ABSTRACT

Nanoengineered Bacteria for Cancer Immunotheranostics

Cancer prevalence has reached alarming proportions, impacting people across all genders, ages, and geographic regions. The World Health Organization (WHO) has reported that cancer, as a significant contributor to global mortality, accounts for roughly one out of every six deaths worldwide. The escalating burden of cancer calls for a comprehensive approach that encompasses various facets, including early detection methods, preventive measures, and efficacious treatment strategies. Despite the challenges posed by cancer, notable advancements have been achieved in recent years towards combating this complex disease. The efficacy of traditional cancer therapies in the ongoing fight against cancer is diminishing due to various factors, including the presence of heterogeneity within cancer cells, the development of treatment resistance, the occurrence of significant side effects, and the progression of metastasis. Consequently, there is a substantial demand to explore novel strategies that exhibit reduced toxicity and minimal adverse effects. Additionally, there remains a continuous need for the improvement of existing techniques and the exploration of innovative approaches to overcome these challenges.

Over the past few decades, the utilization of bacterial therapy for cancer treatment has garnered increasing attention and recognition due to its notable efficacy and comparatively reduced incidence of side effects. Despite being a century-old technique, bacterial therapy is currently experiencing a renaissance, gaining significant momentum as a viable approach in the field of oncology. This therapeutic modality encompasses the utilization of bacteria, either in their natural state or after incorporating genetic modifications. Bacterial therapy has emerged as a highly promising avenue in the field of cancer treatment, presenting opportunities for both standalone utilization and synergistic integration with other therapeutic modalities and has successfully advanced to the stage of conducting human clinical trials. Presently, the majority of investigations and genetic modifications in bacterial therapy predominantly focus on pathogenic bacteria, which raises concerns about the potential generation of virulent revertants. Consequently, there exists a pressing necessity to explore the discovery of non-pathogenic strains in order to circumvent these challenges. Promising results have been observed in studies utilizing non-pathogenic probiotic strains as a viable alternative to pathogenic and genetically modified strains. Consequently, further exploration of these bacterial alternatives and the development of novel modification techniques that ensure the preservation of their inherent characteristics become crucial. Hence, the objective of our project is to delve into this unexplored realm and employ chemical modifications to tailor bacteria according to our specific requirements, while maintaining their morphology and functionality unaltered.

The present work proposes the application of non-pathogenic anaerobic bacteria in bacterial therapy, with an emphasis on enhancing their functionality through chemical modifications. The thesis encompasses two major chapters that introduce innovative strategies for chemically modifying bacteria devoid of genetic modifications. These strategies involve the incorporation of specific properties essential for effective tumor targeting and localization, with the goal of improving tumor regression. The modified bacteria were employed in conjunction with photothermal therapy (PTT), synergistically aiming to achieve accelerated and enhanced tumor reduction outcomes. By developing such a system, it aims to effectively induce tumor regression and improve overall treatment outcomes. The findings elucidated in Chapter 2 demonstrate the chemical modification of probiotic bacteria named *Bifidobacterium bifidum* (BB*)* which inherently possesses anticancer properties. In this study, the bacteria were subjected to modifications aimed at enhancing their efficacy in tumor targeting studies. This was accomplished by incorporating a near-infrared (NIR) agent into the bacteria BB, resulting in the formation of modified bacteria that exhibit fluorescence and photothermal conversion efficiency. The desired outcome was achieved by subjecting BB to incubation with indocyanine green (ICG) dye encapsulated in cremophor EL(CRE), followed by subsequent washing. As a result, the bacteria underwent modification as the nanoparticles penetrated through the bacterial membrane, resulting in the modified bacteria, ICG-CRE-BB, which exhibited the desired augmentation in photothermal conversion efficiency. Subsequently, the modified bacteria were subjected to comprehensive characterization, comparing them with their pure form to analyze their altered properties. This was followed by *in-vitro* studies to evaluate their toxicity and anticancer properties. The *in-vitro* investigations demonstrated a significant increase in temperature when subjected to an NIR laser irradiation, along with minimal toxicity in the absence of laser irradiation. Upon exhibiting promising results *in-vitro*, the modified bacteria were subjected to *in-vivo* studies using Colon26 tumor syngeneic models, wherein they were administered via intratumoral injection into the tumor. The tumor treatment involved the synergistic use of modified bacteria and photothermal therapy (PTT), wherein laser irradiation was applied to the solid tumors. Using an NIR fluorescence bioimager, the accumulation of bacteria was observed explicitly in the hypoxic tumor environment. Furthermore, this observation was confirmed through the application of a colony assay. The outcomes

demonstrated the remarkable tumor localization capability of the bacteria, followed by significant tumor regression. This research offers promising prospects for employing biocompatible chemicals in the chemical modification of bacteria for application in cancer therapy. We firmly believe that further refinement and optimization, particularly in the context of anaerobic probiotic strains, holds the potential for clinical studies to explore the utilization of these modified bacteria.

The third chapter shows an alternative method of modification, namely the incorporation of monoclonal antibodies, which represents a groundbreaking strategy garnering significant attention in contemporary therapies. Notably, various types of checkpoint inhibitors have exhibited promising outcomes in accelerating the process of tumor regression. In this project, we expanded upon the modification approach utilized in the previous chapter and extended it to naturally fluorescent purple photosynthetic bacteria (PPSB). To identify the most suitable PPSB strain, extensive screening processes were conducted, evaluating factors such as toxicity, photothermal conversion, and fluorescence and *Rhodopseudomonas palustris* (RP) emerged as the most favorable candidate. Through a process of incubation and subsequent washing, a checkpoint inhibitor, anti-mouse programmed death ligand monoclonal antibody (anti-PD-L1), was covalently attached to RP (which has exhibited excellent efficacy in selectively targeting cancer cells in prior studies) using biocompatible anchor for membrane (BAM). This resulted in the formation of modified RP with anti-PD-L1 (anti-PD-L1–BAM–RP), enabling us to utilize the inherent fluorescence properties of RP in conjunction with the attached checkpoint inhibitor. A series of characterizations were conducted to compare the modified bacteria with their pure counterparts, aiming to identify any discernible differences. Subsequently, *in-vitro* studies were undertaken to investigate the cytotoxicity of both types of bacteria. They were conducted to assess the cytotoxicity of the modified bacteria and pure bacteria and it was observed that the modified as well as the pure bacteria demonstrated low cytotoxicity. Moreover, upon laser irradiation, the temperature elevation observed owing to the inherent fluorescence properties of the bacteria. Encouraging results prompted further evaluation of the modified bacteria in an *in-vivo* setting, with a focus on exploring their targeting effects and tumor regression capabilities. Tumor syngeneic mouse models were employed for this study, and an 808 nm NIR laser was used to irradiate the tumor after bacterial injection. Remarkably, significant tumor reduction was achieved within a short duration, showcasing the efficacy of this non-toxic anaerobic bacteria. Additionally, the precise localization of bacterial tumors was verified through the utilization of a bioimager and a colony assay. We hold the belief that delving into the realm of anaerobic bacteria and augmenting them with synthetic therapeutic materials holds tremendous potential for the development of an ideal combination in bacterial therapy.

In conclusion, we firmly believe that this research on the chemical modification of non-pathogenic bacteria to augment their therapeutic efficacy for application in cancer treatment holds tremendous potential and represents a paradigm-shifting advancement in knowledge. It is imperative that we explore alternatives to the currently employed pathogenic strains and delve into the untapped potential of non-pathogenic anaerobic strains. Our studies have instilled in us a strong belief that this approach has a bright future and the potential to match or even surpass existing therapies. This novel tool can significantly strengthen our arsenal in the fight against this formidable disease.

Keywords: Cancer, bacteria, near infrared, immunotherapy, chemical functionalization, phototherapy, laser