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Pluronic–lysozyme conjugates as anti-adhesive and antibacterial bifunctional polymers for surface coating

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ABSTRACT

This paper describes the preparation and characterization of polymer–protein conjugates composed of a synthetic triblock copolymer with a central polypropylene oxide (PPO) block and two terminal polyethylene oxide (PEO) segments, Pluronic F-127, and the antibacterial enzyme lysozyme attached to the telechelic groups of the PEO chains. Covalent conjugation of lysozyme proceeded via reductive amination of aldehyde functionalized PEO blocks (CHO-Pluronic) and the amine groups of the lysine residues in the protein. SDS-PAGE gel electrophoresis together with MALDI-TOF mass spectrometry analysis revealed formation of conjugates of one or two lysozyme molecules per Pluronic polymer chain. The conjugated lysozyme showed antibacterial activity towards *Bacillus subtilis*. Analysis with a quartz crystal microbalance with dissipation revealed that Pluronic–lysozyme conjugates adsorb in a brush conformation on a hydrophobic gold-coated quartz surface. X-ray photoelectron spectroscopy indicated surface coverage of 32% by lysozyme when adsorbed from a mixture of unconjugated Pluronic and Pluronic–lysozyme conjugate (ratio 99:1) and of 47% after adsorption of 100% Pluronic–lysozyme conjugates. Thus, bifunctional brushes were created, possessing both anti-adhesive activity due to the polymer brush, combined with the antibacterial activity of lysozyme. The coating having a lower degree of lysozyme coverage proved to be more bactericidal.

1. Introduction

Biomaterials in the human body are prone to bacterial infections, which may lead to the formation of a biofilm. Biofilm formation is preceded by protein adsorption, deposition of cells and bacteria. The complex structure of a biofilm, containing slime and extracellular polymeric matrix, makes it resistant to the host immune system as well as to antibiotic treatment [1]. Infection of implanted biomaterials usually requires secondary surgery [2,3]. Various biomaterials surface modifications have been developed to improve their antibacterial properties of implant surfaces, such as applying bactericidal agents [4], a hydrogel coating releasing bioactive antibodies [5], nitric oxide releasing substrates [6], a coating with furanones [7], chalcones [8], or various polymers [9]. Especially the later polymer brush coatings, have been proven in the past to reduce bacterial adhesion by one or two orders of magnitude [10–12], which makes them a promising tool for biomedical applications. A polymer brush is formed when highly soluble polymer chains are grafted to the surface at high packing density, forcing the polymer chains to extend into the surrounding aqueous medium. Thus, a highly hydrated polymer layer is formed at the surface, which acts as a barrier preventing deposition of particles, including bacteria [10]. Polymer chains can be grafted to the surface by simple physi-sorption, or by covalent bond formation. Chemical attachment makes the brush more stable but it is a complex and time-consuming procedure [13]. The grafting density plays a crucial role in the conformation of the adsorbed polymer layer. At low grafting densities, the polymer chains are coiled resulting in a so-called mushroom conformation of the adsorbed polymer molecule. At higher grafting densities, when the separation between the anchoring points is less than the hydrodynamic radius of the polymer coils, the polymer chains are forced to stretch into the surrounding medium forming a brush conformation [14]. In aqueous media, polyethylene oxide (PEO) is most often used as the soluble polymer. Ethylene oxide (EO) moieties

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in the PEO chain have a good structural fit with water molecules enabling a strong hydrogen bond between the ether oxygen of PEO and the hydrogen of water. Thus the PEO brush coating is highly hydrated. Compression of the PEO chains in the brush increases the osmotic pressure along with a reduction of their conformational entropy. This creates a strong repulsive force against deposition of indwelling particles, including bacteria [13,15,16].

The aim of this study is to design a bifunctional polymer brush coating by conjugation of an antibacterial compound with the polymer molecules so that the brush attains bi-functionality i.e., resistance to particle deposition and selective lethal interaction with microorganisms. For our study, we chose Pluronic F-127 as the PEO-containing polymer. Pluronics are a family of synthetic non-toxic neutral triblock copolymers made up of a central hydrophobic polypropylene oxide (PPO) block that is connected to two hydrophilic PEO side blocks, PEO_n–PPO_m–PEO_n. In aqueous medium this triblock copolymer self-assembles into micelles. After exposure to a hydrophobic surface, the micelles disaggregate in favour of adsorption. It is inferred that the hydrophobic PPO block attaches spontaneously to the surface, whereas the two PEO segments extend into the water phase [17,18]. Lysozyme was chosen as the antimicrobial agent because of its well known bactericidal properties [19,20], physiological abundance, high thermal stability with respect to structure [21], wide pH activity range [22], and well known structure [23,24]. Lysozyme is able to destruct bacterial cell walls by an enzymatic hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues of peptidoglycan in the bacterial cell wall, especially for Gram-positive bacteria [20]. Broadly directed activity makes lysozyme an important antimicrobial agent that can be used to prevent biomaterial associated infections by a wide variety of bacterial strains [25]. The use of polymer–protein conjugates is relatively new in biomedical research and has potential applications in drug delivery systems [26–28], and as novel biocompatible materials for e.g., implants and tissue engineering [29–32]. We used the protein–polymer conjugation approach [26] to synthesize lysozyme functionalized Pluronic molecules.

For lysozyme to exert enzymatic activity, it is desired, if not required, that it is exposed to the solution rather than being adsorbed to the surface. If the conjugate would adsorb with the lysozyme attached to the surface, the lysozyme would barely be accessible for the bacteria due to shielding by the polymer. Moreover, it has been reported that after adsorption on hydrophobic surfaces lysozyme looses its antimicrobial activity [33]. This implies that the conjugate should adsorb in a similar conformation as the unmodified Pluronic, i.e., via attachment of its PPO block, as shown in Fig. 1. Surface coatings consisting of Pluronic–lysozyme conjugates were characterized in terms of thickness, viscoelastic properties, surface composition and their anti-adhesive and antibacterial properties.

2. Materials and methods

2.1. Aldehyde-end functionalization of Pluronic F-127

5 M excess of Dess-Martin periodinane (1,1,1-tris(acetyloxy)-1,1-dihydro-1,2-benziodoxol-3(1H)-one, 97%, Sigma Aldrich, Germany) was added to a solution of 200 mg of Pluronic F-127 (PEO_{99}–PPO_{65}–PEO_{99}), MW = 12.6 kDa (BASF, Sigma Aldrich, Germany) in 20 ml of dry dichloromethane (Sigma Aldrich, Germany) at room temperature and stirred overnight. Thereafter, the reaction mixture was treated with cold diethyl ether ~30 ml (Merck, Germany). The precipitated product was cooled on an ice-water bath for 1 h, filtered off, washed with cold diethyl ether (2 × 20 ml) and dried under vacuum. The product was characterized by proton NMR in order to confirm conversion of the PEO hydroxy end-groups into aldehyde functionalities and the degree of conversion was determined using the Purpald colorimetric assay [34]. First, a calibration curve was recorded, in order to convert measured UV–VIS absorbance into number of aldehyde groups, using formaldehyde (37 wt% in H_2O, Sigma Aldrich, Germany), as described elsewhere [34]. Aldehyde functionalized Pluronic was dissolved in ultrapure water in a known range of concentrations. Next, 100 µl of polymer solution was added to 100 µl of 30 mM Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole, Sigma Aldrich, Germany) solution in 2 M NaOH. After 15 min equilibration, 100 µl of a 30 mM sodium periodate ($NaIO_4$, Sigma Aldrich, Germany) solution in 0.2 M NaOH was added and oxidation

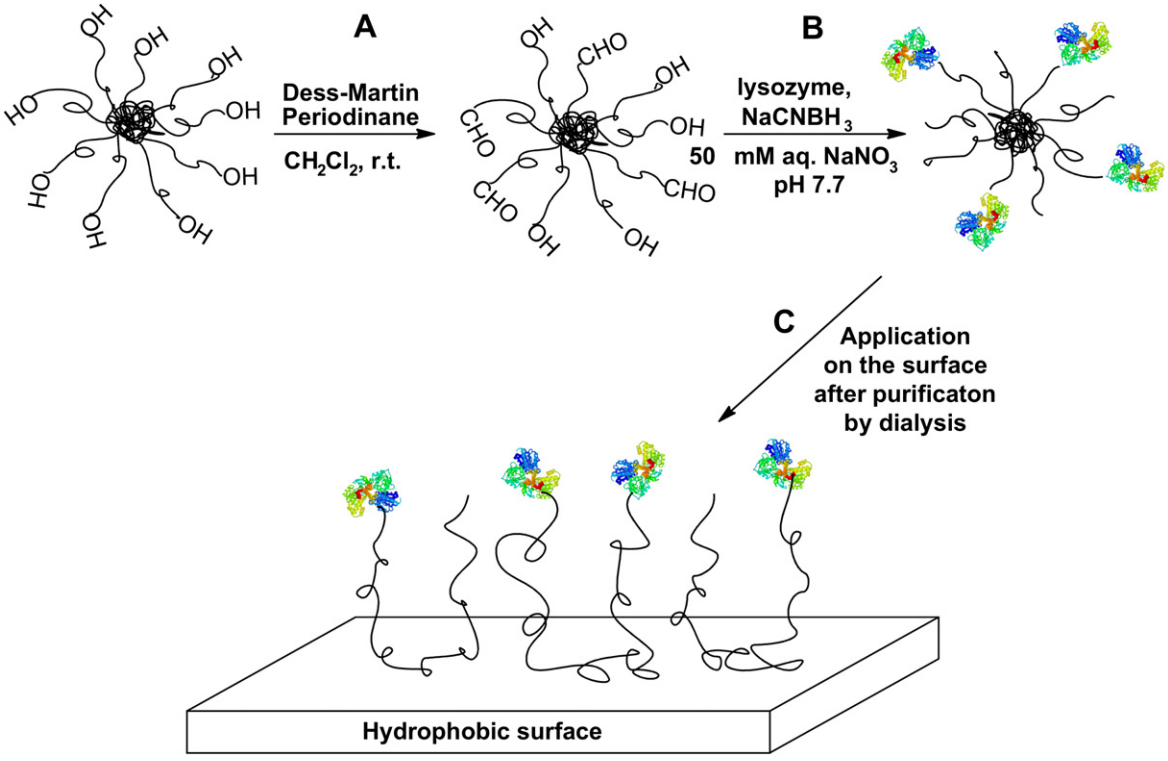

Fig. 1. Reaction scheme for the oxidation of Pluronic (A) and conjugation with lysozyme (B) forming micelles in aqueous medium, and adsorption on a hydrophobic surface into the brush conformation (C).