Overview of results for 2010-2011

1 Introduction

The diagnosis of bacterial infections remains a major challenge in medicine. Although numerous contrast agents have been developed to image bacteria, their clinical impact has been minimal because they are unable to detect small numbers of bacteria in vivo, and cannot distinguish infections from other pathologies such as cancer and inflammation. In this report, we present a new family of contrast agents, termed maltodextrin based imaging probes (MDPs), which can detect bacteria in vivo with a sensitivity two order of magnitude higher than previously reported, and, for the first time, detect bacteria via a bacteria-specific mechanism that is independent of host response and secondary pathologies. MDPs are composed of a fluorescent dye conjugated to maltohexaose, and are rapidly internalized through the bacterial-specific maltodextrin transport pathway, endowing the MDPs with a unique combination of high specificity and sensitivity for bacteria. Here, we show that MDPs selectively accumulate within bacteria at millimolar concentrations, and are a thousand-fold more specific for bacteria than mammalian cells. In addition, we demonstrate that MDPs can image as few as \(10^5\) CFUs in vivo and can discriminate between active bacteria and inflammation induced by either LPS or metabolically inactive bacteria (Xing et al (1)).

Bacterial infections cause significant mortality and morbidity in the world despite the availability of antibiotics. For example, in the United States in 2010, bacterial infections caused 40,000 deaths from sepsis alone and were also the leading cause of limb amputations. A major limitation preventing the effective treatment of bacterial infections is an inability to image them in vivo with accuracy and sensitivity. Consequently, bacterial infections can only be diagnosed after they have become systemic or have caused significant anatomical tissue damage, and at this stage are challenging to treat due to the high bacterial burden. There is therefore a great need for the development of contrast agents that can image small numbers of bacteria accurately in vivo.

2.1 Results

2.2 Synthesis of maltodextrin based imaging probes MDP-1 and MDP-2

We have synthesized perlyene-labeled maltohexaose (MDP-1) and IR786-labeled maltohexaose (MDP-2) to investigate maltohexaose mediated fluorophore internalization by bacteria. MDP-1 and MDP-2 were synthesized according to the scheme shown in Figure 1, by conjugating alkyne functionalized fluorescent dyes onto azide functionalized maltohexaose \(5\), via the click reaction. The azide functionalized

![Figure 1. Synthesis of MDP-1 and MDP-2](image-url)
Maltotetraose was synthesized in 4 steps from maltotetraose. This synthetic strategy introduces the imaging probes on the reducing of maltotetraose, and was selected because MD transporters recognize carbohydrate substrates from the non-reducing end, and tolerate large structural modifications at the reducing end of maltodextrins.

The details of the synthesis of MDP-2 via the click reaction between 5 and IR786 alkyne thioether are briefly described below. The compounds 1 and 3 (1:2 molar ratio) were dissolved in DMF, to which was added Cul and DIPEA. The mixture was stirred at rt for 24 h under nitrogen and the solvent was removed in vacuo. The residue was dissolved in CH2Cl2 and washed twice with water and once with brine. The organic phase was dried over Na2SO4, filtered and evaporated to dryness in vacuo. The residue was purified by flash column chromatography on silica gel (CH2Cl2/CH3OH, 15/1) to afford the acetyl protected intermediate 3 in a 73% yield. This intermediate was deprotected in a mixture of CH3OH and aqueous LiOH for 24 h under nitrogen. The crude MDP-2 was isolated by neutralizing the deprotection solution with Dowex 50W resin, filtering, and concentrating in vacuo, to obtain a residue. MDP-2 was purified by flash column chromatography on silica gel (CH2Cl2/CH3OH/H2O, 5/5/2). MDP-2: HRMS (MALDI) m/z Found: 1724.7398, calculated: 1724.7373 for C80H118N5O34S+ [M]+. MDP-1 was synthesized via a similar strategy. MDP-1: HRMS (MALDI) m/z Found: 1416.4868, calculated: 1416.4852 for C63H83N3NaO32 [M+Na]+.

MDP-2 and MDP-1 were also characterized by H-NMR, and C13-NMR, these data are omitted because of space limitations (see reference 1).

**Figure 2.** MDPs are robustly internalized by bacteria and have a thousand-fold selectivity for bacteria over mammalian cells.

a, Gram negative and gram positive bacteria robustly internalize MDP-1. *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), *Bacillus subtilis* (BS) and *Staphylococcus aureus* (SA) were incubated with 20 µM MDP-1 for 1 hour and the intracellular MDP-1 concentration was determined by perylene fluorescence. MDP-1 is robustly internalized by EC, PA, SA and BS. Results are expressed as mean millimolar concentration per CFU ± standard error of the mean (s.e.m.), for n = 6 per group.

b, MDP-1 has high specificity for bacteria over mammalian cells. Bacterial strains and mammalian cells (rat aortic smooth muscle cells (RASMs), macrophages (MAs) and fibroblasts (FBs)) were incubated with 20 µM MDP-1 for 1 hour. The intracellular MDP-1 concentration was determined by perylene fluorescence and normalized to protein content. Bacteria transport MDP-1 at a rate three orders of magnitude faster than mammalian cells. The results are expressed as mean micromoles per gram of protein ± s.e.m. for n = 6 per group.

**D2.3 MDPs are robustly internalized by bacteria and have high specificity for bacteria**

We have been able to demonstrate that maltotetraose conjugated dyes are robustly internalized by a variety of bacteria, including *P. aeruginosa*, and have very low accumulation within mammalian cells. Bacteria were incubated with 20 µM MDP-1 for one hour, washed with PBS, lysed, and the cellular supernatant was analyzed by fluorometry. Figure 2a demonstrates that MDPs can deliver large quantities of conjugated molecules to bacteria. For example, *Escherichia coli* internalized MDP-1 at a rate sufficient to generate millimolar intracellular concentrations, and pathogenic bacteria such as *P. aeruginosa, Staphylococcus aureus* and *Bacillus subtilis* also robustly internalized MDP-1. To our knowledge, this represents the first demonstration of a targeting strategy that can deliver millimolar concentrations of small molecules to bacteria.
MDPs also have the potential to deliver compounds to bacteria with high specificity because mammalian cells do not express maltodextrin transporters (1). We therefore investigated the specificity of MDP uptake by bacteria. The uptake of MDPs in bacteria and mammalian cells was determined and compared. Bacteria (*E. coli, P. aeruginosa, S. aureus and B. subtilis*) and mammalian cells (rat aortic smooth muscle cells, macrophages and fibroblasts) were incubated with a 20 µM concentration of MDPs for one hour, washed with PBS, lysed, and the cellular supernatant was analyzed for perylene or IR786 fluorescence. Figure 3b demonstrates that MDP-1 has high specificity for bacteria. For example, both gram-positive and gram-negative bacteria internalized MDP-1 at a rate three orders of magnitude faster than mammalian cells. In particular, pathogenic bacteria such as *P. aeruginosa* and *S. aureus* internalized 200-300 µmol of MDP-1 per milligram of protein, whereas rat aortic smooth muscle cells and fibroblasts internalized undetectable levels of MDP-1. Additionally, MDP-2 had a similarly high level of specificity for bacteria over mammalian cells (data not shown). Thus MDPs have a 1000-fold selectivity for bacteria over mammalian cells, and should therefore be able to target bacteria *in vivo* with high specificity.

**D2.4 Maltodextrin conjugates have the potential to be internalized by a wide variety of bacteria**

A key issue with using the maltodextrin transporter for targeting bacteria is their expression profile in bacteria. We therefore performed a protein sequence homology search for the presence of classical maltodextrin transporters, such as LamB and/or MalE in common pathogens. We identified maltodextrin transporters or highly homologous maltose transport systems in *Citrobacter koseri, Enterobacter sp., E. coli species, Klebsiella pneumoniae, Salmonella species, Shigella flexneri, Shigella sonnei, Vibrio vulnificus, and Vibrio cholerae*. The MD uptake pathway was identified in all but 5 species investigated, namely *P. aeruginosa*, *Neisseria gonorrhoeae*, *Acinetobacter baumannii*, *Campylobacter jejuni*, *Haemophilus ducreyi* and *Stenotrophomonas maltophilia*. However, we anticipate that these bacteria may also internalize maltodextrins via non-classical maltodextrin transport systems, and have already demonstrated that *P. aeruginosa* robustly internalizes MDP-1. In addition we investigated if *Neisseria meningitidis*, a gram negative pathogen that does not express classical maltodextrin transporters, internalizes MDP-1 via flow cytometry, following the procedures described above. Figure 3 demonstrates that *Neisseria meningitidis* robustly internalized MDP-1, and internalizes it at a rate similar to that of *E. coli*, supporting the notion that a wide range of bacteria can potentially internalize maltodextrin based imaging agents.

**D2.5 MDP-2 can image bacteria in vivo**
Based on the *in vitro* results described above we hypothesized that the MDPs have the potential to target bacteria *in vivo*. Accordingly, we investigated the ability of MDP-2 to image and target bacterial infections in rats. 

**E. coli** (10⁷ CFUs) were injected into the left thigh muscle of rats, and the right thigh muscle was injected with saline as a control. After 1 hour, MDP-2 (280 - 350 μL of 1 mM MDP-2 in PBS) was injected into the rats via the jugular vein, and the rats were imaged after 16 hours in an IVIS Lumina imaging machine. The rat image is a representative result of six experiments, and identifies the infection site.

**MDP-2** is efficiently cleared from un-infected tissues. *Escherichia coli* (10⁷ CFUs) were injected into the left thigh muscle of rats, and the right thigh muscle was injected with saline as a control. After 1 hour, MDP-2 (280 - 350 μL of 1 mM MDP-2 in PBS) was injected into the rats via the jugular vein. The rats were sacrificed after 16 hours and the biodistribution of MDP-2 was determined by measuring IR786 fluorescence. MDP-2 is cleared from all the major organs and selectively accumulates in infected muscle tissue. Data are plotted as mean fluorescent units (FUs) per gram of tissue ± s.e.m. (n = 6 rats per group).

**Figure 4. MDP-2 can image bacterial infections in vivo.**

**a**, MDP-2 can image 10⁷ *Escherichia coli* CFUs *in vivo*. *Escherichia coli* (10⁷ CFUs) were injected into the left thigh muscle of rats, and the right thigh muscle was injected with saline as a control. After 1 hour, MDP-2 (280 - 350 μL of 1 mM MDP-2 in PBS) was injected into the rats via the jugular vein, and the rats were imaged after 16 hours in an IVIS Lumina imaging machine. The rat image is a representative result of six experiments, and identifies the infection site.

**b**, MDP-2 is efficiently cleared from un-infected tissues. *Escherichia coli* (10⁷ CFUs) were injected into the left thigh muscle of rats, and the right thigh muscle was injected with saline as a control. After 1 hour, MDP-2 (280 - 350 μL of 1 mM MDP-2 in PBS) was injected into the rats via the jugular vein. The rats were sacrificed after 16 hours and the biodistribution of MDP-2 was determined by measuring IR786 fluorescence. MDP-2 is cleared from all the major organs and selectively accumulates in infected muscle tissue. Data are plotted as mean fluorescent units (FUs) per gram of tissue ± s.e.m. (n = 6 rats per group).

*D2.6 Conclusions*

There is a great need for the development of contrast agents that can image bacterial infections with high sensitivity and specificity. In this report we demonstrate that MDPs have a unique combination of robust transport and high specificity, and are able to detect as few as 10⁵ CFUs *in vivo* with high specificity. MDPs have tremendous potential for improving the diagnosis of bacterial infections given their ability to accurately detect small numbers of bacteria *in vivo*.

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