find and utilize more attachment points to the substratum (40). The reference to the internal surfaces of water systems is important since surface roughness is more significant for materials immersed in water, within both static and turbulent flow conditions (41). Attachment takes longer to become irreversible for surfaces subject to an atmospheric environment (42). This is one of the reasons why 316 grade stainless steel is preferred for liquid immersion rather than the 304 grade.

Microbial Type, Size, and Attachment

The characteristics of the contaminating microorganism is important when considering the dynamics of surface attachment. While all microorganisms have the ability to attach to a surface, and most bacteria seek to as a means to stimulate growth, the attachment to interface is controlled by the macromolecular structure of the cell wall (such as fimbriae) and hence there are species variations as well as different community dynamics (such as an association with biofilms). As well as preferring horizontal surfaces, as mentioned above, the larger the surface area then the more likely attachment is to occur (43). The degree of attachment can be assessed using different techniques, such as microbial adhesion to hydrocarbons (MATH), which involves quantifying bacteria adhesion to the hydrocarbon droplets that form; atomic force microscopy; total internal reflection microscopy; quartz crystal microbalance; and the use of liquid flow cells (44). None of these techniques is easily applicable to the typical pharmaceutical laboratory.

Once attachment takes place during the initial phase attachment is reversible (that is organisms can relatively easily return to the planktonic state since they ae only weakly held by electrostatic forces). This occurs due to hydrodynamic and electrostatic interactions (45). After a period of time, attachment becomes irreversible. This takes several hours, and it is the product of Van der Waals interactions taking place between the hydrophobic region of the outer cell wall and the surface (46) plus the release of exopolysaccharides that will eventually form a biofilm matrix (47). The discussion about surface roughness is important at this stage. Nano- and microscale surface roughness enhances the adhesion of bacteria to substrates during the initial steps of colonization as it provides more surface area for cell attachment. The easier it is for cells to colonize the more likely irreversible attachment is to occur.

Some organisms move to an irreversible form of attachment through adhesion, and they do so by producing extracellular polymeric substances, which enables cell–to-cell adhesion and hence the development of a biofilm (48). The substance produced is composed of exopolysaccharides, extracellular proteins, humic substances, nucleic acids, and phospholipids (49). Biofilm formation is of particular concern when it occurs in pharmaceutical water systems.

As well as species variation, species variation also needs to be considered in relation to material type. For example, taking stainless steel and poly (methyl methacrylate) (PMMA), the latter being known as acrylic, Perspex, or plexiglass, then thermodynamics shows that microorganisms adhere better to stainless steel than PMMA (this comes down to differences between materials in terms of their surface energy). This is due to the relative nature of the positive surface charge (the vast majority of bacteria are negatively charged) (50). It follows that the irreversible adhesion of bacteria is discouraged on negatively charged surfaces, while it is promoted on positively charged surfaces. With different species, thermodynamics also explains why an organism like *Pseudomonas aeruginosa* is more likely to adhere to a surface than*Bacillus cereus* (this is a factor of the surface energy of different bacteria). If the surface charge can be altered, especially for use in higher-grade cleanrooms, this could present an antimicrobial means to limit the likelihood of bacterial attachment.

Some bacteria are better equipped to undergo phenotypic changes upon contacting a surface, which promotes adhesion (certain bacteria become morphologically differentiated upon attachment) (51). A proportion are also adept and surface sensing, enabling them to better adapt to the surface conditions. For example, cells of *Staphylococcus aureus* can sense the binding of surface ligands to receptors on one side of its cell body and respond by localizing receptors to the surface-associated region (52). Also of importance is the size of the microbial cell relative to the surface substrata. For example, S. aureus (cells 0.5–1 ?m diameter) are retained in higher numbers on surfaces with microtopography pits of*Ra* 0.8 ?m, as is*Streptococcus oralis* (0·5 - 1.0??m) (53), compared with*Pseudomonas aeruginosa* (cells 1 ?m × 3 ?m) (54). Even with the same species, there are variations of which the most important appears to be bacterial cell surface hydrophobicity. Such findings tally with studies that indicate the effect of surface roughness differing for bacteria of different morphological shapes and hence which possess different surface energies, as with rod and cocci bacteria (55).

SURFACE DAMAGE

All surfaces in use become damaged and regular maintenance is necessary. If damage is substantial or left for long periods untreated, then damages to surfaces will influence the likelihood of microbial adhesion or entrapment. This is shown through studies looking at the strong correlation between adhesion and the damage that causes greater surface roughness (56). The type of damage may also be a factor. For instance, one study determined where damage is linear (as with scratches), then cocci are more likely to become trapped than rod shaped bacteria, which is explainable on the basis of cell alignment and binding energy within or across surface features (57). Another factor is the size of the abrasion: if scrapes are considerably larger than the microbial cells, then retention is not significant; however, if the abrasions are of microbial size dimensions, then retention is far more likely. Research by Riedewald led to the recommendation that "scratches or faults deeper than a multitude of the particle diameter are significantly more difficult to clean than the mother plate material" (58).

Surface damage can also lead to a stress concentration occurring. This is a location in an object where the stress is significantly greater than the surrounding region. This arises from such forms of damage like holes, nicks, and scratches. While not generally related to microbial attachment, it is worth noting for the effect of increasing fatigue strength and potentially the brittleness of the material (59).

A related issue with damage and microbial attachment is with microorganisms themselves creating damage in the form of corrosion. In turn, this fosters conditions for future bacterial attachment. With reference to a point above, areas of welding subject to corrosion are the most vulnerable (60). Corrosion can also be caused by disinfectants, especially those that are chlorine based. Most notable is the 'rouging' of stainless steel, which is the result of the formation of iron oxide, hydroxide, or carbonate either from external sources or from destruction of the passive layer. This oxidation may arise from added chlorine. Where chlorine disinfectant residues are not removed following the targeted disinfectant contact time having elapsed, the resulting reaction is self-perpetuating by the chloride reacting with the chromium in stainless steel to form hypochlorous acid as a byproduct. In turn, the hypochlorous acid oxidizes the iron and forming more chloride. As discussed earlier, increasing the molybdenum content of the stainless steel increases the resistance to chloride attack. Treatment is based on passivation. The passivation process removes exogenous iron, restores a passive oxide layer that prevents further oxidation (rust) (61), and also cleans the parts of dirt, scale, or other welding-generated compounds.

CONCLUSION

This paper has presented the importance of surface finish and surface roughness in the context of specifying equipment for use in pharmaceuticals and healthcare, primarily from the perspective of lowering the likelihood of microbial attachment (whist acknowledging that microbial attachment contains its own set of variables). Literature indicates that specifying an*Ra* value of below 0.8 µm is a means to control this likelihood, with appreciable benefits seen down to 0.16 µm, after which anything smoother ceases to be significant). While Ra is a flawed measure, it remains the established measure of surface roughness and the easiest one for manufacturers to produce against. This paper has considered some other related variables like contact height and the shape of surface defects.

It is important that pharmaceutical specifications include a requirement for*Ra*, especially for surfaces being intended for use with product contact or water system surfaces. Determining stainless steel finish only is not sufficient because stainless steel surface finish notations represent the processing method rather than a direct consideration of roughness. Hence, the necessity of recommending a surface roughness of less 0.8µm when establishing one of the important hygiene criterion for a surface.

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