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EDTA AS A POTENTIAL AGENT PREVENTING FORMATION OF *STAPHYLOCOCCUS EPIDERMIDIS* BIOFILM ON POLICHLORIDE VINYL BIOMATERIALS

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Abstract: Polichloride vinyl (PCV) is a widely used thermoplastic polymer, also in the production of medical devices. In the present study we assess the influence of EDTA *in vitro* on the biofilm structure formed by *Staphylococcus epidermidis* isolates on PCV biomaterials (Nelaton and Thorax catheters). The 6 strains of *S. epidermidis* were isolated from nasopharynx of hospitalised patients. It was found that all isolates were able to form the biofilm on both PCV biomaterials, irrespective of adhesion properties (cell surface properties, ability to slime production, minimal time needed for adhesion). The EDTA showed bacteriostatic effect against planktonic cells of the isolates (MIC = 0.25–0.5 mmol/l; MBC = 10.0–>25.0 mmol/l; MBC/MIC = 20, 30, 40, >50). The adhesion process and also formation of the biofilm was inhibited by EDTA at concentrations 1.0–2.0 mmol/l (2–8 × MIC). The eradication of the mature biofilm was achieved at 2.0–4.0 mmol/l EDTA (4–8 × MIC) for two strains, while for the other four isolates, concentration of EDTA needed for eradication effect was >32 mmol/l (> 128 × MIC). Data obtained in this paper suggest that EDTA may be regarded as a useful agent preventing formation of the *S. epidermidis* biofilm on PCV biomaterials.

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INTRODUCTION

Polichloride vinyl (PCV) is a widely used thermoplastic polymer – a most valuable product of the chemical industry. It is also one of the polymers frequently used in the production of medical devices [9].

Coagulase-negative staphylococci are the part of the natural microflora of the human body. The most wide-spread species of coagulase-negative staphylococci is *Staphylococcus epidermidis*, colonizing skin and mucosa membranes of the nose and throat [28]. This species is also one of the most important etiologic agents of hospital infections associated with the biofilm formation on different indwelling medical devices [7, 28].

Biofilm is the microbial lifestyle in natural and manmade environments; it may form on a wide variety of surfaces, including living tissues, indwelling medical devices, dental water unit systems, industrial or potable water system piping, or natural aquatic systems [6, 13, 21, 23, 24, 25]. According to the definition "this is a structure, an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material" [6]. In the process of biofilm formation, three general steps are involved: 1. adhesion process – bacterial cells attachment to the surface, while they "search" for a place to settle; 2. formation of bacterial microcolonies in the growing biofilm; 3. slime production – covering of the structure by exopolysaccharide matrix [6, 27].

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Biofilms have great importance for public health because of their role in certain infectious diseases (e.g. native valve endocarditis, otitis media, periodontitis or chronic prostatitis) and importance in a variety of device-related infections [6]. The pathogenicity of biofilm includes the following mechanisms: detachment of cells or biofilm aggregates, exchange of plasmids responsible for drug resistance within biofilm cells, or resistance to the host immune system [6]. Besides, treatment of biofilm-associated infections is difficult due to the inherent resistance of microbial cells embedded in the biofilm to standard antimicrobial therapy in contrast to planktonic (free-floating) cells [4, 7]. The antimicrobial resistance is a result of a number factors: 1. binding of the agent; 2. lack of penetration of inhibitors; 3. localization of neutralizing enzymes; 4. low growth rate of the microbes; 5. expression of a resistant phenotype due to surface growth [25].

A wide range of chemical compounds are used in order to remove biofilm structure, e.g. antibiotics, disinfectants or antiseptics and other non-antibiotic agents; their effectiveness depends on the properties of the biofilm-forming bacteria [22, 26]. Some of these compounds, e.g. hydrogen peroxide, sodium hydrochloride or alkaline peroxide, seem to be the most effective in the complete eradication of biofilm in dental unit water systems [22, 26]. Medical devices treatment with antimicrobial agents and antimicrobial locks are some of the methods in the destruction of mature biofilm or prevention of its formation [6].

One of the compounds regarded as a potential agent against biofilm formation is EDTA (ethylenediaminetetraacetic acid) – a widely known metal chelating agent; it forms complexes with Mn^{2+}, Cu^{2+}, Fe^{3+}, Co^{3+}, Ca^{2+} or Mg^{2+} [11]. This compound has been found to inhibit growth of some bacterial species, most probably due to its complexing properties [3, 11].

The aim of this study was to assess the effect of EDTA *in vitro* on adhesion, formation and eradication of *S. epidermidis* biofilm on the surface of biomaterials made from PCV.

MATERIALS AND METHODS

Bacterial strains. The 6 strains of *S. epidermidis* isolated from nasopharynx of hospitalised patients were included in the present study. The specimens (throat and nose swabs) were inoculated onto Chapman agar and agar with 5% of sheep blood and incubated for 48 h for the 35–37°C. The isolated staphylococcal strains were identified using coagulase-test tube and API STAPH microtests.

Determination of slime production. *S. epidermidis* isolates were inoculated onto nutrient agar plates containing sucrose and 8% of Congo red, and then cultured for 24 h at 35–37°C. Slime production was assessed on the basis of the colour of staphylococcal colonies according to the criteria presented by Freeman *et al.* [1], as follows: black colonies with metalic sheen – strains intensively producing slime, dark-pink colonies – strains moderately producing slime, light-pink colonies – strains no-producing slime.

Determination of cell surface properties. The relative cell surface hydrophobicity of the *S. epidermidis* isolates was determined by using modified ammonium salt aggregation test (SAT) [5, 12]. It was assumed that strains autoaggregated were described as very strong hydrophobic, aggregated at 0.4–1.0 M $(NH_4)_2SO_4$ – as strong hydrophobic, at 1.2–1.6 M $(NH_4)_2SO_4$ – as hydrophobic, at > 1.8 M $(NH_4)_2SO_4$ – as hydrophilic.

Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of EDTA. The MIC of EDTA for *S. epidermidis* isolates was performed by the broth dilution method, using dilutions of EDTA in TSB (trypticasein soy broth). Final concentrations of EDTA ranged from 0.001–25.0 mmol/l. Inoculum of bacterial strains was 5×10^5 CFU/ml. After incubation at 35°C for 24 h, the MICs were assessed visually as the lowest EDTA concentration showing complete bacterial growth inhibition. In order to determine the MBC of EDTA for *S. epidermidis* isolates, 10 µl of the staphylococcal culture from each tube that showed thorough growth inhibition, from the last positive one and from the growth control was streaked onto TSA (trypticasein soy agar). After incubation at 35°C for 24 h, the MBCs were assessed visually as the lowest EDTA concentrations at which there was no bacterial growth.

Biomaterials. All assays were carried out on two catheters made from polichloride vinyl (PCV) – Nelaton and Thorax catheters. The biomaterials were cut aseptically into ca. 0.5 cm^2 fragments and placed into Petri dishes.

Determination of adhesion to the biomaterials *in vitro*. The ability of adhesion of the *S. epidermidis* isolates on both catheters was studied *in vitro* by TTC (2, 3, 5 – triphenyltetrazolium chloride) method, based on the ability of living cells to reduce tetrazolium salt to red formazan precipitates [8]. The standarized bacterial suspensions (0.5 according to McFarland standard) in sterile PBS (phosphate-buffered saline) were incubated with appropriate biomaterial for 1, 2, 3, 4, 5, 6, 12 and 24 h at 35–37°C. Nonadherent cells were removed by careful rinsing of catheter fragment with sterile PBS and then resuspended in TSB medium with one drop of 1% TTC, followed by overnight incubation at 35–37°C. The minimal time needed for adhesion process was determined visually by an appearance of red formazan precipitates, both on the catheter surface and in the medium.

Determination of biofilm formation on the biomaterials *in vitro*. The ability of formation of biofilm by the *S. epidermidis* isolates on both catheters was studied *in*

vitro also by TTC method [8]. The standarized bacterial suspensions (0.5 according to McFarland standard) in TSB were incubated with appropriate biomaterial for 24 h at 35–37°C. Nonadherent cells were removed by careful rinsing of catheter fragment with sterile PBS and then resuspended in fresh TSB. Medium changing and catheter washing procedures after overnight incubation at 35–37°C were repeated three times. Finally, one drop of 1% TTC solution was added, followed by overnight incubation at 35–37°C. Biofilm formation was determined visually on the basis of an appearance of red formazan precipitates both on the catheter surface and in the medium.

The effect of EDTA on adhesion and biofilm formation on the biomaterials *in vitro*. This assay was also based on the TTC method. In each experiment, the following concentrations of EDTA were used: 0.5; 1.0; 2.0; 4.0; 8.0; 16.0; 32.0; 64.0; 128 × MIC (0.125–32.0 mmol/l). (I) In order to assay the effect of EDTA on adhesion, the standarized bacterial suspensions (0.5 according to McFarland standard) in sterile PBS containing EDTA were incubated with appropriate biomaterial for 2–24 hours (according to the minimal time needed for adhesion of each strain to biomaterials) at 35–37°C. Then, a drop of 1% TTC solution was added, followed by overnight incubation at 35–37°C. The minimal concentration of EDTA inhibited adhesion process was determined visually as the concentration where the red formazan precipitates were not found, neither on the catheter surface nor in the medium. (II) In order to assay the effect of EDTA on biofilm formation, the bacterial suspensions in TSB containing EDTA were incubated with appropriate biomaterial for 72 h at 35–37°C, with medium changing and catheter washing process as described above. Then, a drop of 1% TTC solution was added, followed by overnight incubation at 35–37°C. The minimal concentration of EDTA inhibited biofilm formation was determined visually as the concentration where the red formazan precipitates were not found, neither on the catheter surface nor in the medium. (III) In order to assay the effect of EDTA on biofilm eradication, the mature 72 h biofilms were incubated in the presence of EDTA for 24 h and then a drop of 1% TTC solution was added, followed by overnight incubation at 35–37°C. The minimal concentration of EDTA eradicated the mature biofilm, determined visually as the concentration at which the red formazan precipitates were not found, neither on the catheter surface nor in the medium.

Table 1. Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and MBC/MIC ratio of EDTA for nasopharyngeal isolates *Staphylococcus epidermidis*.

Strain	MIC (mmol/l)	MBC (mmol/l)	MBC/MIC ratio
A	0.5	>25.0	>50
B	0.5	>25.0	>50
C	0.5	>25.0	>50
D	0.5	10.0	20
E	0.5	15.0	30
F	0.25	10.0	40

Reproducibility of the results. All results were carried out in triplicate. Representative data are presented.

RESULTS

All isolates of *S. epidermidis* possessed hydrophilic cell surface, assessed by SAT test, because the isolates did not aggregate even at 1.8 M $(NH_4)_2SO_4$. The 5 strains showed a very strong ability to slime production, forming black colonies with a metalic sheen on Congo red agar, while one strain did not express the slime production, forming pink colonies. The isolates showed differential ability to adhere to the surface of the PCV catheters, as monitored by minimal time needed for adhesion. On the surface of Nelaton's catheter, the three isolates were adhered after 12 h and other three – after 24 h. Different results were obtained for Thorax catheter: one strain was able to adhere after 2 h, another – after 12 h, and other four – after 24 h. Despite these differences, all above strains showed the ability to biofilm formation on the surface of both catheters, as monitored by an appearance of red tetrazolium formazan precipitates on the catheter surface and also in the medium as the result of cutting off the planktonic cells from the biofilm structure.

Table 2. The influence of EDTA *in vitro* on adhesion, biofilm formation and eradication of the mature structure formed by nasopharyngeal *Staphylococcus epidermidis* isolates on the surface of Nelaton catheters.

Type of process	The concentrations of EDTA (mmol/l) The concentrations as multiplies of MIC values (× MIC)					
	A	B	C	D	E	F
I[a]	1.0 2 × MIC	1.0 2 × MIC	1.0 2 × MIC	1.0 2 × MIC	1.0 2 × MIC	2.0 8 × MIC
II[b]	2.0 4 × MIC	2.0 4 × MIC	2.0 4 × MIC	2.0 4 × MIC	1.0 2 × MIC	2.0 8 × MIC
III[c]	>32 >64 × MIC	4.0 8 × MIC	2.0 4 × MIC	>32 >64 × MIC	>32 >64 × MIC	>32 >128 × MIC

[a] the influence of EDTA on adhesion process; [b] the influence of EDTA on biofilm formation; [c] the influence of EDTA on disruption of mature biofilm

Table 3. The influence of EDTA *in vitro* on adhesion, biofilm formation and eradication of the mature structure formed by nasopharyngeal *Staphylococcus epidermidis* isolates on the surface of Thorax catheters.

Type of process	The concentrations of EDTA (mmol/l) The concentrations as multiplies of MIC values (× MIC)					
	A	B	C	D	E	F
I[a]	2.0 4 × MIC	2.0 4 × MIC	1.0 2 × MIC	1.0 2 × MIC	1.0 2 × MIC	2.0 8 × MIC
II[b]	2.0 4 × MIC	2.0 4 × MIC	2.0 4 × MIC	2.0 4 × MIC	1.0 2 × MIC	2.0 8 × MIC
III[c]	>32 >64 × MIC	2.0 4 × MIC	4.0 8 × MIC	>32 >64 × MIC	>32 >64 × MIC	>32 >128 × MIC

[a] the influence of EDTA on adhesion process; [b] the influence of EDTA on biofilm formation; [c] the influence of EDTA on disruption of mature biofilm

The planktonic cells of the *S. epidermidis* isolates were sensitive to EDTA with the following MIC's values – 0.5 mmol/l for five strains and 0.25 mmol/l for one isolate. The MBC's values for EDTA were estimated as following: 10 mmol/l (two strains), 15 mmol/l (one strain), and >25 mmol/l (3 strains) (Tab. 1). The high values of MBC/MIC ratio indicated that EDTA behaved as a bacteriostatic agent against the isolates of *S. epidermidis*.

EDTA inhibited adhesion and biofilm formation by the *S. epidermidis* isolates on both biomaterials at concentrations of 1.0–2.0 mmol/l (2-8 × MIC), as monitored by the lack of the reduction of TTC. The eradication by EDTA of the mature staphylococcal biofilm formed on the biomaterials was achieved by 2.0–4.0 mmol/l EDTA (4–8 × MIC) only for two strains, while for the other four isolates, EDTA had no effect, even at 32 mmol/l (64 or 128 × MIC), as also monitored by the lack of reduction of TTC (Tab. 2 and Tab. 3).

DISSCUSION

The biofilm is the main form of microbial life in nature. It is constructed of microorganisms adhering to the surface and coated with slime – the exopolysaccharide matrix, protecting the microbial cells against unfavourable environmental factors [4, 7]. Moreover, biofilm is one of the most important factors responsible for the pathogenicity of bacterial strains, e.g. coagulase-negative staphylococci [4, 28]. It can be the source of general infections as the result of cutting off microorganisms from the structure and transferring via the bloodstream to other body sites [4, 7].

It is known that the formation of the biofilm is determined by several factors related both to microbial cells (e.g. cell surface properties, slime production) or biomaterial structure (e.g. chemical composition, roughness). Our data suggest that the slime did not perturb adhesion of *S. epidermidis* isolates to PCV biomaterials, since there was no correlation between the extent of slime production and time needed for adhesion. However, the literature data suggest that the slime may mask some bacterial molecules important for the attachment of *S. epidermidis* strains to the surfaces [2]. Although both catheters used in our study were made from the same polymer – PCV, differential time needed for adhesion of *S. epidermidis* isolates was noted, even in case of strains showing similar adhesion properties, most probably due to differences in the structure those two catheters.

Several studies were undertaken to manage with microbial biofilm on the biomaterials, including the incorporation of antibiotic or non-antibiotic agents (e.g. usnic acid, surfactin, epigallocatechin-gallate, ovotransferin, protamine sulfate) into biomaterials [15, 29]. The impregnation of catheters by antibiotics seems to be an inappropriate way of prevention of biofilm formation, since in contrast to non-antibiotic agents, it can lead to an increase of bacterial resistance to antimicrobial drugs [15]. In this paper, we assessed the influence of EDTA *in vitro* on formation and eradication of *S. epidermidis* biofilm formed on the PCV biomaterials (Nelaton and Thorax catheters).

EDTA, a widely known chelating agent with anticoagulant activity is used for some medical purposes, such as the treatment of hypercalcemia [10, 15]. It is also used in dentistry to remove inorganic debris of the root canal and prepare it for obturation [14, 20]. Only some literature data suggest that EDTA possesses potential activity against microbial biofilm [3, 10, 11, 15, 19]. Moreover, the literature data indicate that the inhibitory effect of EDTA against staphylococcal biofilm was increased in combination with minocycline, ovotransferin and protamine sulfate [16, 17, 18, 29]. Our results showed that the adhesion and formation of the *S. epidermidis* biofilm on the PCV Nelaton and Thorax catheters was inhibited by EDTA at low concentrations (between 1–2 mmol/l). In contrast, the eradication of mature *S. epidermidis* biofilm required, in most cases, much higher concentrations of this agent (>32 mmol/l); only in the case of two strains was the biofilm disrupted in low concentrations of EDTA – 2–4 mmol/l. Our data indicate also that there was no correlation between the ability of slime production by staphylococcal strains and the concentrations of EDTA needed for inhibition of adhesion or biofilm formation and eradication of the mature structure. This observation may suggest that slime is not the effective agent protecting staphylococcal cell against EDTA.

Root *et al.* [19] studied the effect of EDTA *in vitro* on the eradication of biofilm formed by *S. epidermidis* on a

Hickman catheter made from silicone. According to their data, high concentration of EDTA (108 mmol/l) was required to eradicate the staphylococcal biofilm. However, other authors have shown that concentrations of EDTA, higher than 2 mmol/l expressed a toxic effect *in vitro* on the viability of cell cultures [3].

The mechanism most possibly responsible for the inhibitory effect of EDTA on staphylococcal biofilm formation is the chelation of metal ions. It is well-known that the PIA (Polysaccharide Intracellular Antygen) in *S. epidermidis* is a necessary factor involved in the first step of biofilm formation. The appropriate cell surface properties determined by the presence of PIA antigen depends on the level of Mg^{2+} ions [28]. Therefore, the decreased level of Mg^{2+} ions is due to chelation by EDTA, and may inhibit the adhesion procces and, as a result, also the formation of biofilm [15].

CONCLUSIONS

Our data indicate that EDTA may be regarded as a useful agent in the prevention of staphylococcal biofilm formation on PCV medical devices, but not for the disruption of mature biofilm, due to its cytotoxicity at higher concentrations.

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