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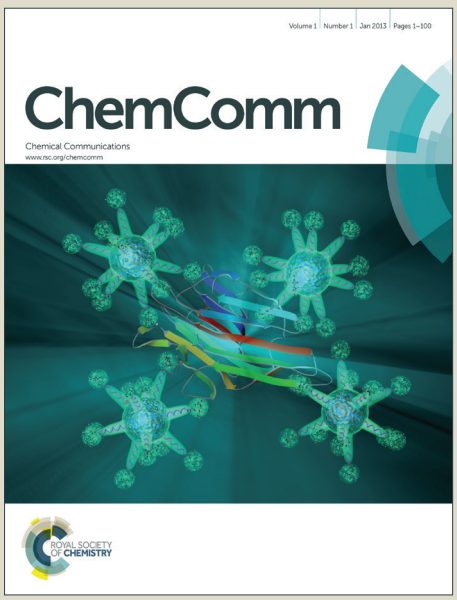

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NanoKeepers: stimuli responsive nanocapsules for programmed specific targeting and drug delivery

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Bacterial resistance is a high priority clinical issue worldwide. Thus, an effective system that rapidly provides specific treatment for bacterial infections using controlled dose release remains an unmet clinical need. Herein, we report on NanoKeepers approach for the specific targeting of *S. aureus* with controlled release of antibiotics based on nuclease activity.

The clinical management of bacterial infections generally includes antibiotic treatment via oral or intravenous administration. Since this is a non-target strategy, high doses of antibiotics are required to obtain therapeutic effects.[1] As results of this practice, toxicity and bacterial resistance have been reported as the most common drawbacks.[2] Bacterial resistance is currently among the top health challenges worldwide.[3] In the last decades, the world was keeping hope in drugs such as carbapenems, as the last weapon to battle resistant bacteria.[4] However, a couple of years ago, several bacteria species have reported resistance to carbapenems, leaving the world defenseless to such infections.[5] Thus, antibiotics should be considered as a limited resource; as more as we use them, their effeciency will be reduced in the future.[6] To preserve the life-saving potential of antibiotics, a more careful use should be implemented, including lowering the dosage by more effecient administration methods.[7] The development of new approaches is therefore required to overcome this unprecedented challenge.[4] Controlled delivery systems based on adequately modified nanocapsules incorporate characteristics such as specific targeting, control of dose and drug release to overcome the side effects of conventional treatments,[8] offering a pre-designed strategy for more efficient therapies.[9] Drug-loaded nanocapsules (DLNCs) are well suited for specific delivery of drugs for therapeutic applications in different human pathologies.[10] The concept of DLNCs relies on introducing a drug into nanocapsules, followed by an adequate sealing of the mesoporous structure in order to avoid leakage. The surface of the DLNCs is then functionalized such as to interact specifically with the target location, upon which the drug is released by an external stimulus. DLNCs in this way improve drug pharmacokinetics, biodistribution, cell-specific targeting and drug kinetics, resulting in enhanced efficacy and improved tolerability.[11]

A limitation of existing DLNCs is the fact that current stimuli-response delivery systems use bulk stimuli, including: light, magnetic field, ultrasound and pH.[12] These stimuli are passive and non-specific approaches. Enzyme activity (mostly proteases) has been reported as alternative stimulus for controlled-drug release approaches,[12] however, the instability of peptides in physiological conditions (non-targeting proteases) may be an issue for peptide-based approaches. For example, it has been reported that only one protease, cathepsin L, is able to cleave more than a third of the human proteome,[13] indicating that unspecific degradation is expected for peptide-approaches.

We have recenty described a novel molecular imaging approach for the specific, non-invasive detection of *S. aureus* based on the activity of its secreted nuclease, micrococcal nuclease (MN).[14] While this diagnostic method is highly advantageous and specific, it does not offer a therapeutic option for bacterial infections. However, once an infection has been detected, a therapeutic approach that rapidly provides specific treatment for bacterial infections would be highly desirable. Herein, we propose what we denote "NanoKeepers" as a therapeutic option based on the nuclease activity of MN as a stimulus-response for specific targeting and controlled drug release.

As opposed to exisiting concepts of DLNCs, NanoKeepers incorporate an engineered oligonucleotide which fulfills three functions: i) to block the nanopore, retain the loaded drug inside the nanocapsule and provide specificity to *S. aureus* nuclease (Figure 1a), ii) to be resistant to endogenous nucleases (serum nucleases), and iii) to provide specific targeting based on nuclease activity going along with active drug release. Upon specific degradation by MN (Fig. 1b), the oligonucleotide is detached from the nanocapsule and

subsequently, the antibiotic is released (Fig. 1c). Combining these three functionalities into one single nanodevice represents a significant progress over currently existing nanoparticle-based approaches.

To demonstrate the applicability of this proof-of-concept study, we have selected *S. aureus* as a clinical model of bacterial infection. *S. aureus* is a major cause of human disease, responsible for several conditions such as endocarditis and septis and has emerged as a major public health threat, with resistance to a variety of antibiotics.[15]

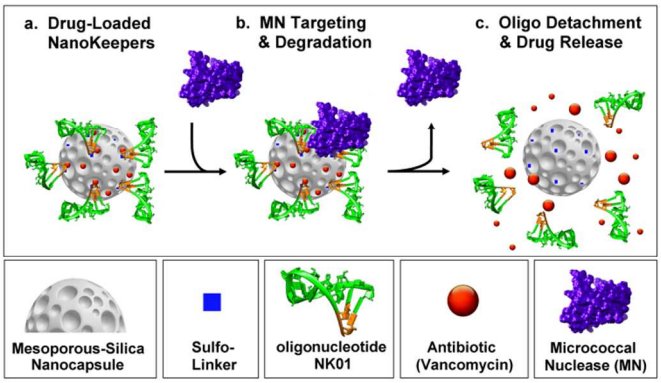

Fig. 1. Scheme of NanoKeepers approach. a) Silica nanocapsules were modified with a sulfo-linker (small blue square) to facilitate the coupling to amine-modified oligonucleotides. Oligonucleotide NK01, composed of a pair of deoxythymidines (orange) and several 2'-*O*-methyl modified nucleotides (green), was designed to maximize sensitivity to micrococcal nuclease (MN). In addition, the forming hairpin structure of NK01 works as a cap to retain the previously loaded antibiotic. b) When NanoKeepers encounter MN (purple), c) the oligo NK01 is detached as result of nuclease activity and the encapsulated antibiotic is released.

The utility of NanoKeepers as a therapeutic approach depends on the ability to identify specific bacterial nucleases and thus, certain types of bacteria. Previously, we have pioneered a molecular approach for the specific detection of *S. aureus* using its secreted nuclease, MN, as a trigger mechanism for activatable-fluorescent probes. This oligonucleotide probe sequence (11mer) has two central thymidines, flanked by 2'-O-Methyl nucleotides (mCmUmCmGTTmCmGmUmUmC), modified at the ends with a fluorophore and a quencher (named as TT probe), where the m stands for 2'O-Methyl nucleotides and TT for DNA thymidines.[14] This TT probe confers specificity to MN (most likely by the TT sequence) while resistance to endogenous nucleases in mouse and human serum is provided by the 2'-O-Methly nucleotides. Based on this knowledge, we integrated a similar TT probe oligonucleotide sequence into mesoporous nanocapsules. To this end, we have engineered the TT probe (Fig. S1a) into a hairpin structure such as to widely block the porous orifice and in this way aim at retaining the loaded drug inside the nanocapsule.

A remaining limitation of the resulting TT hairpin probe was the center location of the TT: shifting the TT location from the center of the TT probe to the 5'-end of the hairpin sequence would foster complete detachment from the nanocapsule upon MN actuation. This change of the TT position, however, bores the risk of affecting the degradation efficiency by MN. We therefore designed a modified oligonucleotide sequence (FAM-TTmCmGmCmUmUmC mGmGmCmGmAmA–Quencher), denoted NK01 (Fig. S1b). Degradation of NK01 through nuclease activity by MN and stability in serum (Fig. S2) were found comparable to those reported by the TT probe,[14] thus, indicating the utility of NK01 for being incorporated into the NanoKeepers approach.

In order to verify in how far NK01 would block and retain a cargo molecule inside the nanocapsules, 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 NK01 oligo had been functionalized with an amine group ($NK01\text{-}NH_2$) at the 5'- end in order to facilitate the coupling to the capsule surface as previously described.[10] Synthesis and characterization of the final capsules are provided in Fig. S3 and S4 (ESI). Briefly, the NanoKeepers were loaded with Rodhamine B and thereafter blocked with the oligo $NK01\text{-}NH_2$. Subsequently, they were incubated in serum samples to mimic endogenous nuclease background (control), and serum samples spiked with MN. The samples were analyzed by a spectrofluoremeter with appropriate filters for Rodhamine B.

As depicted in Fig. 2, a release of Rhodamine B occurs in serum samples spiked with MN, indicating specific release based on MN activity. In samples containing only serum, a minimal release of Rhodamine B is observed, revealing a favorable low-leakage capsule stability at physiological conditions. These results demonstrate the viability of the concept of NanoKeepers as drug delivery systems based on nuclease activity with specific targeting for MN and, hence, their physiological applications.

To corroborate the specificity of NanoKeeper-NK01, a sub-optimal sequence was evaluated, denoted NK02 (NH_2-UmUmmCmGmCmUmUmCmGmGmCmGmAmA). In the NK02 oligonucleotide, the TT DNA motif originally present in NK01 was replaced by two 2'-O-Methyl Uracils (UmUm) in an attempt to reduce the degradation efficiency of MN. NK02 was then incorporated into the nanocapsules following the aforementioned approach.

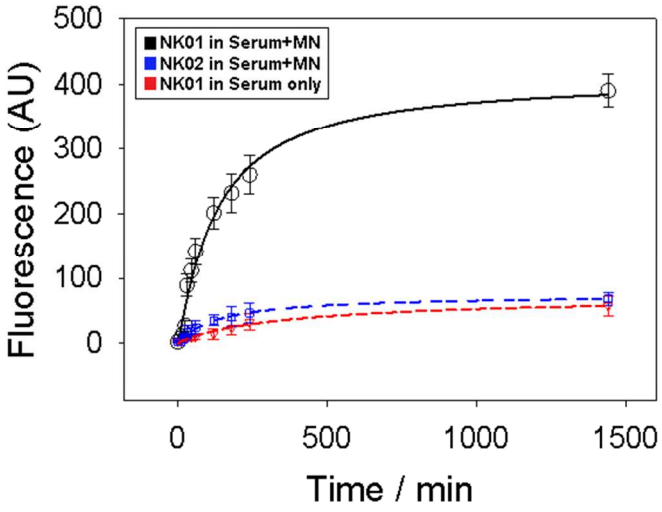

Fig. 2. Evaluation of NanoKeepers under physiological conditions. NanoKeepers loaded with Rhodamine B and capped with NK01 oligo (NanoKeepers-NK01) were incubated with serum, to determine their stability and cargo retention under physiological conditions (red dashed line). NanoKeepers-NK01 were incubated with serum samples containing MN to evaluate the release of cargo by the specific targeting of MN (black line). Non-specificity studies were carried out by capping Rhodamine B NanoKeepers with a control oligomer (NanoKeepers-NK02). This sub-optimal construct was incubated with serum samples containing MN (blue dashed lines). All the measurements were carried out in triplicate; the results show average fluorescence intensity and the error bars represent standard deviations.

Indeed, no release of cargo molecules (Rhodamine B) by NanoKeepers containing the NK02 sequence was observed owing to the presence of MN (Fig. 2) and signal levels resembled rather those of NK01 in serum only. This demonstrated that on one hand NK02 was similarly capable of closing the nanopore, however, no opening was triggered by actuation of MN. Hence, the specific recognition and then cleavage of the TT DNA motif of NanoKeeper-NK01 were indeed the responsible mechanism for the delivery of the cargo molecules in presence of MN. This observation confirmed nuclease activity as the trigger mechanism for drug delivery in the NanoKeepers approach.

The therapeutic efficacy of NanoKeepers was subsequently evaluated in bacterial cultures. NanoKeepers were loaded with vancomycin (a common antibiotic for treating *S. aureus* infections) and capped with NK01 oligonucleotide. These vancomycin-loaded NanoKeepers were characterized to confirm almost leakage-free stability and release only by specific actuation of MN (Fig. S5).

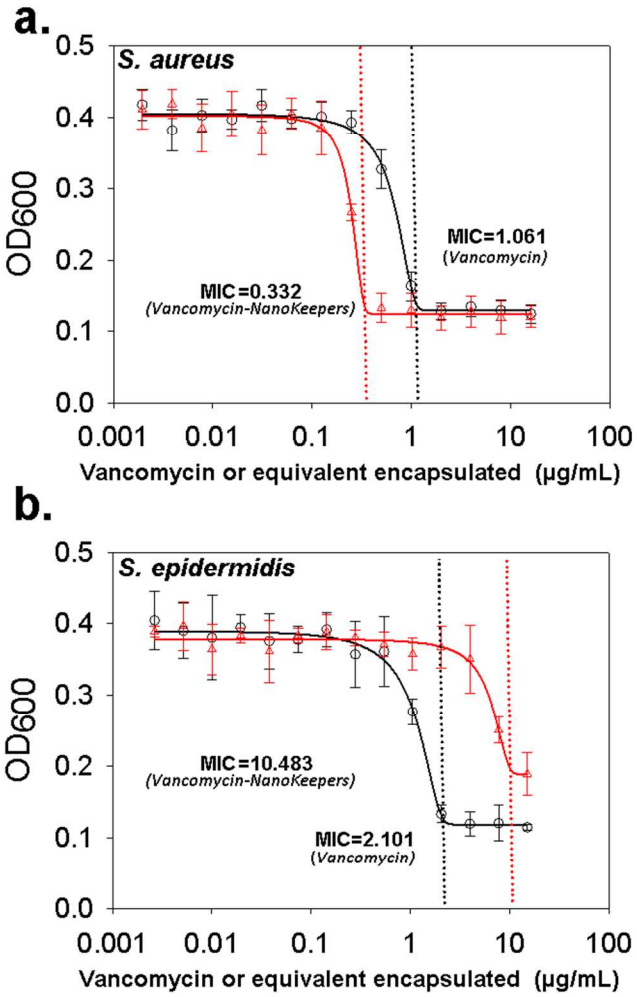

Fig. 3. Therapeutic efficacy of NanoKeepers. Susceptibility of a) *S. aureus* and b) *S. epidermidis* cultures were challenged against serial two-fold dilutions of vancomycin-NanoKeepers (red lines) or vancomycin only as control (black lines). After a 24 h incubation period at 37˚C, the cell viability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

The concentration of vancomycin encapsulated was calculated to be 15.5 pmol/mg. During 24h, the susceptibility of two cultures was then observed, namely that of *S. aureus* which produces a genus of Staphylococcus that has been reported to produce undetectable levels of MN.[14]

The bacterial susceptibility was determined by minimal inhibitory concentrations (MICs) in 96-well microtiter plates using the microdilution method according to the Clinical and Laboratory Standards Institute.[16] However, rather than Mueller-Hinton broth, tryptic soy broth (TSB) was used for susceptibility testing as it is the common medium used for Staphylococcal cultures.

As can be seen in Fig. 3, the MICs of vancomycin-NanoKeepers for *S. aureus* and *S. epidermidis* (control) were found to be 0.332 µg/mL and 10.483 µg/mL, respectively. These results mean a 31-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 1-2 µg/mL (Fig. 3) which is in agreement with previous studies.[17] Importantly, *S. epidermidis* was only affected by high doses of vancomycin-Nanokeepers (10.483 µg/mL). This suggests that at an appropriate dose (e.g. at 1 MIC = 0.332 µg/mL), NanoKeepers approach could be safe for non-targeted bacteria or cells while efficiently killing the targeted bacteria *S. aureus*. 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 NanoKeepers. Time-kill curves have been previously used *in vitro* to provide more detailed information about the time course of the antimicrobial effects[18]. Fig. 4 depicts the effect of 1 MIC concentration of vancomycin-loaded NanoKeepers and the change in density of viable bacteria as a function of time. As can be seen, vancomycin-loaded NanoKeepers are an effective therapeutic strategy against *S. aureus*, with a moderate response after 2 h; followed by a strong and prolonged bactericidal activity from 6 to 24 h (red dashed line), and as compared to the *S. aureus* growth curve (black line). In contrast, the viability of *S. epidermidis* was not affected by vancomycin-NanoKeepers (green line), being similar to the control *S. epidermidis* growth curve (blue line). These results clearly demonstrate the bactericidal efficacy of NanoKeepers for *S. aureus* and the limited effects on the viability of non-targeted bacteria. The results also confirm the safety and efficacy of NanoKeepers as a therapeutic approach.

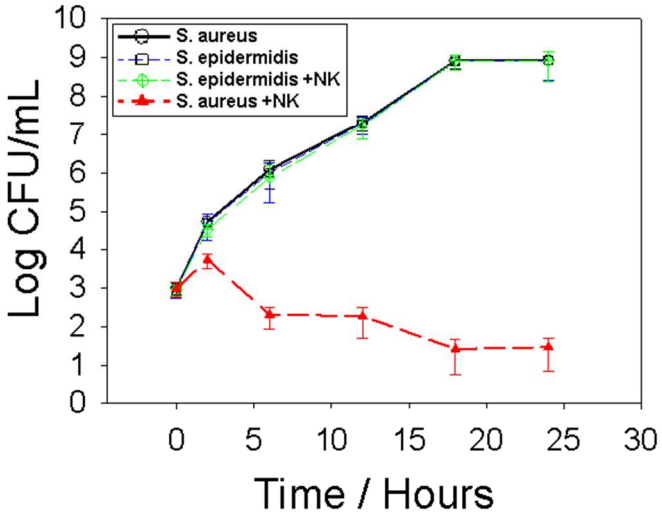

Fig. 4. Time-kill curves for *S. aureus* and *S. epidermidis* cultures. Vancomycin-loaded NanoKeepers at 1 MIC concentration was used to evaluate the change in density of viable bacteria over time. After each time indicated in the figure, the log of CFU/mL was determined. All the measurements were performed in triplicate; the error bars indicate the standard deviations.

Conclusions

In conclusion, we have developed a new and safer therapeutic strategy for *S. aureus* with high efficacy, specificity and active drug delivery. The described NanoKeepers allow in principle administering antibiotics at a lower dosage as well as the use of stronger therapeutic compounds or combination of drugs (polytherapy) in a safer manner. In this way, the approach proposed may contribute to diminishing bacterial resistance. We anticipate that the concept of NanoKeepers 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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(ESI) available: Detailed experimental procedures. Fig. S1-S5. See DOI: 10.1039/c000000x/

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