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Bacterial attachment on poly[acrylonitrile-*co*-(2-methyl-2-propene-1-sulfonic acid)] surfaces

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ABSTRACT

The influence of material properties on bacterial attachment to surfaces needs to be understood when applying polymer-based biomaterials. Positively charged materials can kill adhered bacteria when the charge density is sufficiently high [1] but such materials initially increase the adherence of some bacteria such as *Escherichia coli* [2]. On the other hand, negatively charged materials have been shown to inhibit initial bacterial adhesion [3], but this effect has only been demonstrated in relatively few biomaterial classes and needs to be evaluated using additional systems. Gradients in surface charge can impact bacterial adhesion and this was tested in our experimental setup.

Moreover, the evaluation of bacterial adhesion to biomaterials is required to assess their potential for biological applications. Here, we studied the bacterial adhesion of *E. coli* and *Bacillus subtilis* on the surfaces of acrylonitrile-based copolymer samples with different amounts of 2-methyl-2-propene-1-sulfonic acid sodium salt (NaMAS) comonomer. The content related to NaMAS based repeating units n_{NaMAS} varied in the range from 0.9 to 1.5 mol%.

We found a reduced colonized area of *E. coli* for NaMAS containing copolymers in comparison to pure PAN materials, whereby the bacterial colonization was similar for copolymers with different n_{NaMAS} amounts. A different adhesion behavior was obtained for the second tested organism *B. subtilis*, where the implementation of negative charges into PAN did not change the overall adhesion pattern. Furthermore, it was observed that *B. subtilis* adhesion was significantly increased on copolymer samples that exhibited a more irregular surface roughness.

INTRODUCTION

Bacterial attachment and subsequent biofilm formation on surfaces give rise for difficulties in the use of materials in biomedical applications. Biofilms are surface associated bacterial communities. On the surface of biomedical devices biofilms mean a risk for bacterial infections and they may further serve as a reservoir for plasmids carrying antibiotic-resistance genes.

Many attempts were made to prevent biofilm formation on biomedical materials. One starting point is the modification of existing materials by incorporation of antimicrobial substances, such as silver salts, chlorhexidine and antibacterial peptides, or antibiotics to kill bacteria on the surface of the material. On the other hand the prevention of biofilm formation

might be achieved by the development of materials that are inherently resistant to biofilm formation. The chemical composition of the material itself, but also its physical properties such as surface charges, hydrophilicity and surface roughness can influence the attachment of bacteria. Materials with charged surfaces may inhibit initial bacterial adhesion. In this study we used negatively charged copolymers (P(AN-*co*NaMAS)) prepared from acrylonitrile (AN) and sodium-2-methyl-2-propene-1-sulfonate (NaMAS). The standard production methods for commercially available AN-based copolymers are radical polymerizations in *N,N'*-dimethylformamide (DMF) or *N,N'*-dimethylsulfoxide (DMSO) as solvent. This makes the resulting material potentially toxic for cells as these solvent residues cannot be completely removed. Therefore, a water born suspension polymerization was applied for the synthesis of AN-based copolymers in which only water, the necessary monomers, and the water soluble initiators were present. The resulting materials were characterized by several physico-chemical methods to explore the impact of the processing on the material properties [4].

For the evaluation of the influence of material surfaces to bacterial adhesion we have developed an approach to study and quantify bacterial attachment to polymeric materials under standardized conditions.

EXPERIMENTAL DETAILS

Materials:

The water born synthesis and the characterization of a series of P(AN-*co*-NaMAS) was reported recently [4]. Four different polymers with a content related to the NaMAS based repeating units n_{NaMAS} of 0.0 mol% (PAN), 0.9 mol% (P(AN-*co*-NaMAS)09), 1.2 mol% (P(AN-*co*-NaMAS)12) and 1.5 mol% (P(AN-*co*-NaMAS)15) as determined by ^{1}H-NMR spectroscopy were obtained with number averaged molecular weights ranging from 34000 to 51000 g·mol^{-1} and a polydispersity around 5 (table I).

Table I: Composition, molecular weight, thermal properties and surface characteristics of P(AN-*co*-NaMAS) copolymers

Sample ID	n_{NaMAS} [a] [mol%]	M_n [b] [g·mol^{-1}]	T_g [c] [°C]	θ_{Adv} [d] [°]	R_q [e] [µm]
PAN	0.0	34000	97	57.7± 5.3	0.110 ± 0.014
P(AN-*co*-NaMAS)09	0.9	51000	99	61.2 ± 3.7	0.084 ± 0.007
P(AN-*co*-NaMAS)12	1.2	36000	105	63.6 ± 4.2	0.063 ± 0.042
P(AN-*co*-NaMAS)15	1.5	46000	105	57.4 ± 5.3	0.071 ± 0.014

a) Content related to the NaMAS based repeating units in P(AN-*co*-NaMAS) determined by ^{1}H-NMR spectroscopy and the last two-digit number was given accordingly; **b**) Number averaged molecular weight as obtained from gel permeation chromatography (GPC); **c**) Glass transition temperature (T_g) determined by DSC measurements; **d**) Advancing water contact angle achieved via contact angle measurements using captive bubble method; **e**) Surface roughness analysed by optical profilometry.

Test specimen with a diameter of 13 mm and a thickness of 1 mm were produced via a sintering method at temperatures in the range from 125 °C to 140 °C under a load of 2000 kg utilizing a custom made tool. The disc shaped samples were sterilized with ethylene oxide (45 °C, exposure time: 180 min) prior to biological tests or surface characterization.

As shown in table I, all copolymers exhibited similar thermal properties with a glass transition in the range of 100 to 104 °C, almost identical surface characteristics with advancing contact angles around 58 to 63° and a surface roughness of R_q = 0.06 – 0.08 µm as determined by optical profilometry [4].

Bacterial attachment to material surfaces:

As material properties cannot be possibly reflected and assessed by just one indicator organism, two model organisms known for their medical relevance and their biofilm forming capacity were chosen for the bacterial adhesion experiments. These selected bacterial strains with different Gram-characteristics are *E. coli* (K-12 W3110) and *Bacillus subtilis* (DSM 10). They were routinely grown on Luria-Bertani (LB) or 3-(N-morpholino)propane sulfonic acid (MOPS) minimal medium (modified) [6] agar plates or in broth with 130 rpm (both at 37 °C). MOPS minimal medium was used in biofilm experiments for standardizing the cultivation conditions. Bacterial adhesion was tested on ethylene oxide sterilized acrylonitrile-based copolymer test specimen submerged in glass flasks with diluted (OD 0.03) bacterial cell suspensions from overnight cultures. After an initial sedimentation/growth phase of 1 h without shaking the polymer samples were incubated at 37 °C with 60 rpm shaking for 4 h or 18 h. After inoculation the test samples were removed and incubated in phosphate buffered saline (PBS) for three minutes. Non-adhering bacteria were removed by multiple washing in PBS (room temperature, 8 times). Fixation of attached bacteria was done with 3 % para-formaldehyde (PFA) overnight at 4 °C. PFA was removed and the material samples were washed six times with PBS before storage in 50 % EtOH at 4 °C. The samples were dried for 10 minutes at 46 °C before dehydration in an ethanol series of 50 %, 80 % and 96 % (all steps of 3 min duration). After air drying, samples were examined using a Nikon N*i*-U Microscope with NIS-elements imaging software (Nikon, Japan). Bacterial attachment on all materials was tested in three or more replications for every material and both reference organisms. Surface colonization by attached bacteria was documented by incident light microscopy. A minimum of 30 pictures was taken for every tested material sample. Surface colonization was assessed on digitalized images and the area covered by bacteria on the surface quantified using ImageJ 1.47 software [7]. Statistical calculations were done with an R-script [8].

DISCUSSION

Bacterial attachment on the surfaces of biomedical materials is undesired. In our studies we analysed medical grade P(AN-*co*NaMAS)s in respect to bacterial adhesion. To assess the attachment of different bacterial species to the negatively charged P(AN-*co*NaMAS) test specimen and the uncharged pure PAN samples as reference the ratio of the surface covered by bacteria to the total sample surface was analysed.

The results of our experiments (table II) indicate that reference organisms *E. coli* and *B. subtilis* demonstrate a different adhesion behavior to the materials tested.

Table II: Mean area of P(AN-*co*-NaMAS) copolymers covered by bacteria

Sample ID	Mean area[a] [%] covered by	
	E. coli	***B. subtilis***
PAN	5.62 ± 0.32	2.44 ± 0.29
P(AN-*co*-NaMAS)09	1.82 ± 0.15	2.60 ± 0.62
P(AN-*co*-NaMAS)12	1.12 ± 0.16	4.33 ± 0.50
P(AN-*co*-NaMAS)15	1.27 ± 0.15	1.58 ± 0.26

a) Calculation of the ratio mean and standard deviation of the area proportion covered by bacteria based on the analysis of 30 up to 120 digitalized pictures of colonized surfaces.

Material surfaces of acrylonitrile-based copolymers show a reduced adhesion of *E. coli* cells in comparison to the reference (figure 1).

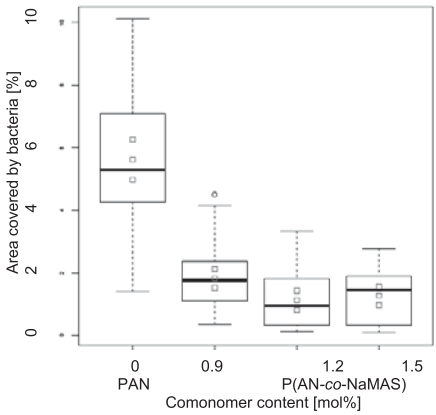

Figure 1: Colonization of *E. coli* on P(AN-*co*-NaMAS) copolymers.
Median (black bar), mean value and doubled standard-deviation (red squares), 25 % and 75 % percentiles (small edges of the rectangle), minimum and maximum values (thin black bars) and outlier (circle) for the calculated area covered by bacteria are displayed.

In comparison to the pure PAN materials the incorporation of 2-methy-2-propene-1-sulfonic acid sodium salt (NaMAS) seems to cause a reduction in the attachment of *E.* coli

whereby no statistically significant differences in bacterial colonization were recorded between copolymers with varying n_{NaMAS} amounts.

A different adhesion pattern has been observed for the second reference organism *B. subtilis* (figure 2). Bacterial adhesion to the copolymers with 0.9 and 1.5 % n_{NaMAS} did not differ from the cell attachment to the uncharged PAN.

On the contrary, spread of *B. subtilis* cells significantly increased on copolymer samples with 1.2 % n_{NaMAS}. These materials exhibited a significantly higher standard deviation of the surface roughness values (*Rq*) (table I). Therefore, we attributed the observed differences in the average surface area covered by the Gram-positive *B. subtilis* cells seem to correlate to the variability of surface roughness.

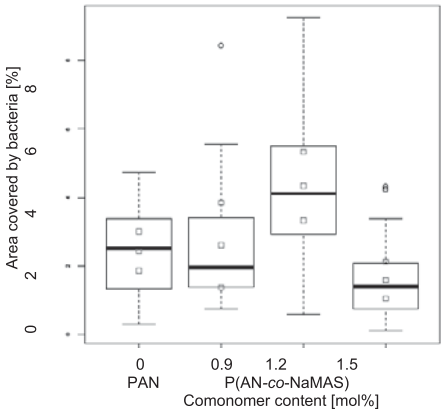

Figure 2: Colonization of *B. subtilis* on P(AN-*co*-NaMAS) copolymers.
Boxplot legend see Figure 1.

A different attachment behavior was observed for the two bacterial strains on the tested biomaterial samples. In comparison with the uncharged pure PAN reference material, an influence of negatively charged P(AN-*co*-NaMAS) copolymers on initial bacterial adhesion was only observed in tests with *E. coli* strain. The area colonized by *E. coli*, however, did not significantly change with increasing n_{NaMAS} content.

Varying levels of attachment were observed for the two tested bacterial species. This finding is not surprising because Gram-positive and Gram-negative bacteria possess different surface properties and macromolecular surfaces and therefore use diverse attachment mechanisms (e.g. surface proteins, flagella and exopolysaccharides).

B. subtilis attachment seems to be influenced by the interaction of two parameters: electrical charge and surface roughness. The influence of negative charge of the copolymers on bacterial attachment could not be revealed clearly. Further experiments shall clarify the influence of single parameters (e. g. surface roughness, wettability, electrical charge) on bacterial adhesion. It would be especially interesting to analyze the influence of the surface roughness on bacterial adhesion.

CONCLUSIONS

The chosen experimental setup with two different reference microorganisms enables a preliminary estimation of the tendency of materials to be colonized by microbial cells. Our first results suggest that negatively charged P(AN-*co*NaMAS) may inhibit initial bacterial adhesion. This further shows that tests with only one bacterial strain are not sufficient for a general assessment of material susceptibility to microbial colonization. Further studies with additional bacteria strains should be carried out to evaluate the first results. A set of reference microbial strains for the standardized investigation of diverse (bio)materials should be generated. This would enable the estimation of the influence of physical and chemical surface parameters on bacterial colonization. Results of such microbiologically standardized tests could support the development of new materials for biomedical applications.

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