CHAPTER 5

General Discussion

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The aim of this thesis was to explore the advantages and disadvantages of photothermal nanoparticles as an infection-control strategy. To this end, polydopamine nanoparticles were synthesized and evaluated for their merits in a prophylactic and therapeutic clinical mode. Without further modification, polydopamine, photothermal nanoparticles provided merits for the prophylactic control of bacterial infections due to bacteria that might have entered the body per-operatively and in the early post-operative period. Despite the possibility to localize NIR irradiation to an infection site to activate heat generation, spreading of heat could cause collateral tissue damage. Accordingly, nanoparticle dosing, NIR irradiation power and duration must be well tuned in clinical applications. Self-targeting of modified or encapsulated photothermal nanoparticles to yield accumulation of nanoparticles in an infectious biofilm enhanced local heat generation to an extent that allowed therapeutic application as well.

Nanoparticle-based photothermal therapy has advanced most in cancer therapy [1,2], yielding high specificity and few systemic side effects [3], although most applications of photothermal therapy are still in their early research stages (preclinical studies in animals) [4,5]. Some pioneers have moved forward to clinical trials [6], such as a study based on cytokine tumor necrosis factor-alpha conjugated gold nanospheres [7] that has advanced first-in-human to phase II trials. Near-infrared photo-immunotherapy has also been used in phase I/II clinical trials in humans, targeting epidermal growth factor receptors and has been successfully completed [8]. The application in bacterial infection-control follows directly from the developments in cancer therapy and is particularly promising as it is advocated to kill bacteria irrespective of the strain involved or its possible antibiotic-resistance, while it was not a priori expected to induce thermo-resistance [9].

The penetration of NIR irradiation is deep, but still limits the range of application of photothermal therapy [10]. Its penetration depth ranges up to several millimeters in live tissues, depending on e.g. wavelength, energy, area of irradiance, coherence, pulsing and the type of tissue involved [11,12]. Penetration of NIR radiation into cartilage ranged up to 5 mm over NIR wavelengths between 1000 and 2500 nm [13]. Peripheral nerves and the brain cortex could be penetrated less up to around 0.4 mm [14] at a wavelength of 830 nm. Skin penetration ranged up to 2 mm [15]. Accordingly, it can be concluded that NIR penetration through tissue is still a limiting factor in the use of photothermal therapy [9,16-18]. Endoscopic optical fiber diffuser and implantable wireless LED [19-21] are currently under investigation in animals and humans in order to find ways to reach deeper tissue for photothermal therapy. Thus, the optimism about the clinical application of photothermal nanoparticles to cure bacterial infections may need to be dampened.

In addition to the above limitations, many different examples from environmental and industrial biofilms show that bacteria have the ability to become heat resistant up to temperatures of around 95 °C in terrestrial geothermally-heated habitats (**Figure 1**), like hot springs [22]. By definition, thermophiles grow at temperatures between 60 °C to 80 °C above which they are called “hyperthermophiles” [23].

Figure 1. Colorful bacterial mat in a geothermally heated pool in Rotorua, New Zealand (photo by Henk J. Busscher, with permission).

Thermoresistant bacteria have genomically evolved over the ages as a response to environmental changes through horizontal gene transfer, gene mutation and genome reduction and gene loss (see **Figure 2** for an overview of mechanisms). Heat shock proteins represent a well-known example of proteins brought to expression in temperature sensitive genes. Many heat shock genes sensitive to high temperatures have been reported, such as *htpG, htpR*, *groES* and *groEL* in *Escherichia coli* 27 [24,25], *orp37* and *orp3S* in *Staphylococcus aureus* [26], *DnaK* and *GroEL* in *Acinetobacter baumannii* [27], and *GroEL* or *DnaK* in *Enterococcus faecium* [28]. Typically, thermophilic bacteria have higher stability of protein structures and enzymatic activities that can be achieved by amino acid substitution [29], creation of hydrophobic cores [30] and burying of polar groups [31].

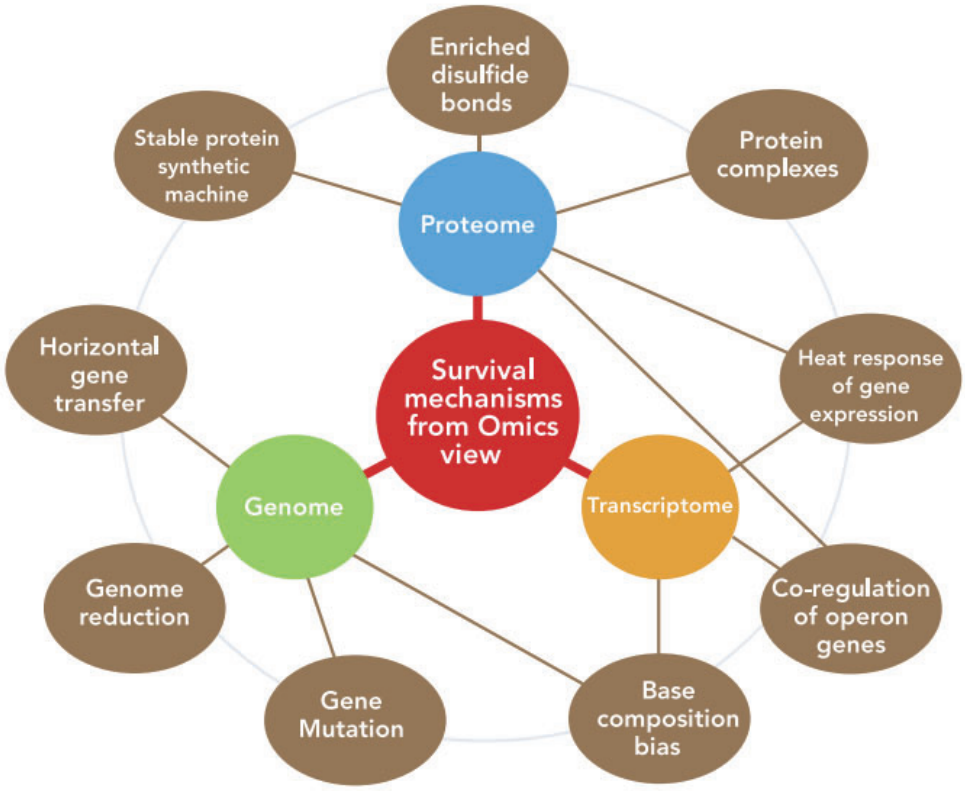

Figure 2. Summary of survival mechanisms of thermophilic bacteria, according to the views from an omics-perspective [23] (reprinted with permission from American Physical Society (APS)).

In view of our findings in **Chapter 2** on the development of thermo-resistance in human pathogens, we have carried out a series of pilot-experiments, exposing serial sub-cultures of ESKAPE-panel pathogens to increasing temperatures (**Figure 3**) in order to expand further on induction of thermal resistance.

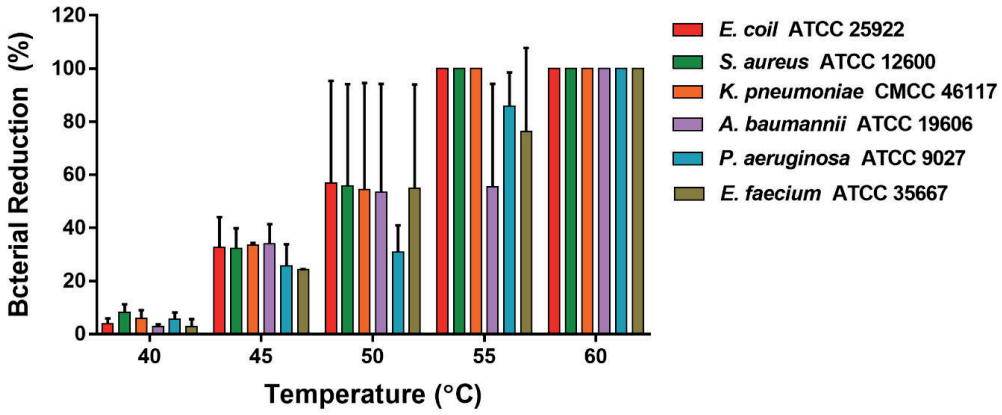

Figure 3. The percentage reduction in CFUs of ESKAPE panel strains when sub-cultures are exposed to gradually increasing temperatures, as compared with the bacterial strain grown at 37 °C. The temperature was step-wise increased by 5 °C, the strains were exposed to each temperature for 24 h, while sub-culturing at each temperature three times before growth at a higher temperature. All data were expressed as means ± SD values over triplicate experiments with separately and independent bacterial cultures.

Growth at 40 °C hardly affected the number of CFUs grown in suspension, while in general bacterial killing increased with increasing temperatures. Yet already at 45 °C, it can be seen that *Pseudomonas aeruginosa* and *E. faecium* lag behind in being heat-killed, suggesting development of heat-resistant sub-populations (note that also other studies reported thermo-resistance in *E. faecium*, see **Table 1**). This trend is continued at 55 °C and at this temperature also comprises *A. baumannii*. At 60 °C, all ESKAPE-panel pathogens remained to be killed by exposure to this temperature without surviving sub-populations. Collectively, current findings exclude human pathogens from the definition of thermophiles [23], but stronger thermo-resistance may not be excluded upon prolonged and more extensive sub-culturing as a prelude of what might happen when photothermal therapy would be wide-scale used clinically.

Table 1. Percentage survival of *E. faecium* when cultured for 2 h at elevated temperatures [28] (reprinted with permission from Springer).

Time (h)	Temperature				
	52 °C	55 °C	60 °C	65 °C	70 °C
0	100	100	100	100	100
1	76.9	32.0	0.5	0.12	0.035
2	16.2	7.5	0.11	0.02	0.001

At this point however, it is most important to establish whether extensive serial sub-culturing bacteria more than three times at 45 °C will further increase survival at relatively low

temperatures that are yet harmful to humans and whether other ESKAPE-panel pathogens will exhibit heat-resistance as well upon more prolonged serial sub-culturing at such temperatures. Also, it would be of interest to study possible changes in gene expression to these studies.

The limiting temperatures to which the human body can be exposed depend a lot on humidity and are limited to around 35 °C in air with 100% humidity to 70 °C in dry air (around 10% humidity), both with strict limits to duration of exposure. Particularly exposure temperatures in dry air are fairly high, which is due to self-regulation mechanisms, such as the production of sweat to cool the skin. Locally however, self-regulation of internal body temperature is lacking and tissue damage will occur already in a lower temperature range between 39 °C and 42 °C and higher [32,33]. Such temperatures can be expected to yield heat inactivation of proteins and enzymes, denaturation and DNA damage [34,35]. Since these temperatures encompass the temperatures required for killing human pathogens, this type of damage must be balanced against the effects of the infection itself. Summarizing, it can be concluded, that particularly the development of thermo-resistant sub-populations at a relatively low, but clinically important limiting temperature of around 45 °C is worrisome for future application and needs further investigation.

Future research

Apart from gaining further insight into the possible development of thermo-resistance amongst human pathogens along the experimental pathway suggested above, the pathway to human clinical translation of photothermal infection control, as in its clinical translation in cancer therapy, is not easy. So far, experiments have only been carried out in infected-wound healing models in mice. Although such animal experiments are clearly a good first choice, it is arguably overly used in the meantime and it is now urgently needed to involve other, deeper infections in the animal evaluation of the merits of photothermal nanoparticles for bacterial infection-control. Therefore, larger animal models might be required to determine the limits of NIR irradiation efficiency in activating heat generation.

Such experiments need to be done with urgency, before entering the long and troublesome pathway of regulatory approval and permission for human clinical studies needed for translation and marketing of photothermal nanoparticles for clinical control of bacterial infections.

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