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Delivery of cyclodextrin polymers to bacterial biofilms — An exploratory study using rhodamine labelled cyclodextrins and multiphoton microscopy

Hanna Thomsen[a,c], Gábor Benkovics[d,e], Éva Fenyvesi[d], Anne Farewell[b,c], Milo Malanga[d,*], Marica B. Ericson[a,*]

[a] Biomedical Photonics Group, Department of Chemistry and Molecular Biology, University of Gothenburg, Sweden
[b] Microbiology Division, Department of Chemistry and Molecular Biology, University of Gothenburg, Sweden
[c] CARe, Centre for Antibiotic Resistance Research, University of Gothenburg, Sweden
[d] CycloLab Ltd. Budapest, Hungary
[e] Department of Organic Chemistry, Faculty of Science, Charles University in Prague, Czech Republic

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ABSTRACT

Cyclodextrin (CD) polymers are interesting nanoparticulate systems for pharmaceutical delivery; however, knowledge regarding their applications towards delivery into complex microbial biofilm structures is so far limited. The challenge is to demonstrate penetration and transport through the biofilm and its exopolysaccharide matrix. The ideal functionalization for penetration into mature biofilms is unexplored. In this paper, we present a novel set of rhodamine labelled βCD-polymers, with different charge moieties, *i.e.*, neutral, anionic, and cationic, and explore their potential delivery into mature *Staphylococcus epidermidis* biofilms using multiphoton laser scanning microscopy (MPM). The *S. epidermidis* biofilms, being a medically relevant model organism, were stained with SYTO9. By using MPM, three-dimensional imaging and spectral investigation of the distribution of the βCD-polymers could be obtained. It was found that the cationic βCD-polymers showed significantly higher integration into the biofilms, compared to neutral and anionic functionalized βCDs. None of the carriers presented any inherent toxicity to the biofilms, meaning that the addition of rhodamine moiety does not affect the inertness of the delivery system. Taken together, this study demonstrates a novel approach by which delivery of fluorescently labelled CD nanoparticles to bacterial biofilms can be explored using MPM. Future studies should be undertaken investigating the potential in using cationic functionalization of CD based delivery systems for targeting anti-microbial effects in biofilms.

1. Introduction

Bacterial biofilms exist in aggregate formats on the surfaces of, *e.g.*, wounds, catheters, medical implants, and throughout many aspects of nature (Donlan, 2002). Covered in a protective exopolysaccharide (EPS) matrix, biofilms are able to survive harsh environmental conditions. Heterogeneity within the biofilm allows for flow of nutrients while protecting dormant "persister" cells. Penetration of antibiotics is hampered by the EPS matrix and may not reach deeper layers of the biofilm. Despite eradication of the top-most planktonic individual bacteria (Davies, 2003), the biofilm can remain adhered and continue growth through planktonic dispersal and signaling for matrix growth (Lewis, 2001). Novel antimicrobial drug delivery systems have been introduced (Smith, 2005), comprising, *e.g.*, liposomes (Jones et al., 1997; Kim et al., 1999) or polymer nanoparticle drug carrier systems (Cheow et al., 2010; Forier et al., 2014), but penetration of external compounds through the biofilm and EPS matrix is complex. Thus in order to develop efficient drug delivery strategies to target microbial biofilms, new mechanistic approaches are required.

Cyclodextrins (CDs) have been introduced for improving drug delivery in many applications (Uekama et al., 1998). For example, encapsulation can enhance penetration of drugs (Loftsson and Duchene, 2007; Loftsson et al., 2007), enable solubility of poorly soluble pharmaceutical compounds and improve stability in

* Corresponding authors.
E-mail addresses: malanga@cyclolab.hu (M. Malanga), marica.ericson@chem.gu.se (M.B. Ericson).

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biological media (Uekama et al., 2006). Native CDs and their semi-synthetic derivatives, such as 2-hydroxypropyl-βCD or sulfobutylether-βCD are approved excipients in many drug formulations (Loftsson and Brewster, 2012). Positively charged CD derivatives, such as quaternary ammonium βCD, are primarily used as drug penetration enhancers (Kis et al., 2003). Water soluble crosslinked CD polymers (CDp) are also interesting for drug delivery (Renard et al., 1997). CDs used for encapsulation of bactericidal compounds applied to microbial biofilms have improved antibacterial effectivity in recent reports (Hegge et al., 2012), and further exploration of how CD based delivery systems can be adopted to target complex microbial biofilms structures should be undertaken.

Visualization of microbial biofilms has so far been limited to conventional optical microscopy methods (Hannig et al., 2010; Jefferson et al., 2005; Palmer and Sternberg, 1999). Large heterogeneous micro-colonies can grow up to 100 μm in depth, presenting an optically complex structure making optical microscopy challenging. Confocal laser scanning microscopy has been used to acquire 3D information of biofilms (Jefferson et al., 2005; Palmer and Sternberg, 1999), but light scattering and photobleaching limits the applicability. Instead, multiphoton laser scanning fluorescence microscopy (MPM) shows advantages compared to confocal microscopy for 3D visualization of optically dense matter (Centonze and White, 1998; Helmchen and Denk, 2005). MPM is most often based on two-photon excitation (2PE) (Denk et al., 1990; Helmchen and Denk, 2005; Zipfel et al., 2003), by which two photons are absorbed simultaneously to produce molecular excitation. The energy of the absorbed photons are approximately half that of the energy of photons absorbed in a one-photon process, thus enabling excitation with near infrared (NIR) wavelength region. This highly unlikely process results in inherent confocality and less photodamage to biological samples. MPM has been successfully adopted for studies of biological tissues (Bender et al., 2008; Mank et al., 2008; Simonsson et al., 2011; Wang et al., 2010), but is yet unexplored for antimicrobial research.

Nanoparticulate systems have been explored for delivery to biofilms (Li et al., 2014; Nevius et al., 2012; Peulen and Wilkinson, 2011; Wang et al., 2016); however, it is unclear what parameters are beneficial for uptake in the biofilm. Here visualization techniques such as MPM can be useful. To trace the nanoparticles using MPM; fluorescence labelling is required. Rhodamine B (RB) is a xanthene dye, commonly used as fluorescence tracker due to its high quantum yield and large extinction coefficient. Conjugation of RB onto CD scaffolds generates a conjugate that preserves the spectroscopic properties of the free dye and shows enhanced water solubility (Malanga et al., 2012, 2016). Covalently linked RB-CD conjugates have a wide range of applications, from solid surfaces (Nishimura et al., 2008) through nanoparticles (Diaz-Moscoso et al., 2010) to live cells. For applications where constant, pH-independent fluorescence reporting is required, RB–CD conjugation through thioureido bond formation is preferred. Commercially available RB isothiocyanate (RBITC) can be coupled to 6-monodeoxy-6-monoamino-βCD scaffolds (Malanga et al., 2012). Not only the native βCD but also randomly methylated, 2-hydroxypropylated (Malanga et al., 2012), randomly phosphorylated βCD (Agostoni et al., 2015) and βCDp (Malanga et al., 2014) can be tagged by RBITC. The properties can be further tuned by incorporating charged moieties making RB-labelled CDp interesting compounds for exploring distribution in biological systems.

Herein, we present an approach by which the delivery of RB labelled CDp to bacterial biofilms is explored using MPM. The impact of charged moieties was investigated. RB labelled βCDp with neutral charge RBβCDp(n), anionic sulfobutylether-βCD polymer, SBE-RBβCDp(a), or cationic quaternary ammonium-βCDp, QA-RBβCDp(c), were prepared. These different charged RBβCDp, were applied to mature *Staphylococcus epidermidis* biofilms, being a medically relevant Gram-positive bacterium known to form biofilms on medical devices and prosthetic implants (O'Gara and Humphreys, 2001). Spatially resolved 3D visualization and spectral analysis were applied to study penetration and localization of the RBβCDp within these microbial communities.

2. Results and discussion

2.1. Synthesis and characterization of rhodamine labelled βCD polymers

A set of RB labelled βCD polymers were prepared based on the post-branching functionalization of RBβCDp(n). Two novel epichlorohydrin-branched polymers bearing simultaneously a fluorophore and multiple charges, QA-RBβCDp(c) and SBE-RBβCDp(a), were prepared. Table 1 shows the properties of all three βCDp compounds. The properties are similar, apart from the functionalization groups providing the charge. Interestingly, the measured average size was found larger for the charged particles. The determined MW of 50 kDa, should account for a nanoparticulate carrier system corresponding to approximately 5 nm, as is the case for the RBβCDp(n), but the introduction of charged moieties increases the electrostatic repulsion and thereby the measured size in the DLS measurements.

The synthetic route for obtaining the three different polymers is schematically illustrated in Fig. 1. The starting point for all compounds was a one-step copolymerization reaction between unlabelled βCD and RBITC labelled βCD adapted from a previously described procedure (Malanga et al., 2014). This allowed incorporation of RBITC label into the CD polymer in 0.05% (w/w) as determined by UV–vis titration, to obtain neutral RBβCDp(n). The βCD content of the polymer (around 70% w/w) was determined by ^{1}H NMR spectroscopy (Renard et al., 1997), the average molecular weight (~50 kDa) was measured by static-light scattering as previously described (Puskás et al., 2013). Negatively charged sulfobutylated CDp, *i.e.*, anionic SBE-RBβCDp(a), was prepared by reacting RBβCDp(n) with 1,4-butane sultone in basic aqueous solution. Positively charged quaternary ammonium CDp, *i.e.*, cationic QA-RBβCDp(c), was obtained by using glycidyltrimethylammonium chloride as alkylating agent in alkaline conditions and extensively dialyzed by using a low cut-off (100–500 Da) dialysis

Table 1
Properties of RBITC labelled CDp prepared for this study.

	Charge (mV)	Group	DS[a]	MW (kDa)	Average size (nm)	RBITC labelling (w/w)
RBβCDp(n)	Neutral 0 ± 3	–	–	50	5.3	0.05%
SBE-RBβCDp(a)	Anionic −19 ± 2	sulfoalkyl	~2	50	11.4	0.05%
QA-RBβCDp(c)	Cationic +9 ± 2	quaternary ammonium	~1	50	15.7	0.05%

[a] DS – Degree of Substitution.