Colloids and Surfaces B: Biointerfaces 126 (2015) 98–105

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Mechanistic approaches on the antibacterial activity of poly(acrylic acid) copolymers

Günther Gratzl[a,b,*], Sarah Walkner[c], Sabine Hild[d], Achim Walter Hassel[c,e], Hedda Katrin Weber[b], Christian Paulik[a]

[a] Johannes Kepler University Linz, Institute for Chemical Technology of Organic Materials, Altenberger Str. 69, 4040 Linz, Austria
[b] Kompetenzzentrum Holz GmbH, Altenberger Str, 69, 4040 Linz, Austria
[c] Competence Centre for Electrochemical Surface Technology, Viktor Kaplan Str. 2, 2700 Wiener Neustadt, Austria
[d] Johannes Kepler University Linz, Institute of Polymer Science, Altenberger Str. 69, 4040 Linz, Austria
[e] Johannes Kepler University Linz, Institute for Chemical Technology of Inorganic Materials, Altenberger Str. 69, 4040 Linz, Austria

ARTICLE INFO

Article history:
Received 7 July 2014
Received in revised form 3 November 2014
Accepted 8 December 2014
Available online 15 December 2014

Keywords:
Poly(acrylic acid)
Antibacterial
Antimicrobial
Bactericidal
Block copolymer
Mechanism

ABSTRACT

The availability of polymeric antimicrobially active surfaces, which are mainly based on cationic surface effects, is limited. We have previously reported the discovery that, in addition to cationic surfaces, anionic surfaces based on poly(acrylic acid) (PAA) copolymers have a bactericidal effect. In this study, poly(styrene)-poly(acrylic acid)-diblock copolymers (PS-b-PAA) are used to describe the major variables causing the material to have a bactericidal effect on *Escherichia coli* ATCC 25922 in aqueous suspensions. Upon contact with water, the surface structure of the copolymer changes, the pH value decreases, and the PAA-block migrates toward the surface. Systematically modified antimicrobial tests show that the presence of acid-form PAA provides maximum antimicrobial activity of the material in slightly acidic conditions, and that an ion-exchange effect is the most probable mechanism. Antimicrobially inactive counter-ions inhibit the bactericidal activity of the copolymers, but the material can be regenerated by treatment with acids.

1. Introduction

The number of publications describing antibiotic-resistant germs has recently increased rapidly [1–3]. Su et al. [4] reported that 98.4% of *Escherichia coli* (*E. coli*) isolates from municipal wastewater treatment plants were resistant against at least one antibiotic agent tested, and 90.6% of these isolates were resistant against at least three antibiotic agents. Since such tendencies are very common for many bacteria, alternatives to antibiotics with nonspecific killing mechanisms are in the focus of current research [5–8].

Leaching acids, such as acetic acid and fatty acids, are examples of currently used alternatives whose antimicrobial activity has been known for a long time [9,10]. It was shown that both acid concentration and pH value can be the cause of the bactericidal effect of acids [10,11]. Some anionic agents, such as fatty acids and anionic peptides, are expected to act very similar to cationic peptides, by first interacting with the membrane phospholipids and then inserting a non-polar side chain into the cell membrane with final lysis of the membrane [12,13]. In other cases, such as acetic acid or sorbic acid, the acidic agent was shown to interact with, but not insert and lyse, a membrane system [14]. It is believed that protonated forms of these agents and other short-chain organic acids can somehow cross the cell membrane and acidify the cytoplasmic pH level [15,16]. However, the ability of acids to cross membranes freely has been questioned, and researchers now expect membrane interactions with the acidic agents [14,17,18]. Interactions of negatively charged acidic groups with negatively charged membrane components are expected to result from the formation of salt bridges between the acids with divalent counter ions, such as Mg^{2+} and Ca^{2+}, which balance the membrane charge on the surface of bacteria [12]. Multivalent carboxylic acids, such as ethylenediaminetetraacetic acid (EDTA) [19,20] and citric acid [21], are also expected to interact with these membrane-charge-balancing divalent counter ions by forming chelates, which destabilizes the membrane and can cause cell death [15].

Another aspect of the antimicrobial effect of acidic agents is that they can reduce the pH value, which interferes with the pH

* Corresponding author at: Johannes Kepler University Linz, Institute for Chemical Technology of Organic Materials, Altenberger Str. 69, 4040 Linz, Austria.
Tel.: +43 732 2468 5498; fax: +43 732 2468 8816.
E-mail address: guenther.gratzl@jku.at (G. Gratzl).

http://dx.doi.org/10.1016/j.colsurfb.2014.12.016
0927-7765/

homeostasis of bacteria and can cause cell death after prolonged exposure [22]. Low external pH values (<3.5) result in a reduction of the intracellular pH value to <4.5, where essential proteins denature, the DNA depurinates and cell membranes start to lyse [23]. However, *E. coli* in particular has developed various mechanisms to resist acidic conditions for some time, which can be activated by slow reduction of the pH value [23], but all bacteria that usually live in moderate environments die after prolonged exposure to low pH values.

Leaching acidic agents such as acetic acid, lactic acid, EDTA and sorbic acid are commercially used as additives for polymeric food-packing materials [24]. Unlike leaching acids, covalently bonded acids such as acrylic acid in poly(styrene)-poly(acrylic acid)-diblock copolymers (PS-b-PAA) are well investigated in terms of their adhesion-reducing effects on germs [25–27], while bactericidal effects of PAA-containing copolymers [28–30] remain poorly studied [31–33]. It only was shown that the PAA-content correlates with the antimicrobial activity of the material [28,29] while the nanostructure, the copolymer partner of PAA and the chain length of the copolymers is of minor importance [28].

In this study we describe the properties of the material (PS-b-PAA) and the germs and present which factors (pH, electrolytes, salts, changes in the polymer structure) influence the materials antimicrobial activity. All tests were performed on *E. coli*, a gram-negative bacterium which occurs widely in nature and often used for mechanistic studies [6,34,35].

2. Experimental

2.1. Material

In this study water-sensitive reagents were stored in a glove box with controlled nitrogen atmosphere (MBraun MB 150 BGII, H_2O < 1 ppm, O_2 < 1 ppm). The polymers produced were treated exclusively with 18 MΩ water (Thermo TKA-LAB-HP).

2.1.1. Synthesis of poly(styrene)-poly(acrylic acid)-diblock copolymers

Diblock copolymers were produced as explained earlier [28]. In brief, polymers were prepared by anionic polymerization of styrene and *tert*-butyl acrylate, obtaining poly(styrene)-poly(*tert*-butyl acrylate) block copolymers (PS-b-PtBA). Subsequent acidic hydrolysis reaction with *p*-toluenesulfonic acid in 1,4-dioxane yielded PS-b-PAA.

2.1.2. Sample preparation

A solution of about 10 wt% was prepared by dissolving the PS-b-PAA copolymer in 1,4-dioxane under stirring at 50 °C. When the polymer was dissolved, the solution was cast by placing 1 mL of the solution dropwise on a cover slip. Overnight the solvent evaporated and the sample was vacuum-dried. The final layer had about 100 mg on an 18 mm × 18 mm glass-surface.

2.1.3. Counter-ion loading and regeneration

For several tests, the counter-ions NH_4^+, Ag^+ and Mg^{2+} were loaded on PS-b-PAA copolymers. Ammonium was loaded by placing the cover slip with the cast polymer for 1 h directly in 2 mL of an ammonium hydroxide solution (30–33%, diluted to 0.01 mol L^{-1}, Riedel-de-Haën). The polymer was then washed with water (18 MΩ) and vacuum-dried. For silver and magnesium loading, the ammonium-loaded polymer was used to exchange cations from a silver nitrate solution (volumetric solution, 0.028 mol L^{-1}, Riedel-de-Haën) or a prepared solution of magnesium chloride hexahydrate (99%, Acros Organics), each for 1 h. Regeneration was achieved with an ammonium-loaded polymer surface and a prepared solution of citric acid (99%, dissolved to a solution of 10 wt%, Sigma Aldrich) under shaking for 1 h. The polymer surfaces were then washed with water (18 MΩ), vacuum-dried and used for antimicrobial testing.

2.1.4. Nuclear magnetic resonance spectroscopy

PS-b-PtBA copolymers were analyzed by nuclear magnetic resonance (NMR) spectroscopy (Bruker digital Avance III 300 MHz NMR-spectrometer) to determine PS-b-PAA contents as described in [28]. $CDCl_3$ (≥99.8%, for NMR spectroscopy, Merck) was used as a solvent and internal reference.

2.1.5. Size exclusion chromatography

The molecular weight was determined by size exclusion chromatography (SEC) of PS-b-PtBA copolymers in THF. The polymers were separated with Phenomenex Phenogel columns and detected with a Bischoff RI-detector 8110 and a Wyatt MiniDawn light scattering detector at a flow rate of 0.350 mL min^{-1}. Polystyrene standards were used for calibration, and the molecular weights were determined by means of the Zimm plot method.

2.2. Surface–water interactions

2.2.1. Atomic force microscopy

For atomic force microscopy (AFM) an Asylum Research MFP 3D SA AFM with Olympus cantilever (OMCL-AC240TS, resonance frequency ~70 kHz, spring constant ~1.7 N m^{-1}, tip radius <10 nm) was used. The measurements in air were performed in AC mode with a 2 V free amplitude and a set point of about 60–80%. The measurement in water was carried out with a 1 V free amplitude and a set point of about 60–80%. The resonance frequency in water was about 30 kHz.

2.2.2. Contact-angle determination

The contact angles were determined with a Dataphysics Contact Angle System OCA 20. At least 10 contact-angle values with 10 μL deionized water after 10 s attachment time were used for calculations.

2.2.3. Solubility test

To determine whether the polymer is soluble in water, 100 μL of water were placed on the cast copolymer surface for 6 h. Subsequently, 10 μL were transferred to a cover slip and stored until the water had vaporized. The residual polymer film on the cover slip was analyzed with AFM. A blind control was performed on PS, where no film residue was detectable.

2.2.4. Electrochemical pH changes

The change in pH value over a given time period was measured using the pH microelectrode presented in [36,37]. The microelectrode was calibrated with diluted sulphuric acid solutions in the relevant pH range, and pH values were determined with a standard pH glass electrode calibrated with standard buffer solutions. The test was performed by placing 100 μL of commercially available spring water on the polymer sample and transferring 10 μL of the drop to carrier after 1 h, 2 h and 3 h. By immersing the pH μ-electrode into the drop on the carrier, the open circuit potential (OCP), and thus the pH value of the solutions was measured.

2.3. Antimicrobial tests

For antimicrobial tests, petri dishes with a Luria/Miller medium (25 g L^{-1} LB medium, for microbiology, Roth) and agar agar (10 g L^{-1}, for bacteriology, Roth) were prepared.

E. coli ATCC 25922 were stored at −60 °C; before use, the germs were cultured in LB medium over night and sub-cultured on an LB-agar medium at 37 °C. Bacterial suspensions were prepared by