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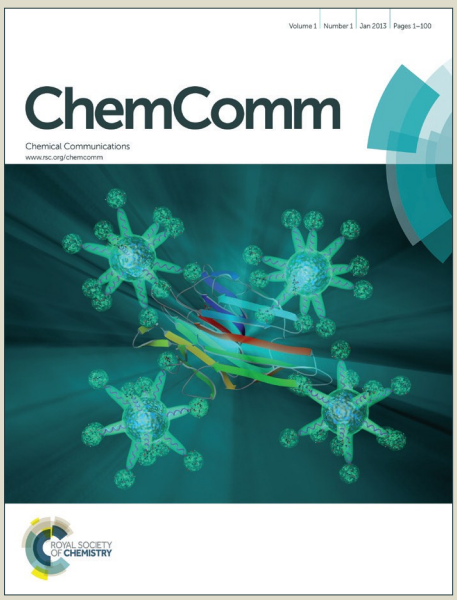

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Antibiotic loaded nanocapsules functionalized with aptamer gates for targeted destruction of pathogens

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In this study, we designed aptamer-gated nanocapsules for the specific targeting of cargo to bacteria with controlled release of antibiotics based on aptamer-receptor interactions. Aptamer-gates provided a specific decrease in minimum inhibitory concentration (MIC) values of vancomycin for *Staphylococcus aureus* when mesoporous silica nanoparticles (MSNs) were used for bacteria-targeted delivery.

Microbial infections are leading cause of mortality worldwide despite the antibiotics that are highly effective in inhibiting them. The major reasons for clinical failure of antibiotic therapy are associated with low penetration efficiency to infection sites, the side effects of antibiotics, as well as the evolution of antibiotic resistance in bacteria.[1] According to a recent World Health Organization (WHO) survey, very high rates of resistance was determined among common-health associated infections worldwide.[2] Several microorganisms evolved clinically significant resistance against almost every available antibiotic. Yet the development of new classes of antibiotics has lagged far behind our growing need. Therefore, developing new and multidimensional strategies to combat microbial infections is warranted.[3] Here, we aim to contribute to these strategies by developing a novel tool in targeted antibiotic delivery to solve the problem of antibiotic resistance due to sublethal doses.[4]

Various types of nanoparticles have been employed in the delivery of antibiotics by non-targeted or targeted strategies.[5] Antibiotics encapsulated in nanoparticles have shown great potential in replacing the administration of antibiotics in free form.[6] In encapsulation formats, inorganic nanocontainers provide protection to antibiotics against physical deactivation and improves antibiotic pharmacokinetics and biodistribution. Studies showed that liposome carriers enhanced *Staphylococcus* inhibition of vancomycin.[7] In addition, nanoparticles can be modified to target or respond to a particular bacteria, and thereby facilitate the selective concentration or release of the antibiotic at infection sites. Thus, the delivery of antibiotics with nanoparticles augments the level of the bioactive drug at the site of infection while reducing the dosage and the dosing frequency. The end results are improved therapeutic effects as well as decreased drug side effects in patients. Surface-charge-switching polymeric nanoparticles were successfully used as vancomycin carrier to the cell walls of bacteria which decrease the pH around the infected tissues.[8] A core-shell supramolecular gelatin nanoparticles loaded with vancomycin was released only at infection sites after degradation by gelatinase which is over-expressed in the infected areas.[9] In a similar approach, lipase-secreting bacteria was targeted by vancomycin-loaded and lipase sensitive hydrogel. However, the main obstacle in translation of targeted antimicrobial treatment in clinical application remained as the lack of highly selective and robust targeting molecules.[10]

Aptamers are functional oligonucleotides frequently employed as specific targeting agents for drug delivery and biosensor development.[11] The novel concept of gatekeeping has been introduced and applied to the design of a variety of stimuli-responsive nanodevices.[12] Mesoporous silica nanoparticles (MSNs) have been demonstrated to be a convenient nanocarrier for stimuli-responsive and controlled drug delivery with many types of applications[13-15] as well as a variety of sensor applications.[16-18] Aptamer-gated mesoporous silica nanocapsules have been proposed to have potential in overcoming major challenges associated with nanocapsules.[19, 20] Nanovalves were developed to respond to redox, pH, temperature, light for opening/closing the pores.[21] Model drug cargo molecules were delivered specifically to breast cancer cells. Due to dual function

of the aptamer gate as targeting and controlling the release rate, the cargo molecules were release over a designed time interval.[22, 23] The aptamer-gate mechanism was designed by using nucleolin binding aptamer (AS1411) which are over-expressed on the cell surface of cancer cells.

To obtain *S. aureus* specific molecular gate, the aptamer sequences were converted to hairpin structure. Among three potential aptamer sequences as reported by Cao *et al.*,[24] the specific aptamer sequence of this study was experimentally determined from the response of switch probe designs. The interaction of *S. aureus* surface antigens with the aptamer sequence disrupts the hairpin structure and leads to rearrangement of the structure, separating quencher and fluorophore from each other. This leads to an increase in fluorescence that depends on the presence of the target (Fig. S1). Among the three probes, SA20hp responded with highest fluorescence peak in the presence of 10^4 *S. aureus* cells. Based on these results, we have selected SA20hp as the molecular gate aptamer in the rest of the study. The response of SA20hp probe was also challenged with *S. epidermidis* and only a slight response was obtained at about 82 % lower than that of *S. aureus* cells. In order to verify how far aptamer gate molecules would block and retain a cargo molecule inside the nanoparticles, we have used MCM-41 type silica particles. The pores (average diameter 2.7 nm) were modified with a sulfo-linker for allowing subsequent amine coupling as the aptamer had been functionalized with an amine group (aptamer-NH_2) at the 5´- end in order to facilitate the coupling to the nanoparticle surface as previously described.[22] Fig. 1 is a schematic depiction of strategy as used in this study. The antibiotic vancomycin was absorbed into the pores of mesoporous silica nanoparticles and capped with SA20hp gatekeeper molecule. When the nanoparticles bind to their target molecules on the surface of *S. aureus* cells via gatekeeper aptamer sequences, the cargo antibiotic is released to kill bacteria. Otherwise, vancomycin is entrapped in the pores and thus inactive. The mechanism of gatekeeper function of aptamer hairpins were previously explained. [25, 26]

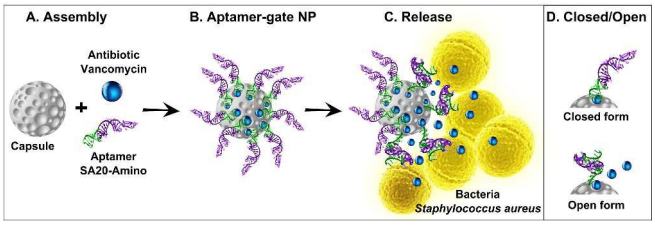

Fig. 1 The general scheme of aptamer-gated specific antibiotic delivery approach.

Synthesis and characterization of the final nanocapsules are provided in Fig. S2. The particles were 177.5 nm of diameter on the average and the hexagonal pore structure of 2.7 nm were confirmed with TEM analysis. The concentration of encapsulated vancomycin was calculated to be 15.5 pmol/mg by absorbance measurements at 280 nm, following our capping procedure through immobilization of 11.8 ± 1.3 pmol aptamers per mg nanoparticles.

The encapsulation ability and release kinetics of aptamer-capped nanoparticles were investigated by monitoring vancomycin release by UV absorbance and Maldi-MS analysis. The nanoparticles were loaded with vancomycin and thereafter blocked with the aptamer hairpin structures. Subsequently, they were incubated in serum samples to mimic endogenous background, and serum samples spiked with *Staphylococcus* cells. These vancomycin-loaded and aptamer-capped nanoparticles were characterized to confirm almost leakage-free stability and release only by specific actuation of aptamer-ligand interaction. The leakage was about 5 % ± 0.2 (n=3) at the end of 24 hours incubation (Fig. S3, red line). Next, the release of entrapped vancomycin was triggered by addition of 10^4 cells of bacteria *S. aureus*. This concentration was chosen in order to warrant binding and opening of the majority of hairpin aptamer molecules resulting in the release of vancomycin. Indeed, the addition of *S. aureus* cells increased the release dramatically, as anticipated (Fig. S3, black line). The cumulative amount of vancomycin increased up to 5 hours and levelled off afterwards, which corresponded to about 12.1 ± 0.2 of encapsulated vancomycin molecules in one mg of nanoparticles as determined by UV absorbance analysis at the end of 24 hours. This proved that the aptamer nanovalves were generally functional in the sense that the hairpin aptamer blocked the pores prior to addition of cells, whereas the presence of *S. aureus* cells triggered their opening and hence release of vancomycin. The half-life of *S. aureus*-triggered release was estimated to be 45 min. A control experiment with the same nanoparticles, but *S. epidermidis* cells addition was performed for 24 hours. The vancomycin release was significantly lower than release by *S. aureus* at about 15% (Fig. S3, blue line). Goodness of fit in different release kinetic models (Power Law, Zero-order and Higuchi´s equations) was evaluated in order to understand the mechanism of drug release (Table S2). The *in vitro* release mechanism in aptamer-capped nanoparticles was therefore identified to be a Fickian diffusion mechanism. The n values were less than 0.5 for all the batches indicating that the release rates exhibit a combined mechanism of diffusion partially through a swollen matrix and partially through water-filled pores.[27]

The detection and quantification of vancomycin during a release determination study can be easily followed by simple UV spectrophotometry absorbance due to peptide bonds.[28] However, a more detailed analysis of vancomycin is not possible with UV method in the presence of other peptide bond-containing molecules. For example, blood serum contains many protein components. In such circumstances, a sensitive and accurate method is required for further characterization of release kinetics. Matrix-assisted laser desorption /ionization mass spectroscopy (MALDI-ToF-MS) is an established technique for protein identification and characterization due to its sensitivity, accuracy, reproducibility and robustness.[29] The method is simple in concept and design with limit of detection at femtomol levels.[30] The release kinetics of vancomycin upon 10^4 *Staphylococcus aureus* addition was also confirmed by a qualitative MALDI-ToF-MS analysis which is more reliable and sensitive compared to UV absorbance method.[29] Fig. 2A shows protonated ion peal

signal/noise ratio of vancomycin in a typical vancomycin release experiment from aptamer-gated nanoparticles. The UV methods revealed the release of the most of encapsulated vancomycin molecules in the first 5 hours (Fig. 2, right panel). The vancomycin release behavior was similar in both Maldi-MS analysis and UV method.

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial agent inhibiting the growth of a microorganism after overnight incubation. The therapeutic efficacy of aptamer-gated vancomycin nanoparticles was subsequently evaluated in two strains of *Staphylococcus* cultures. Mesoporous nanoparticles were loaded with vancomycin (a common antibiotic for treating *S. aureus* infections) and capped with *S. aureus* binding aptamer hairpin. During 24h, the susceptibility of two cultures was monitored, namely that of *S. aureus* which is a genus of *Staphylococcus* that has specific affinity to the aptamer sequence of this study and that of *S. epidermidis* which is another strain of *Staphylococcus* that has not any affinity to the aptamer sequence.[24]

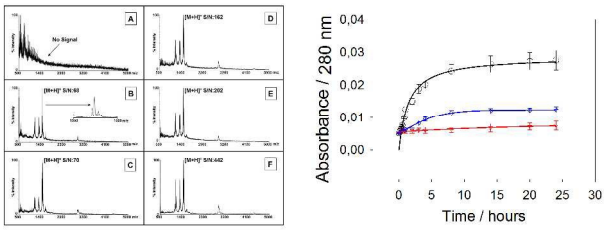

Fig. 2 Vancomycin release time-course after addition of 10^4 *S. aureus* cells into samples with aptamer-capped vancomycin-nanoparticles. Upper panel shows mass spectra at (A) initial time (before addition of bacteria) and at (B) 15 sec. (C) 180 sec. (D) 5 min. (E) 60 min. (F) 35 hours after the addition of the bacteria cells. On the right panel, vancomycin release monitored by absorbance spectra.

As can be seen in Fig. 3, the MICs of vancomycin-nanoparticles for *S. aureus* and *S. epidermidis* (control) were found to be 0.420 µg/mL and 6.295 µg/mL, respectively. Further control experiments were performed with vancomycin-nanoparticles capped with a scrambled sequence and with nanoparticles only and *Salmonella* aptamers only samples (Fig. S3). These results mean a 15-fold increased efficacy between *S. aureus* and *S. epidermidis*, thus indicating specific targeting in the case of *S. aureus* and lower toxicity for *S. epidermidis.* The MIC for vancomycin only, used as control in our study, amounted to 1.1-2.4 µg/mL (Fig. 3) which is in agreement with previous studies.[31] Importantly, *S. epidermidis* was only affected by high doses of vancomycin-nanoparticles (6.295 µg/mL). This suggests that at an appropriate dose (e.g. at 1 MIC = 0.420 µg/mL), aptamer-gated vancomycin nanoparticles approach could be safe for non-targeted bacteria or cells while efficiently killing the targeted bacteria *S. aureus*.

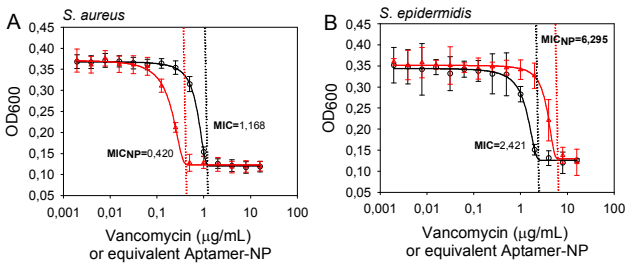

Fig. 3 Therapeutic efficacy of aptamer-gated antibiotic nanoparticles. Susceptibility of a) *S. aureus* and b) *S. epidermidis* cultures were challenged against serial two-fold dilutions of vancomycin-nanoparticles (red lines) or vancomycin only as control (black lines). After a 24 h incubation period at 37˚C, the cell viability was determined by measuring optical density (OD) at 600 nm, and the minimum inhibitory concentrations (MICs) were calculated. All the measurements were performed in triplicate; the error bars indicate the standard deviations.

We verified this hypothesis by comparing kill curves and growth curves of *S. aureus* and *S. epidermidis* in presence and absence, respectively, of vancomycin-loaded aptamer-gated nanoparticles. Time-kill curves have been previously used *in vitro* to provide more detailed information about the time course of the antimicrobial effects.[32] Fig. 4 depicts the effect of 1 MIC concentration of vancomycin-loaded nanoparticles and the change in density of viable bacteria as a function of time. As can be seen in Fig. 4, vancomycin-loaded nanoparticles with aptamer caps can be considered an effective therapeutic strategy against *S. aureus*, by inhibiting bacterial growth after2 h; followed by a strong and prolonged bactericidal activity from 2 to 24 h (blue dashed line), and as compared to the *S. aureus* growth curve (black line). In contrast, the viability of *S. epidermidis* was only slightly affected by vancomycin-nanoparticles (green line), being similar to the control *S. epidermidis* growth curve (red line). The growth curve of *S. epidermidis* in presence of NP was inhibited about 12 % compared to control growth curve.

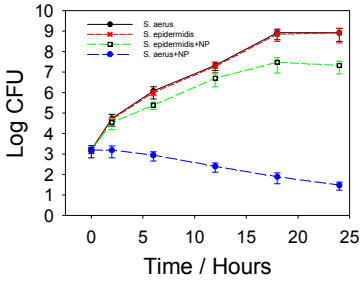

Fig. 4 Time-kill curves for *S. aureus* and *S. epidermidis* cultures after treating with aptamer-nanoparticles. Vancomycin-loaded and aptamer-gated nanoparticles at 1 MIC concentration (as determined in Fig. 3) was used to evaluate the change in density of viable bacteria for 24 hours. After each time indicated, the log of CFU/mL was determined by culturing method.

Aptamers for antibiotic delivery were demonstrated in a limited number of reports. A nanocarrier for antibiotic neomycin was developed by using aptamers for on-demand delivery,[33] in which neomycin aptamers immobilize the antibiotic molecules through affinity binding and released with temperature increase in vitro. Thus, aptamers were used for affinity-immobilizing

antibiotic molecules rather than targeting them. In a similar study, tetracycline-loaded hydrogels were designed for controlled release, using aptamers for enhanced antibiotic loading and sustained release.[34] In the present study, we designed an antibiotic delivery system that can effectively target the antimicrobial molecules and that achieve controlled release at the same time. The described aptamer-gated silica nanoparticles allow in principle administering antibiotics at a lower dosage as well as the use of stronger therapeutic compounds or combination of drugs in a safer manner. In this way, the approach proposed may contribute to diminishing bacterial resistance.

In this report we showed that antibacterial activity of vancomycin increased when loaded in nanoparticles-aptamers composites. Similar results were reported with other types of nanoparticle-antibiotic associations. Xiang *et. al.* described previously how vancomycin derivatives forms a multivalent inhibitor on the cell surface.[35] Aptamers in this study targeted nanoparticles on the surface of *Salmonella* and the interaction with cell surface antigens resulted in the release of vancomycin, which could accumulate to higher concentrations at the bacteria-nanoparticle contact site, resulting in more efficient inhibition of cell wall biosynthesis and hence a decreased MIC value.

Conclusions

MIC results clearly demonstrate the bactericidal efficacy of aptamer-gated nanoparticles for *S. aureus* and the limited effects on the viability of non-targeted bacteria. In fact, we have recently reported a similar approach that use micrococcal nuclease as biomarker, and a substrate oligonucleotide gating system.[36] Comparing these two strategies of capping for effective targeting of vancomycin to *S. aureus*, use of aptamers as capping agents provides alternative opportunities. Since aptamers are well-established technology to obtain high affinity oligonucleotides, it is relatively more available for any kind of pathogenic microorganism or other human conditions. We anticipate that the concept of aptamer-gated is not limited to bacterial infections, but that it bears the potential to be transformed into therapeutic alternatives for other infectious diseases (e.g. virus and fungi) or cancer treatment.

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Notes and references

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Electronic Supplementary Information (ESI) available: Experimental procedures, Fig. S1, S2, S3 and Table S1, S2,. See DOI: 10.1039/c000000x/

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