A NANOPARTICLE-BASED EPIGENETIC MODULATOR FOR EFFICIENT GENE MODULATION

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ABSTRACT OF THE THESIS

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Modulation of gene expression through chromatin remodeling involves epigenetic mechanisms, such as histone acetylation. Acetylation is tightly regulated by two classes of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). Molecules that can regulate these enzymes by altering (activating or inhibiting) their functions have become a valuable tool for understanding cell development and diseases. HAT activators, i.e. *N*-(4-Chloro-(3-trifluoromethyl)phenyl)-2-ethoxybenzamide (CTB), have shown a therapeutic potential for many diseases, including cancer and neurodegeneration. However, these compounds encounter a solubility and a membrane permeability issue, which restricts their full potential for practical usage, especially for in vivo applications. To address this issue, in this work, we developed a nanoparticle-based HAT activator CTB, named *Au-CTB*, by incorporating a new CTB analogue onto gold nanoparticles (AuNPs) along with a poly(ethylene glycol) moiety and a nuclear localization signal (NLS) peptide to assist with solubility and membrane permeability. We found that our new CTB analogue and Au-CTB could activate HAT activity. Significantly, an increase in potency to activate HAT activity by Au-CTB proved the effectiveness of using the nanoparticle delivery platform. In addition, the versatility of Au-CTB platform permits the attachment of multiple ligands with tunable ratios on

the nanoparticle surface via facile surface functionalization of gold nanoparticles. Due to its high delivery efficiency and versatility, Au-CTB can be a powerful platform for applications in epigenetic regulation of gene expression.

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Chapter 1

Introduction

1.1 Therapeutic gene modulation and epigenetics

Gene expression is the process when genetic information on DNA is used to synthesize functional gene products, i.e. RNA and proteins. The notable steps in gene expression are transcription and translation. Transcription is the process of encoding DNA to RNA. Then, RNA is translated into a peptide, and later becomes a functional protein. Each of these steps can be regulated through a variety of mechanisms. Regulation of gene expression is very important for controlling the production of gene products, especially proteins, leading to a control over the structure and function of the cells. Consequently, gene regulation is the fundamental basis for cellular differentiation and morphogenesis. In general, all cells in a multicellular organism possess the same set of genes (genotype). Differences in gene expression profiles give rise to the production of different proteins that control the cell in order to differentiate them into different cell types, creating different ultrastructures (shapes), and eventually produces different proteins or enzymes that catalyze specific metabolic pathways, which makes the characteristic of the cell (phenotype). In short, gene expression determines phenotypes from genotypes. Moreover, the control of gene expression also relates to the versatility and adaptability of an organism to environmental stimuli. [1–3]

Abnormality in gene expression leads to cellular dysfunction, thereby causing the underlying diseases. Treatment targeting the direct cause of the diseases at the gene level can potentially restore the normal equilibrium of gene expression by correcting, up- or down-regulating dysfunctional genes. To date, such therapeutic strategies have been demonstrated in two different approaches, i.e. the introduction of a therapeutic and exogenous gene to the misfunctional cells or the alteration of the expression of

an endogenous gene at any stage in gene regulation. Traditional gene therapy involves introducing an exogenous gene (so called therapeutic DNA) encoding a functional, therapeutic gene product that either replaces or corrects a mutated gene. In principle, the therapeutic DNA, which gets inside the target cells, expresses by cell machinery of the host cell to produce the therapeutic protein that treats the disease directly. Information regarding gene therapy was widely discussed in literature. [4–7] Unlike traditional gene therapy, therapeutic modulation employs a variety of therapeutic agents, i.e. small molecules, RNAi, to alter the expression of an endogenous gene by targeting factors that involve in the process of gene regulation. Therapeutic modulation of endogenous genes can be carried out at any stage of gene regulation, ranging from pre-transcription to post-translation. Endogenous gene modulation at the upstream levels, i.e. chromatin or DNA, is potentially more effective than targeting at the downstream levels, i.e. mRNA or proteins, because, in theory, there are fewer copies of DNA to target than RNA or proteins. Correction or up-regulation of a mutated or under-expressed endogenous gene at the upstream levels ensures the correct expression of the gene, thereby producing normal proteins. [8]

Chromatin remodeling plays a pivotal role in regulating the gene expression of biological processes, i.e. cell proliferation, differentiation. [9] Deregulation of chromatin remodeling has an effect on losing transcriptional regulation, which is necessary for maintaining proper cellular functions leading to various diseases, including cancer. [2,10–14] In eukaryotic cells, DNA is packed with histone proteins to form a DNA/protein complex called chromatin. The fundamental unit of chromatin is a nucleosome, which is composed of an octamer of the four core histones (H3, H4, H2A, H2B) wrapping around 147 base pairs of DNA. The core histones have a globular structure with extended N-terminal "tails". These histone tails are subjected to modification. Modifications of histone tails dictate the higher-order of chromatin structures leading to a global chromatin environment and "orchestrate" the recruitment of enzyme complexes to perform biological tasks. [9] The different composition of modifications allows recruitment of different non-histone proteins, resulting in different enzymatic activities occurring at the specific region of chromatin. This may explain how modification of histone tails

can regulate transcription, thereby regulating gene expression. Histone modification is implicated in epigenetics. Epigenetics is defined as heritable changes in gene expression and activity without changing into DNA sequences, rather it is caused by the gene (DNA base pair) being "turned off" or "turned on" due to chemical modifications, i.e. histone modifications. [1,3,11,15,16] The understanding in epigenetics can help answer many biological questions, including how cells with identical genotype differentiate into different cell types, how they maintain differentiated states, and how environmentally induced changes can pass on from generation to generation without the presence of the original stimuli. Therefore, epigenetic research has a significant impact and has become a central issue in biological studies of development and diseases.

The major mechanisms that regulate epigenetic modifications are DNA methylation and histone modifications. Histone modifications, as mentioned above, regulate chromatin structure to permit gene activation or repression/silencing. One of the well-studied histone modification mechanisms is acetylation. Acetylation occurs at the lysine residues on the N-terminal of histone tails. The positive charge from the lysine residues binds to the negative charge from the phosphate groups of DNA to tighten nucleosomes, resulting in a closed chromatin structure (so-called heterochromatin), which precludes transcription due to the inaccessibility of transcription factors, marking gene repression. When these lysine residues are acetylated, the interaction between DNA and histone reduces due to neutralization of the positive charge, leading to an open chromatin structure (so-called euchromatin) that allows accessibility of the DNA to the transcriptional machinery, and activates gene expression. Histone acetylation has a reversible process, namely histone deacetylation. Two classes of enzymes that mediate acetylation and deacetylation are histone acetyltransferases (HATs) and histone deacetylases (HDACs). The HAT enzymes catalyze acetylation by transferring acetyl groups from acetyl-CoA to the lysine residues of the histone tails, resulting in a euchromatin state. On the other hand, HDACs remove the acetyl groups inducing chromatin to return to a heterochromatin state. HATs and HDACs work in balance to regulate the dynamic nature of chromatin structures, like a switch turning a gene ON and OFF. These enzymes are well-studied at both the functional and structural levels and are found to be

involved in key cellular processes, for instance, cell cycle control, proliferation, DNA repair, and differentiation. Dysfunction or deregulation of HATs and HDACs brings about atypical gene expression, which affects cellular phenotypes, thereby causing the underlying diseases, i.e. cancer, neurological diseases, etc. Therefore, these enzymes have become appealing targets for developing modulators that can regulate gene expression as a consequence of modulating the enzymatic activity of these histone-modifying enzymes.

Modulators of HATs and HDACs have been developed and utilized in a wide range of applications both as biological tools to study fundamental problems in epigenetics, and as therapeutic agents for many diseases, such as cancer. In cancer, deregulation of histone acetylation patterns in the chromatin is caused by either i) dysfunction of HATs and HDACs in targeting a particular gene loci, ii) overexpression of HDACs, or iii) misexpression of HAT due to mutations causing the suppression of important genes. To restore the normal cell physiology, the development of modulators that can inhibit HDACs or activate HATs is required. HDAC inhibitors are the very first class of HAT/HDAC modulator studied. There are several classes of HDACi categorized based on their chemical structure, i.e. hydroxamates, cyclic peptides, aliphatic acids, and benzamides. HDAC inhibitors have shown significant promise, both *in vitro* and *in vivo*, in the treatment of many diseases, especially cancer. To date, there are two HDAC inhibitors that have been approved by the FDA, suberanilohydroxamic acid (SAHA, vorinostat) and romidepsin (FK228), for the treatment of cutaneous T-cell lymphoma (CTCL). [17, 18] The other HDAC inhibitors are also in various stages of clinical trials. [19] Over the past decades, inhibitors of HDACs have been extensively studied due to new mechanistic understandings of the pathogenesis of cancer and other diseases revealing its link to HDAC functions. Additionally, these molecules are capable of interfering with HDAC activity, thereby providing a significant effect on the chromatin structure, which is found to be beneficial in endogenous (therapeutic) gene modulation and combined therapies. Mechanistically, HDACi indirectly induce hyperacetylation through inhibition of HDAC activity to deacetylate histones, causing chromatin to remain active (euchromatin). This attractive feature of HDACi encourages researchers

to combine HDACi with other functional molecules that target gene loci on the DNA, i.e. transcription gene modulators (sequence-specific DNA-binding molecules), in order to improve the efficacy of the functional molecules. For example, Sugiyama et. al. recently demonstrated the substantial increase in endogenous expression of pluripotent genes, *Oct-3/4* and *Nanog* in mouse fibroblasts by combination of SAHA and the DNA binding hairpin pyrrole-imidazole polyamides (PIPs). [20] Rationally, SAHA could epigenetically induce chromatin to open in the promoter region of *Oct-3/4* and *Nanog*; hence, PIPs could effectively bind to the gene loci, resulting in an increased expression of *Oct-3/4* and *Nanog*. [20] Furthermore, combination therapy of HDACi with other anticancer agents has become a growing research area because the results have demonstrated an enhancement in therapeutic efficiency. The modulators have been combined with radiation therapy, chemotherapy, differentiation agents, epigenetic therapy, and new targeted agents to achieving the synergistic effect. [21–23] Generally, HDACi-treated cells are "sensitized" because their chromatin remain "open", facilitating anticancer drugs that target DNA, i.e. topoisomerase II inhibitor (doxorubicin), to intercalate with DNA, resulting in an implementing effect of the drug to kill cancer cells. Many studies reported a combination of various HDACi, i.e. SAHA, TSA, with anticancer drugs, i.e. doxorubicin, paclitaxel, and showed significant increase in cell apoptosis. [24–26]

Although HDACi show a great promise as therapeutic inventions for many ailments, many reports criticize about the use of HDACi due to the non-specific nature of the modulators. Moreover, inducing hyperacetylation by indirect inhibition of HDACs can potentially improve the chance to disturb key cellular functions regulated by HDACs. [27] To date, there are eighteen HDACs identified with functional redundancy and often associated as a complex rather than functioning individually which makes specific targeting impossible. [28] This encourages scientists to keep looking for the new modulators of HDACs as well as HATs that show specificity towards these specific histone-modifying enzymes. In the last decade, there was a new class of modulator, namely HAT activators, has been discovered. [29] Unlike HDAC inhibitors, HAT activators induce hyperacetylation by activating HAT enzymes. The first small molecule

HAT activator was discovered by Balasubramanyam et. al. [29] This HAT activator, *N*-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide (CTPB), is an amide derivative of anacardic acid, which can be extracted from cashew nutshell liquid. The molecule showed specificity to activate p300 without affecting activity of HDACs, as well as other regulatory factors in the basal transcription machinery. The specificity of this modulator towards a specific enzyme, p300, makes it become a valuable tool in fundamental study, as well as drug development. Inspired by CTPB, several synthetic analogues of CTPB were reported and showed different level of variation in potency of HAT activation upon molecular modification. [30–32] For example, a simplified analogue of CTPB, *N*-(4-Chloro-(3-trifluoromethyl)phenyl)-2-ethoxybenzamide (CTB), has been reported with a slightly higher potency than that of the parent compound (CTPB). [31] The structural basis and mechanism of HAT activation by CTPB and CTB were studied by using computational approaches [33, 34], docking analysis [35], surface-enhanced Raman spectroscopy [31, 36], as well as evaluation of structural-activity relationship by altering chemical entities of the lead compound. [31, 32] The results collectively showed that CTPB and CTB functions by binding to the protein (p300) to induce a structural change of p300, resulting in promoting acetyl-CoA recruitment, thereby enhancing hyperacetylation (HAT activity). Not only does the protein induce structural change upon binding to CTPB/CTB, but also the molecules themselves change their conformations to facilitate binding. From the studies, the polar groups on the molecules, C=O, C-F, N-H, O-C_2H_5 were identified as the binding sites to the protein via hydrogen-bonding with the side chain of amino acids.

Initially, the use of CTPB was limited only *in vitro* due to the severe solubility and membrane permeability issue. This issue was addressed by several strategies, including molecular modification and nanoparticle delivery. In particular, Souto et. al. modified CTB to possess a polar functional group, i.e. hydroxyl, at the original pentadecyl position, but they varied the length of the alkyl chain. [32, 37] Those derivatives showed slight improvement in membrane permeability. [32] On the other hand, nanoparticle-based delivery of a CTB derivative, TTK21, to solve permeability issue was demonstrated by Chatterjee et. al. [30] TTK21 was covalently conjugated to carbon

nanospheres and successfully localized the TTK21-loaded nanoparticles in the nucleus, leading to the enhancement of acetylation compared to treatment with small molecule TTK21 in HeLa cells. Moreover, this study extended to the application in treatment of a neurodegenerative disease, Alzheimer′s disease. TTK21 showed enhanced differentiation and maturation of hippocampal neurons resulting in promoting long term memory of the in vivo mice model. For application in cancer therapy, CTB has shown to increase acetylation of a down-regulated tumor suppressor gene (p53), thereby induced apoptosis in MCF-7 breast cancer cells in a significantly higher extent than lung fibroblast cells (MRC-5, a non-tumorigenic control of this study). [38]

1.2 Gold nanoparticles in drug delivery applications

Over decades, nanoparticles making up of a vast variety of different materials with different elemental composition, size, shape and physical or chemical properties are synthesized and utilized in many biomedical applications, for instance, drug delivery. The term 'nanoparticle' typically applies to particles between 1 and 100 nm. [39] Generally, the synthesis of nanoparticles involves the use of stabilizing molecules (referred to as ligand) to bind to their surface, which allow larger nanoparticles to grow against aggregation, by stabilizing the nuclei with a repulsive force, thereby achieve general control of growth of the nanoparticles over rate, final size or geometric shape. The ligand molecules not only perform their function in stabilizing the growth of the particles during synthesis, preventing the aggregation of the nanoparticles, but also being subjected to functionalize with functional molecules, i.e. biologically active molecules, which allow these nanoparticles to use for specific applications in the field of biomedicine.

Gold nanoparticles (AuNPs) have a long-standing use as promising drug delivery vehicles for molecular and biomolecular therapeutics among other nanocarriers, including liposomes, polymer micelles and vesicles, dendrimers, nanocapsules, and other metal nanoparticles, because they possess a number of desire properties. [40–45] First of all, AuNPs are inert, non-toxic, and biocompatible, which are essentially desirable properties for ideal drug delivery vehicles. Secondly, the synthesis of AuNPs is easy and well-established for a wide range of sizes from 1 nm up to more than 100 nm with strictly controlled dispersity. The control over size and dispersity are crucial for drug delivery applications. In specific, suitable sizes are needed for integrating biomolecules such as proteins and DNA, into biological systems. Furthermore, a variety of shapes, such as spherical, rod-like, core?shell, etc., can be prepared. Shape and size are the critical key parameters that control the optical and electronic properties of gold, i.e. surface plasmon resonance (SPR), which have found useful in many biomedical applications, for instance, biosensors or photothermal therapy. For example, gold nanorods, nanoshells, and other gold nanocrystals can absorb light in the near infrared (NIR) region and induce localized heat. This feature is particularly useful for photothermal therapy of

cancer because cancer cells have shown to be sensitizing by heat. Moreover, AuNPs, as well as other nanoparticles, exhibit the high surface area-to-volume ratio; consequently, they permit dense loading of functional materials, including, targeting and therapeutic materials, onto a single nanoparticle. For example, a 2 nm gold nanoparticle can be loaded $\sim$ 100 ligands by covalent conjugation onto its surface, providing a $\sim$ 110 nm^2 for surface interaction. [46] The total amount of materials loading on AuNPs is tunable by varying size of the particle. Finally, one of the most important aspects of AuNPs is their ease of functionalization due to the facile and well-established surface functionalization strategies. The surface of AuNPs can be tailored with a variety of functional molecules. This highly tunable and multivalent surface structure enables the incorporation of multiple therapeutic drugs or biomacromolecules on the surface of a single nanoparticle, creating functional monolayer-protected clusters. [39, 45, 46] Additionally, a variety of functional monolayers can be functioned to release the payloads using internal or external stimuli such as, pH, enzyme, heat, and light. [40–42, 47–49] Thus, this monolayer platform is an appealing feature for using AuNPs as drug and biomolecule delivery systems.

Ligand systems on the surface of AuNPs are not only aspired for attaching the functional molecules to perform various applications, but also help improve the stability of the nanoparticles by preventing aggregation. [42, 46] Functional ligands can be attached to the nanoparticles as a surrounding layer, called self-assembly monolayer (SAM), to form monolayer-protected clusters (MPCs). The MPCs can be formed either by fabrication of nanoparticles in the presence of ligands or post-functionalization of the pre-formed nanoparticles using ligand exchange reaction established by Murray et. al. [50] The original approach to prepare MPCs of gold is reduction of $HAuCl_4$ (precursor) by citrate producing citrate-protected AuNPs. [41, 43, 46] In this method, citrate acts as both the reducing agent and stabilizer. The size of nanoparticles can be controlled by the feed ratios of $HAuCl_4$ to sodium citrate. Another popular method to prepare MPCs employs biphasic synthetic strategy known as Brust-Schiffrin method. [51] In this method, the initial two phases consisting of alkanethiols (stabilizing ligands) presented in organic phase and $HAuCl_4$ in aqueous phase was added a phase transfer

agent, tetraoctylammonium bromide (TOAB), to transfer $AuCl_4^-$ from the aqueous phase to the organic phase. The organic phase is subsequently reduced by sodium borohydride ($NaBH_4$) to afford alkanethiol-protected MPCs. The monolayers of MPCs can be further elaborated to form mixed monolayer-protected clusters (MMPCs) via Murray ligand exchange reactions. This popular approach for preparing multifunctional AuNPs that possess more than one of functional ligands on the surface. This strategy provides the advantages in the restriction of size and dispersity of the particles owing to the employment of discrete pre-formed nanoparticles with the desirable core sizes. Many strategies have been developed for post-functionalization of gold nanoparticles as drug delivery vehicles are developed, including covalent binding, drug encapsulation, electrostatic adsorption, and other non-covalent assemblies. [39–43, 45–47]

Covalent attachment to AuNPs is the most common type of integration of molecular and macromolecular payloads to the surface of gold nanoparticles. [45–47] Covalent conjugation onto AuNP surface utilizes the well-known thiol-gold chemistry. Thiol functionality has showed the highest affinity to gold, as well as other noble metal. Countless reports in drug delivery systems based on functionalized AuNPs have demonstrated the use of this thiol-gold chemistry to bound functional molecules that have inherent thiol groups on the molecule. In the absence of thiol on the desired molecules, thiol functionality can be introduced by attaching the desired molecules to a 'linker' or 'spacer' molecules that possess thiol functionality through bioconjugation. A wide variety of bioconjugation strategies have been reported for every class of molecules, including small molecules, proteins, RNAi, and DNA. [47] To design bioconjugation, one of the important design principles is that the conjugation should preserve the activity of the conjugated molecule. Therefore, one needs the knowledge about structure-activity relationship of the conjugated molecules to successfully conjugate and maintain the activity of the functional molecule. Zubarev et al showed an interesting design of bioconjugation bearing no functional group to directly attach to AuNPs. [52] In this work, a small molecule chemotherapeutic drug, paclitaxel, was covalently conjugated to 2 nm AuNPs. The author rationally selected hydroxyl group at the C7 position of paclitaxel according to the structure-activity relationship reported for the drug. The conjugation strategy

included the attachment of an oligo(ethylene glycol) linker to C7-OH of paclitaxel following by coupling of the PEGylated-paclitaxel to phenol-functionalized AuNPs. Interestingly, the AuNP-paclitaxel possessed a function of self-therapeutics, which means the drug needed not to be release to perform its therapeutic functions. [52] Different designs of conjugation of different small molecule drugs onto AuNPs were extensively reported. [45, 53, 54] Interesting, these studies made the incorporation of poly(ethylene glycol) (PEG) as linkers become a conventional benchmark for covalent conjugation of functional molecule onto AuNPs. PEG is a linear polymer comprising of repeat ethylene units. The modification of molecules with PEG is called PEGylation. PEGylation of poorly soluble drugs, including small organic molecules or proteins, proved to help with their water solubility. Moreover, PEG also provides steric stabilization to AuNPs when attaching on their surfaces. The end-functional OH groups of the PEG chain can mono-, homo-, and heterobifunctionalize with different functional groups, i.e. NH_2, COOH, to facilitate conjugation of functional molecule to AuNPs. [39, 55] Utilization of AuNPs, especially mixed monolayer-protected clusters (MMPCs), for drug delivery applications offers a distinct set of advantages extended beyond the general nanoparticle system offered, such as improve water solubility of the drug. MMPCs have been used to design efficient receptors for molecular recognition of biomolecules. [46] The presence of multiple ligands on MMPCs allows increase in the affinity and selectivity of the recognition process because they can self-template to complementary surfaces of the guest molecule. The attachment of functional organic molecules on the nanoparticle surface as multivalent recognition elements targeted at biomolecular surfaces allows the modulation of proteins and nucleic acids in the way that is impossible through the traditional use of nanoparticles as support elements. One interesting application is recently published by Patel, et. al. [56] In this work, AuNPs have been surface functionalized with three ligands that imitate the natural transcription factors: DNA-binding domain, activating domain, and nuclear localization signal domain, to create a platform, called Nanoscript, for modulation of gene expression at transcriptional level. Basically, the transcription factors function by binding their DNA-binding domain to the desired gene

sequence on DNA, and then the activating domain recruits the transcriptional machinery complex (e.g. a series of enzymes) to initiate transcription. In such application, administration of the combination of the individual domain is unlikely to achieve to initiate such complex biological process, i.e. transcription.

1.3 Conclusion

Therapeutic gene modulation has become an emerging subject to study over the past decades due to the potential to fix deregulate genes at the upstream level. The concept of epigenetics also becomes a fast-growing area of research because of the link between epigenetics to development of cell, i.e. proliferation and differentiation, and diseases, such as cancer. Modulation of chromatin structure to regulate gene expression is one of the main focuses in epigenetic gene modulation. Epigenetic modulators, such as HAT activators and HDAC inhibitors, have a potential to become future therapeutics playing crucial role in restoring histone acetylation levels in disease conditions associated with hypoacetylation. The synergistic effects of these modulators with other type of epigenetic modulators, or even with other classes of therapeutic agents have a great potential to advance therapeutic efficacy. One important challenge is how to combine these modulators with other therapeutic agents to give high therapeutic efficiency. The use of nanoparticle delivery systems have been set as a new standard for delivering of multiple drugs by co-delivery systems. Multiple drugs can be loaded to nanoparticles, i.e. gold nanoparticles, which could enhance the therapeutic effect of the drug by overcoming issues in solubility, cell permeability, and stability of the drugs, minimizing toxicity, as well as providing a mean to control ratios of each drug to get the most effective synergistic effect of the drug combination. Importantly, nuclear targeting is of significant due to target enzymes are located in the nucleus. Nanoparticles can allow attaching the targeting moiety to promote localization of the systems at the desirable region. Therefore, utilization of nanoparticle-based delivery system can expect the great promise in delivering epigenetic modulators to efficient modulation of gene expression.

Chapter 2

A Nanoparticle-based epigenetic modulator

2.1 A Nanoparticle-based histone acetyltransferase (HAT) activator

2.1.1 Introduction

Histone acetyltransferases (HATs) are an interesting target for drug development due to the link between histone acetylation and pathologies. HAT activators functions to activate HAT enzymes to acetylate histone, resulting in loosen chromatin state (transcriptionally active). Dysfunction of cells due to gene silencing can be restore balance by treating with HAT activators. Moreover, induced chromatin to stay in loosen state by hyperactylation is also beneficial for many applications in gene modulation. A small molecule activator of HAT, namely CTB, was reported as a potent HAT activator, which can regulate the function of HAT to induce hyperacetylation, resulting in loosen chromatin state. To date, CTB and its derivative have been tested for potential drug candidates for therapies of cancer and neurodegeneration. However, CTB suffers from the severe solubility and membrane permeability issue, which is a huge barrier for application of this modulator to in vivo system. [29–31, 57] The use of nanoparticle-based delivery system to overcome this challenge was reported. Chatterjee et. al. [30] demonstrated the use of carbon nanospheres to covalently conjugate a CTB derivative, TTK21, through an amide linkage between the secondary amine of the benzamide and carboxylic acid on the surface of the nanoparticles. They successfully showed localized the TTK21-loaded nanoparticles in the nucleus and observed the enhancement of acetylation compared to treatment with small molecule TTK21 without nanoparticles in HeLa cells. Moreover, this study extended to the application in treatment of a neurodegenerative disease, Alzheimer?s disease. TTK21 showed enhanced differentiation

and maturation of hippocampal neurons resulting in promoting long term memory of the in vivo mice model. Therefore, nanoparticle-based delivery system can be a powerful tool to overcome this intrinsic solubility and permeability problem and permit the practical use of CTB in biological applications.

Moreover, induction of hyperacetylation allows versatility of CTB to potentially combine with other therapeutic agents to achieve synergistic effect and improve therapeutic efficiency. [21,23] For example, several case studies showed that hyperacetylation-induced by HDAC inhibitor could sensitize cancer cells, and by synergistically treat with other anticancer drugs that target DNA, i.e. doxorubicin, the growth inhibition and potentiation of apoptosis were observed. [26]

To harness the full potential of CTB, there is a common challenge in delivering the molecule into the cell, specifically in the nucleus where those HAT enzymes locating. To further application of CTB for combination treatment, the challenge is to deliver CTB with other functional molecule to deliver correct stoichiometric amount of each component and/or at the same site. Based on this concern, the need for a delivery platform that provides i) solubility and permeability, ii) versatility to incorporate other functional molecule, iii) minimal cytotoxicity. Herein, we developed a nanoparticle-based platform to deliver CTB with facile surface functionalization to allow potential co-delivery with other therapeutic molecules with CTB.

2.1.2 Results and Discussion

Construction of nanoparticulate CTB

The nanoparticulate CTB, named Au-CTB, was prepared by assembling three functional components on a single AuNP: 1) *N*-(4-chloro-3-(trifluoromethyl)phenyl)-2- ethoxybenzamide (CTB) as the HAT activator 2) TAT peptide as a nuclear localization signal (NLS) or cell-penetrating peptide (CPP), and 3) poly(ethylene glycol) (PEG) as a water-solubilizing and stabilizing moiety (Figure 2.1). The first component was prepared by synthesizing a new CTB analogue that possessed the following criteria: carrying functionality that can be used for anchoring to the surface of AuNPs, alleviating

the solubility issue of CTB, and most importantly, preserving the potency of CTB as HAT activator. To this end, we decorated a thiol-terminated PEG chain (PEG-SH) to the CTB structure to get the new CTB analogue, so called thiol-PEGylated CTB (Figure 2.3, compound 7). Due to the absence of functional groups that can attach to AuNPs, CTB requires the thiol functionality as the anchoring group enabling the covalent attachment of the molecule to gold nanoparticle surface via Au-S chemisorption (126 kJ/mol). [54,58,59] The PEG moiety was employed to improve the solubility of CTB. In addition, the PEG portion also provides several advantages to the system: (i) minimizing opsonization and clearance by reticulo-endotherial system (RES), (ii) reducing non-specific binding and protein adsorption, (iii) offering stability over the wide range of pH, temperature and ionic strength. [39, 46, 52–55, 60] To preserve the potency of HAT activation, we rationally selected the C3 position of CTB as the site of PEG-SH attachment based on the structure-activity relationship reported for CTB (Figure 2.2). [29, 31, 32] Certain modifications at the C3 position of CTB analogues with different aliphatic chain lengths, as well as end-functional groups, i.e. hydroxyl, have shown to preserve the function as a HAT activator. [32] On the contrary, we avoided conjugation at the functional groups that are responsible for binding to the HAT enzymes, such as CF_3, Cl, NH, C=O, OC_2H_5. [31, 33, 34, 36]

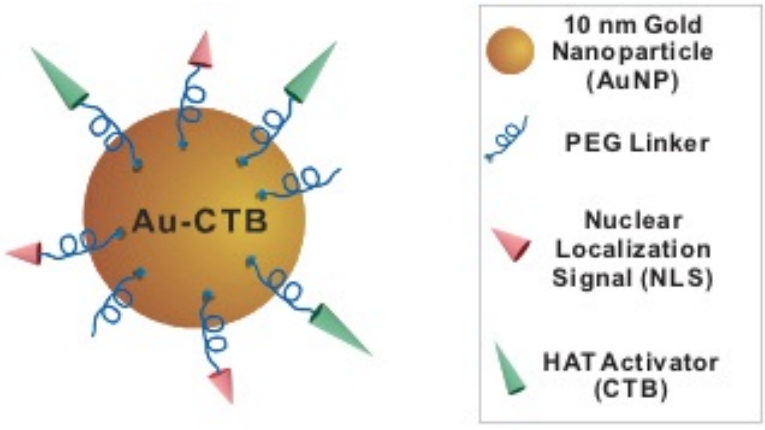

Figure 2.1: Design of Nanoparticulate HAT Activator CTB (Au-CTB)

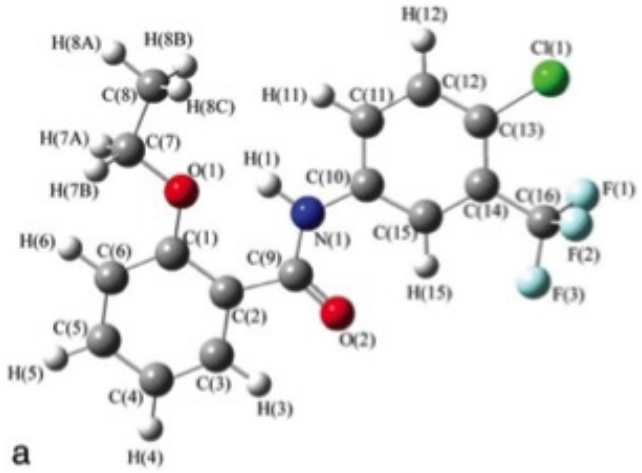

Figure 2.2: Chemical structure of *N*-(4-chloro-(3-trifluoromethyl)phenyl)-2-ethoxybenzamide (CTB). Reprinted from reference [34]. Copyright 2011 Wiley Periodicals, Inc.

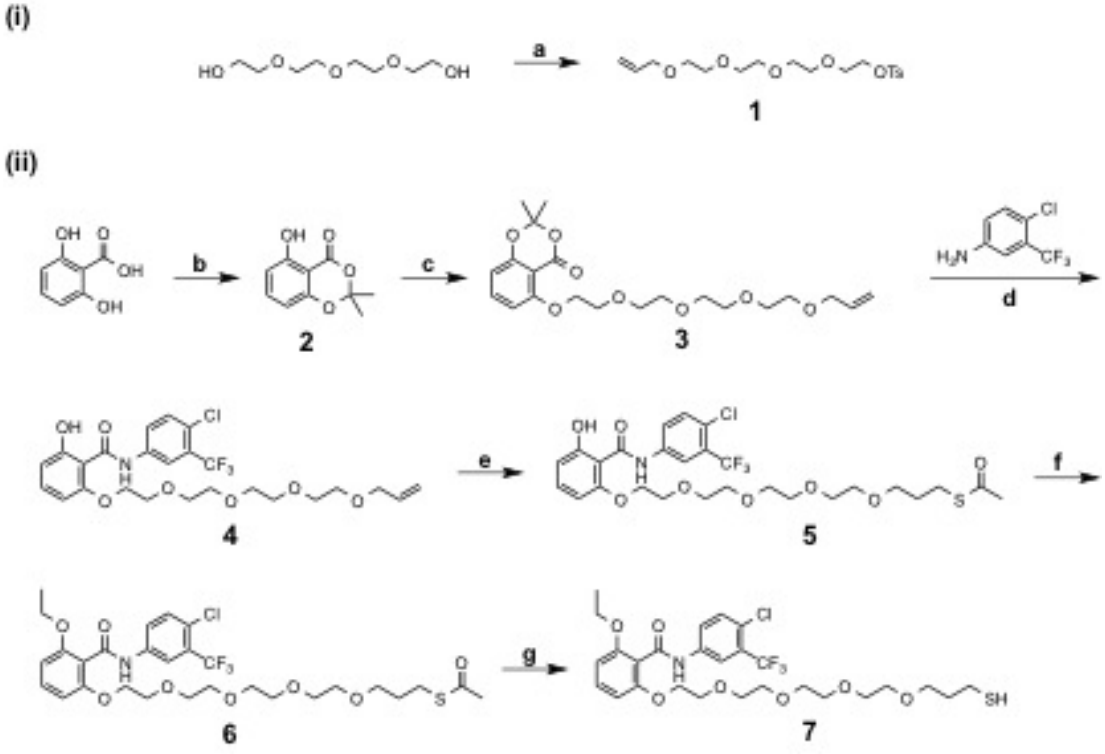

Figure 2.3: Synthesis of thiol-PEGylated CTB. (i) Preparation of heterobifunctional PEG linker: (a) (a-1) NaH, allyl bromide, DMF, 25 h, (a-2) TsCl, DMAP, Et_3N, CH_2Cl_2, 16 h. (ii) Synthesis of thiol-PEGylated CTB: (b) TFA/TFAA, acetone, 24 h; (c) **1**, K_2CO_3, acetonitrile, 80°C, 24 h; (d) 4-chloro-2-(trifluoromethyl)aniline, DMPU, *n*-BuLi, THF, 80°C, 2 h; (e) AIBN, thioacetic acid, toluene, 100-110°C, 16 h; (f) Et_2SO_4, K_2CO_3, acetone, 48 h; (g) TBACN, MeOH, $CHCl_3$.

The synthesis of thiol-PEGylated CTB was illustrated in Figure 2.3. The synthesis divided into two parts: (i) preparation of heterobifunctional PEG linker (**1**) and (ii) synthesis of thiol-PEGylated CTB (**7**). The synthesis began with the protection of 2,6-dihydroxybenzoic acid using acetone and TFAA/TFA to afford 2. 2,6-dihydroxybenzoic acid was used as a synthon to synthesize the CTB analogue because the protected product, 1,3-benzodioxinone (**2**), has a free hydroxyl group available at the C3 position for further modification. [32,37] Conjugation of the PEG linker to the aryl ring was inspired by the reaction of the tosyl PEG and phenolic hydroxyl group in basic condition, reported by Balaz et. al. [61] In a separate synthesis, the heterobifunctional PEG linker (**1**) was designed to possess a tosyl group at one end for attaching to the phenolic OH of (**2**), while the allyl group on the other end would be transformed into thiol. After PEG conjugation, 5-tetraethyleneglycol substituted benzodioxinone (**3**) was deprotected by heating with pre-treated aniline, 4-chloro-3-(trifluoromethyl)aniline, with *n*-BuLi in DMPU at 80°C to afford salicylamide (**4**). The alkene on PEG portion of the salicylamide (**4**) was then converted into thioacetate via free radical mediated nucleophilic addition across the double bond using AIBN to form thioacetate (**5**). Subsequently, the compound **5** was alkylated to get thioacetate-protected CTB (**6**). The typical protocols to deprotect thioacetate employ harsh acid or basic conditions, i.e. refluxing in 1M HCl [50, 54]or treating with 1M MeONa [62]. We found that these deprotection procedures are not suitable for converting thioacetate of compound **6** to the free thiol due to the sensitivity of the amide bond toward hydrolysis in acidic and alkaline conditions. Therefore, we utilized a mild deprotection condition using catalytic tetrabutylammonium cyanide in the presence of MeOH reported by Holmes et. al. [63] to afford thiol-PEGylated CTB (**7**).

A Nuclear localization signal (NLS) peptide was included in this platform as the second component for facilitating delivery of the functionalized nanoparticles (Au-CTB) into the nucleus. An NLS peptide is a short amino acid sequence that mediates the transport of molecules into the nucleus. The NLS peptides are essential for shuttling macromolecules or particles with a size larger than 9 nm (40-60 kDa) through the nuclear pores because only molecules smaller than 9 nm can freely transport through the

pores. [64–66] We utilized a TAT peptide, CALNNAGRKKRRQRRR, as NLS domain. To conjugate the TAT peptide to AuNPs through thiol-gold chemistry, a thiol group was introduced to the TAT peptide by EDC/NHS coupling between an amino group on the peptide and the carboxylic acid of carboxyl-PEG-thiol via EDC/NHS coupling. EDC/NHS coupling is a widely used bioconjugation method for conjugating TAT and other peptides to linker molecules, i.e. COOH-PEG-SH. [47, 67] Lastly, the homofunctional PEG (thiol-terminated PEG, PEG-SH) was also included in the system in order to add stability to the nanoparticle system, as well as to make use of the aforementioned advantages of PEG.

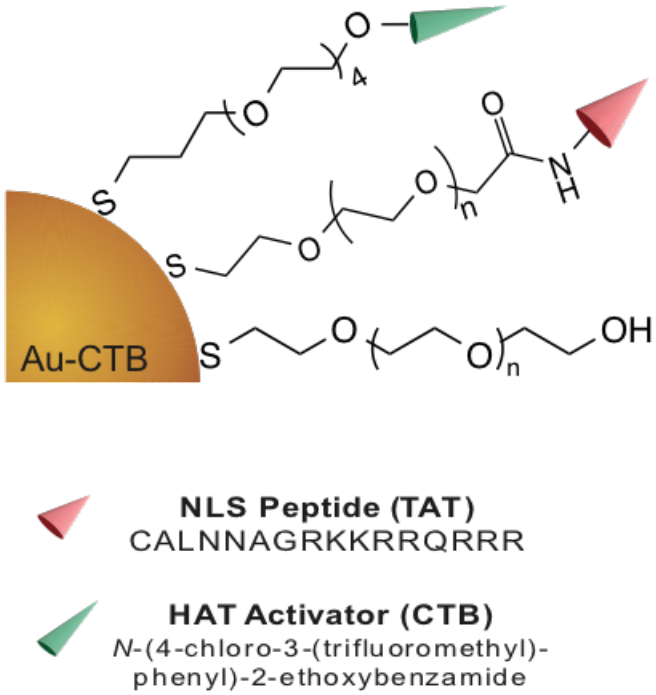

Figure 2.4: Assembly of nanoparticulate CTB (Au-CTB) via thiolate PEG spacers

The three functional components (CTB, TAT, PEG) were then assembled onto a single nanoparticle via a ligand exchange reaction. All the components bearing free thiol groups were readily adsorbed onto the nanoparticle by gold-thiol chemisorption (Figure 2.4). [39–41, 43, 45, 47] The amount (concentration) of each component on a nanoparticle could be controlled by adjusting the concentration of the separate solutions of each component. In this work, we fixed the content of the TAT peptide to be 20% in order

to allow enough transporting effect of TAT to shuttle Au-CTB into the nucleus. The concentration of CTB in the formulations of Au-CTB was empirically selected to be 0%, 33%, 66% and 100% to study the effect of CTB concentration toward HAT activity. Activation of HAT by other CTB analogues reported to have concentration-dependent manner. [29, 30] The PEG content was added up to get 100% of the formulations.

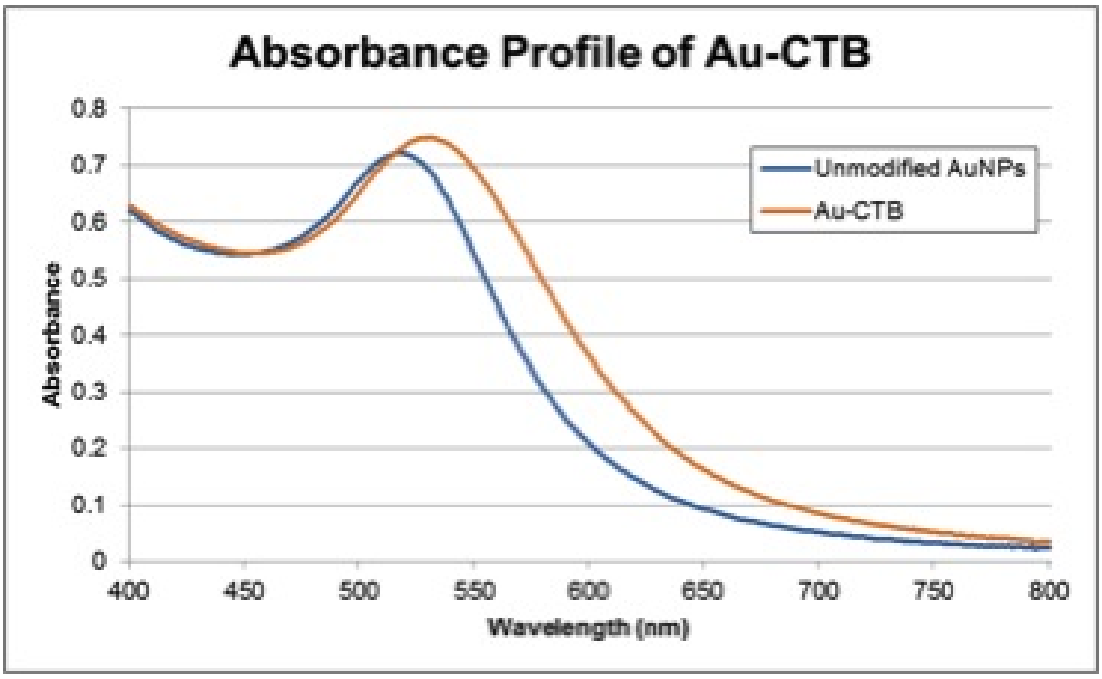

Figure 2.5: Surface plasmon resonance (SPR) band of unmodified gold nanoparticles (AuNPs) (blue) and nanoparticulate CTB (Au-CTB) (orange).

The nanoparticulate CTB was characterized by dynamic light scattering (DLS) and UV-Visible spectroscopy for physical properties of the particles. DLS data reviewed the hydrodynamic diameter of Au-CTB to be 22.6 ± 2.7 nm. The increase in size (the size of unmodified AuNPs are 10 nm) was the indication that the functional ligands were bound on the nanoparticle surface. The UV-Vis spectra could be used to monitor the stability of the nanoparticles. The stability of gold nanoparticles can be observed from the SPR band. Aggregation or instability of gold nanoparticles causes the redshift from the original SPR peak due to lowering in SPR energy caused by delocalization of electrons between surfaces of the aggregated particles. In addition, the intensity would decrease and the peak would broaden. Figure 2.5 showed that our nanoparticles were stable

after the ligand exchange. Surface functionalization of AuNPs shifts the SPR band due to the interaction between the ligand field and the surface electron cloud. [68] As expected, we observed the redshift of 15 nm after functionalization with the functional ligands. The phenomena could be explained by two effects: first, conjugating the functional ligands onto the surface of AuNPs altered the electronic environment of the nanoparticle surface; second, the change in the reflective index of the solvent could influence the shift in the SPR band, according to Mie theory, upon adding DMF solution of the ligands. [68]

HAT activation of Au-CTB

A series of experiments were conducted in order to evaluate the effectiveness of our newly synthesized CTB analogue and thenanoparticulate CTB. Firstly, we tested for the HAT activity of HeLa cells treating with thiol-PEGylated CTB (compound **7**) and its nanoparticulate formulation (Au-CTB) toward using a HAT Activity Assay Kit. In principle, HAT enzyme catalyzes the transfer of acetyl groups from Acetyl-CoA to the histone peptide, thereby generating two products - acetylated peptide and CoA-SH. The CoA-SH was used for quantification of HAT activity by a fluorometric technique. We first confirmed the activity of compound **7** before attaching it to the nanoparticles. Figure 2.6 showed that compound **7** modified at the C3 position with poly(ethylene glycol) enhanced HAT activity in HeLa cells. To compare the influence of nano particle platform, we checked HAT activity of Au-CTB compared with CTB (Compound **7**) at different time points. We checked at 12, 24, and 48 h after treatment and found that the HAT activity reached the extremities at 24 h in both forms (molecular and nanoparticulate) as shown in Figure 2.7. The highly insoluble and membrane impermeable nature was reported for the parent compound, CTPB, and its analogues. [30,32,37] In principle, delivery of CTB by covalently attaching to nanoparticles could enhance binding to its target protein due to multivalency effect, resulting in an increase of potency of CTB. [46] This principle explained the higher CTB-induced HAT activation of the nanoparticulate form (Au-CTB) than that of the molecular form (compound **7**) as shown in Figure 2.7. To further investigate versatility of Au-CTB, we prepared four

gold nanoparticulate formulations of CTB (Au-CTB) with three different CTB contents; 0%, 33%, 66%, and 100%, having the molecular CTB with the same amount used in 100% Au-CTB formulation as reference (Figure 2.8). As expected, we observed the concentration-dependent HAT activity, which was in agreement with reported literature for the concentration-dependent HAT activation of CTB and its analogues. [29–31] This experiment showed that our Au-CTB platform permitted the control over the surface ligand ratio, which is an important feature for future applications in combination therapy.

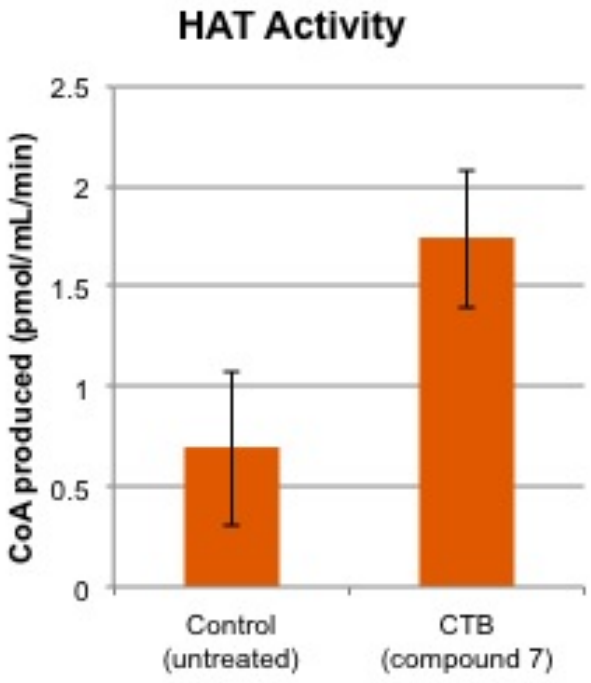

Figure 2.6: HAT activity of thiol-PEGylated CTB (Compound **7**).

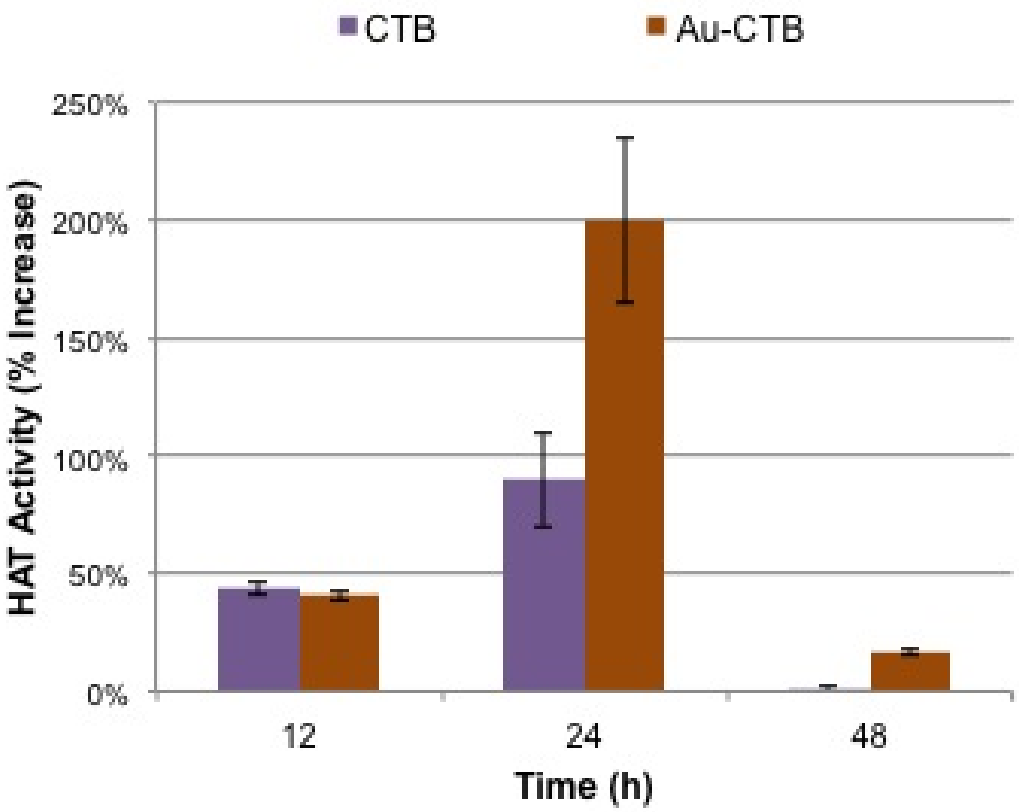

Figure 2.7: Time course experiment of CTB-induced HAT activation. The comparison of HAT activity representing by percent increase in HAT activity of free CTB (CTB) and nanoparticulate CTB (Au-CTB) with the same amount of CTB.

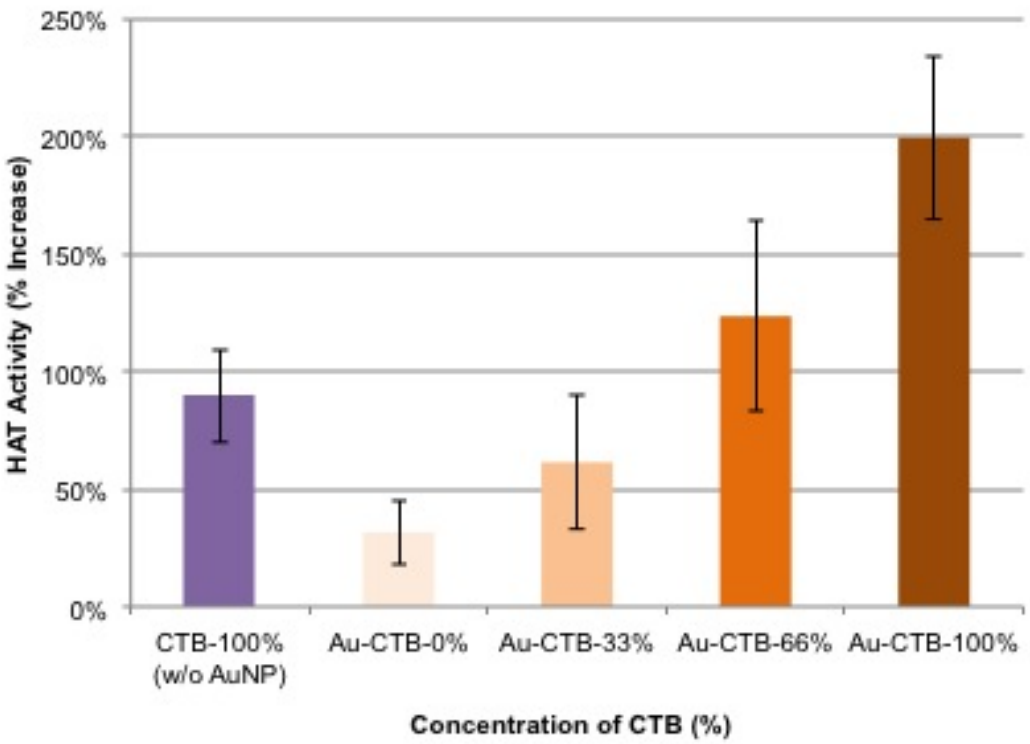

Figure 2.8: Concentration-dependence of HAT activation by Au-CTB. HAT activity of nanoparticulate CTB (Au-CTB) and four Au-CTB formulations with different CTB contents (0% in Au-CTB-0%, 33% in Au-CTB-33%, 66% in Au-CTB-66%, 100% in Au-CTB-100%). Note: The % content reported with respect to the total amount of functional ligands (CTB, NLS, PEG) on AuNPs.

Cytotoxicity of nanoparticulate CTB

Our nanoparticulate CTB (maximum loading, 100% CTB) was tested non-toxic against HeLa cells at the maximal HAT activation (24 h) using MTS assay. Figure 2.9 showed that cell viability of Au-CTB compared with untreated control.

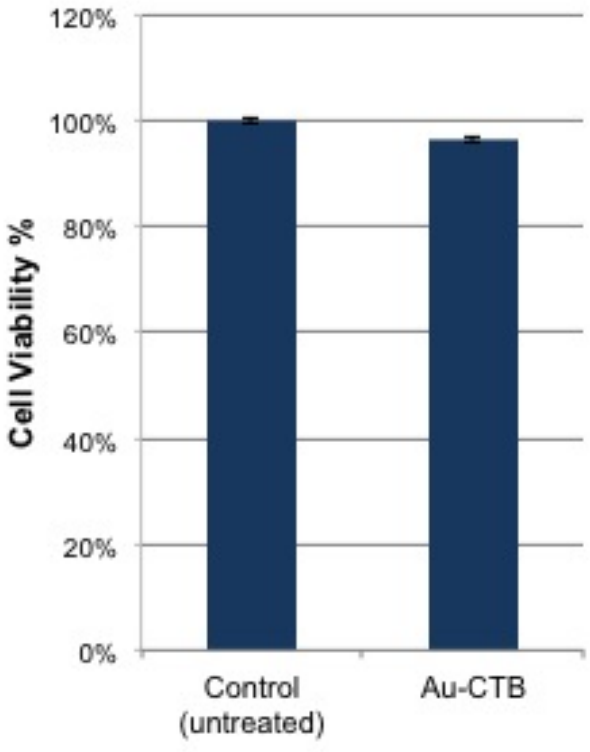

Figure 2.9: Cytotoxicity of Au-CTB.

2.1.3 Conclusion

In summary, we fabricated a nanoparticle-based platform to efficiently deliver a HAT activator CTB to induce hyperacetylation. We first synthesized a new derivative of a known HAT activator molecule CTB. The new CTB, namely thiol-PEGylated CTB, was introduced PEG moiety to improve the solubility of the compound and act a spacer. We end-terminated our PEGylated CTB with thiol functionality as an anchor to AuNPs. Secondly, we constructed nanoparticulate CTB (Au-CTB) composing of thiol-PEGylated CTB, TAT peptide, and PEG ligands. All of these components have their own function in the system: i) CTB acts as key functional molecule to activate HAT enzymes, ii) TAT-peptide uses for improving cell permeability and nuclear localization, iii) additional PEG ligands on the surface of AuNPs help with solubility and prevent non-specific binding. Finally, by making use of facile surface chemistry of AuNPs, we could prepare a series of Au-CTB formulations with different concentration of CTB and tested their HAT activity. The HAT activity confirmed the benefit

of using nanoparticles as delivery vehicle to enhance delivery of CTB. Thus, this Au-CTB platform can be a valuable tool for applications in epigenetic therapy as well as other therapeutic applications that require the modulation of chromatin structure for synergistic functions.

2.1.4 Materials and Methods

General procedures

All reactions were conducted in oven-baked glassware with magnetic stirring under nitrogen or argon atmosphere. All reagents and solvents (anhydrous) were purchased from Sigma-Aldrich, Acros Organics, Alfa Aesar and used without further purification. Analytical thin layer chromatography (TLC) was performed on silica gel matrix on TLC Al foil plates with fluorescent indicator 254 nm. Visualization was accomplished with UV light and iodine stain. Purification of reaction products was carried out by flash column chromatography over Teledyne Isco Redisep Rf silica gel (60 A, 40-60UM). The proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on either a Varian-300 instrument (300 MHz), Varian-400 instrument (400 MHz), or Varian-500 instrument (500 MHz) spectrometer with a set temperature of 25°C. Chemical shifts (δ) of the compounds were recorded in ppm corresponding to the internal standard, tetramethylsilane (TMS). NMR solvent standards were used as follows: for ^{1}H-NMR, $CDCl_3$ = 7.26 ppm. Data were reported as chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants (J) in Hz, and integration. Mass spectrometry was recorded on a Finnigan LCQ-DUO mass spectrometer.

Synthesis of thiol-PEGylated CTB

***p*-toluenesulfonyl tetraethylene glycol allyl ether (1).** Compound **1** was prepared according to literature with slightl modification. [62, 69] Tetraethylene glycol (32.11 g, 0.17 mol) was dissolved in 70 mL DMF and cooled to 0°C under an inert atmosphere. NaH (60% in mineral oil, 1.32 g, 33 mmol) was added in portions. After the

addition, the reaction was stirred at room temperature for 1 h. Then, allyl bromide (4 g, 33 mmol) was added dropwise. The mixture was allowed to proceed overnight. Then, the reaction was quenched with water, followed by washing with hexane and extracting with diethyl ether to yield colorless liquid oil (intermediate compound). The intermediate compound was used for next step without further purification. To an ice-cold solution of the intermediate compound (12.61 g, 54 mmol) in 100 mL dichloromethane, *p*-toluenesulfonate (30.78 g, 0.16 mol) was added. Then, Et_3N (73.41 mL, 0.54 mol) was added dropwise at 0°C using an addition funnel. The reaction was stirred overnight at ambient temperature. Upon completion, the reaction mixture was filtered and the precipitant was washed with aqueous saturated solution of $NaHCO_3$ and dried over $MgSO_4$. After filtration, the precipitant was concentrated *in vacuo* to a yield brown crude mixture. The crude mixture was purified by column chromatography on silica gel using dichloromethane/ethyl acetate (4:1) as eluent to afford yellow liquid oil. Yield. 30%. ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 7.80 (d, J = 8.4 Hz, 2H), 7.34 (d, J = 8.6 Hz, 2H), 5.98-5.83 (m, 1H), 5.31-5.14 (m, 2H), 4.16 (t, 2H), 4.01 (dt, J = 5.7, 1.4 Hz, 2H), 3.72-3.54 (m, 14H), 2.44 (s, 3H); MS (m/z): calculated for $C_{18}H_{28}O_7S$, 388.16; found, 411.12 for $[M+Na]^+$.

5-hydroxy-2,2-dimethyl-4*H*-benzo[*d*-1,3]dioxin-4-one (2). Synthetic procedure of compound **2** was adopted from the literature. [70–72] To a suspension of 2, 6-dihydroxybenzoic acid (3 g) in trifluoroacetic acid (28.8 mL), trifluoroacetic anhydride (18 mL) and acetone (3.6 mL) was added at 0°C. The suspension was warmed slowly to room temperature. After 24 h, the clear yellow solution was observed, and then the volatiles were evaporated under reduced pressure. A saturated aqueous solution of $NaHCO_3$ was added to the residue. Then, the aqueous solution was extracted with three portions of ethyl acetate. The combined organic layers were washed with water and brine, and then dried over $MgSO_4$. The solvent was evaporated after filtrating $MgSO_4$ to afford a yellow crude mixture. Chromatography over silica gel with hexane/ethyl acetate (4:1) as the eluent gave a white solid product. Yield. 35%. ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 10.34 (br, s, 1H), 7.41 (t, J = 8.3 Hz, 1H,), 6.63 (dd, J

= 8.5. 0.9 Hz, 1H), 6.44 (dd, J = 8.2, 0.9 Hz, 1H), 1.75 (s, 6H); MS (m/z): calculated for $C_{10}H_{10}O_4$, 194.06; found, 217.00 for $[M+Na]^+$.

5-((3,6,9,12-tetraoxapentadec-14-en-1-yl)oxy)-2,2-dimethyl-4*H*-benzo[*d*-1,3] dioxin-4-one (3). Compound **3** was synthesized according to the literature. [61] Compound **2** (0.45 g, 2.34 mmol) and *p*-toluenesulfonyl tetraethylene glycol allyl ether 3 (1 g, 2.57 mmol) were reacted in 15 mL acetonitrile in the presence of K_2CO_3 (0.36 g, 2.57 mmol). The reaction mixture was refluxed under inert atmosphere. After 24 h, the mixture was allowed to cool down to room temperature. The mixture was diluted with diethyl ether. The solid byproduct was filtered out. The precipitant was concentrated *in vacuo*. The crude mixture was chromatographed on silica gel column using dichloromethane/ethyl acetate (4:1) as eluent to yield yellow liquid product. Yield. 99%. ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 7.40 (t, J = 8.3 Hz, 1H); 6.62 (dd, J = 8.5, 0.9 Hz, 1H); 6.54 (dd, J = 8.1, 0.9 Hz, 1H); 5.96-5.84 (m, 1H); 5.31-5.13 (m, 2H); 4.22 (t, J = 5.0 Hz, 2H); 4.00 (dt, J = 5.7, 1.4 Hz, 2H); 3.95 (t, J = 5.0 Hz, 2H); 3.84-3.56 (m, 12H); 1.68 (s, 6H); MS (m/z): calculated for $C_{21}H_{30}O_8$, 410.19; found, 433.05 for $[M+Na]^+$.

2-((3,6,9,12-tetraoxapentadec-14-en-1-yl)oxy)-*N*-(4-chloro-3-(trifluoromethyl)phenyl)-6-hydroxybenzamide (4). The synthesis followed a reported protocol. [32,37] To a solution of 4-chloro-3-(trifluoromethyl)aniline (1.92 g, 9.79 mmol) and DMPU (2.46 g, 19.19 mmol) in 25 mL THF was added *n*-BuLi (3.92 mL of 2.5 M in hexane, 9.79 mmol) at 0°C. The reaction mixture was stirred at room temperature for 30 mins. In a separate flask, compound **3** (0.8 g, 1.95 mmol) was dissolved in 25 mL THF. The solution of compound 3 was then added to the aniline solution. After addition, the reaction was proceeded at 80°C for 2 h. Water was added to quench the reaction. Then, the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with 10% aqueous solution of hydrochloric acid, followed by water and brine, then dried over $MgSO_4$. After solvent evaporated, the crude mixture was purified by column chromatography (silica gel, 4:1 dichloromethane/ethyl acetate

mixture) to yield yellow liquid product. Yield. 31%; ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 13.32 (s, 1H), 10.69 (s, 1H), 8.07 (s, 1H), 7.95 (d, J = 8.7, 1H), 7.48 (d, J = 8.7 Hz, 1H), 7.31 (t, J = 8.4 Hz, 1H), 6.67 (d, J = 8.4 Hz, 1H), 6.42 (d, J = 7.6 Hz, 1H), 5.99-5.81 (m, 1H), 5.30-5.13 (m, 2H), 4.34-4.28 (m, 2H), 4.06-3.96 (m, 4H), 3.77-3.71 (m, 2H), 3.63-3.50 (m, 10H). MS (m/z): calculated for $C_{25}H_{29}ClF_3NO_7$, 547.16; found, 570.1 for $[M+Na]^+$.

***S*-(1-(2-((4-chloro-3-(trifluoromethyl)phenyl)carbamoyl)-3-hydroxyphenoxy)-3,6,9,12-tetraoxapentadecan-15-yl) ethanethioate (5).** To a solution of compound **4** (0.34 g, 0.61 mmol) in 30 mL toluene was added AIBN (0.03, 0.18 mmol) and purged with argon. Thioacetic acid (0.23 mL, 0.18 mmol) was added. The solution was stirred at 100-110°C overnight under argon atmosphere. Upon completion, toluene was evaporated. The residue was re-dissolved in fresh toluene, and the procedure was repeated for 3 times. The crude mixture was then loaded into a chromatographic column over silica gel using dichloromethane, following by 15:1 and 10:1 dichloromethane/ethyl acetate mixture) to yield yellow liquid oil. Yield. 65%. ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 10.69 (s, 1H), 8.05 (d, J = 2.2 Hz, 1H), 7.97 (dd, J = 8.7, 2.3 Hz, 1H), 7.49 (d, J = 8.9 Hz, 1H), 7.32 (t, J = 8.3 Hz, 1H), 6.69 (dd, J = 8.4, 1.0 Hz, 1H), 6.43 (dd, 1H), 4.32 (t, J = 4.4 Hz, 2H), 4.02 (t, J = 4.7 Hz, 2H), 3.80-3.38 (m, 14H), 2.92 (t, J = 7.1 Hz, 2H), 2.31 (s, 3H), 1.91-1.74 (m, 2H); MS (m/z): calculated for $C_{27}H_{33}ClF_3NO_8S$, 623.16; found, 646.0 for $[M+Na]^+$.

***S*-(1-(2-((4-chloro-3-(trifluoromethyl)phenyl)carbamoyl)-3-ethoxyphenoxy)-3,6,9,12-tetraoxapentadecan-15-yl) ethanethioate (6).** Following the protocol for alkylation from the literature [32, 37], To a suspension of compound **5** (0.25 g, 0.4 mmol) and K_2CO_3 (0.14 g, 0.99 mmol) in 10 mL acetone was added Et_2SO_4 (77.8 uL, 0.6 mmol). The reaction was stirred for 48 h at ambient temperature. An aqueous saturated solution of NH_4Cl was added to the reaction mixture, and then extracted with diethyl ether. The combined organic layers were washed with brine and dried over $MgSO_4$. After filtration, solvent was evaporated under reduced pressure. The flash

column chromatography was used to purify the mixture using dichloromethane/ethyl acetate (4:1) as eluent to afford yellow liquid oil. Yield. 84%. ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 8.61 (s, 1H); 8.02-7.89 (m, 2H); 7.37 (d, J = 8.6 Hz, 1H); 7.19 (d, J = 8.7 Hz, 1H); 6.50 (m, 2H); 4.14 (t, 2H); 4.00 (quartet, J = 7.0 Hz, 2H); 3.71 (m, 2H), 3.58-3.37 (m, 12H); 3.34 (t, J = 6.2 Hz, 2H); 2.82 (t, J = 7.2 Hz, 2H); 2.23 (s, 3H); 1.72 (quintet, J = 6.7 Hz, 2H); 1.30 (t, J = 6.9 Hz, 3H); MS (m/z): calculated for $C_{29}H_{37}ClF_3NO_8S$, 651.19; found, 674.7 for $[M+Na]^+$.

***N*-(4-chloro-3-(trifluoromethyl)phenyl)-2-ethoxy-6-((15-mercapto-3,6,9,12 -tetraoxapentadecyl)oxy)-benzamide (7).** The protocol for deprotection of thioacetate was adopted from a reported procedure [63]. Briefly, the thioacetate-protected PEG-CTB (21.3 mg, 0.03 mmol) was dissolved in 1 mL chloroform/methanol (1:1) mixture. The reaction vessel was purged with argon before the addition of tetrabutylammonium cyanide (8.7 mg, 0.03 mmol). The reaction mixture proceeded for 24 h. The reaction was monitored by mass spectrometry due to the same Rf value of the reactant and the product. After reaction completed, the reaction mixture was partitioned with chloroform and water. The aqueous layer was then extracted with chloroform. The combined organic layers were washed with saturated NH_4Cl solution and dried over $MgSO_4$. The purification was employed flash column chromatography on silica gel using hexane/ethyl acetate (1:1) as the eluting solvent. Yield. 83%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 8.56 (s, 1H), 7.94 (m, 2H), 7.38 (d, J = 8.5 Hz, 1H), 7.20 (t, J = 8.3 Hz, 1H), 6.50 (dd, J = 8.3, 3.9 Hz, 2H), 4.26-4.08 (m, 2H), 4.00 (quartet, J = 6.9 Hz, 2H), 3.78-3.65 (m, 2H), 3.62-3.28 (m, 14H), 2.49 (quartet, J = 7.3 Hz, 2H), 1.74 (quintet, J = 6.4 Hz, 2H), 1.35-1.23 (m, 4H); MS (m/z): calculated for $C_{27}H_{35}ClF_3NO_7S$, 609.18; found, 632.4 for $[M+Na]^+$.

Construction of nanoparticulate CTB

Nanoparticulate CTB was constructed by attaching thiolate ligands (CTB, TAT peptide, PEG) on to a 10 nm citrate-stabilized gold nanoparticles (AuNPs) (Ted Pella).

CTB and PEG-SH had thiol functional group ready for conjugation, while the TAT peptide was conjugated to a thiolate linker molecule prior to conjugating to AuNPs. The TAT peptide (with terminal OH group) was conjugated to a linker molecule, 1kDa Thiol-PEG-Carboxy (SH-PEG-COOH) (Creative PEGWorks). The PEG molecule was dissolved into a 50 mM solution in ethanol. Then, 50 mM of 1-ethyl-3-(3 - dimethylaminopropyl)carbodiimide) (EDC) (Sigma) and 50 mM of *N*- hydroxysuccinimide(NHS) (Acros Organics) was added to this solution and then placed on a shaker for 1 h to activate the carboxyl group. Afterwards, a 5 mM solution of TAT peptide was added to the solution and allowed to react at room temperature for 2 h. Then, a DMF solution containing a desired ratio of the three functional ligands (CTB, PEG, TAT) was added dropwise to 1mL solution of 7.5 nM AuNPs. The ratio of TAT was fixed at 20%, while the CTB ratio was varied (33%, 66%, and 100%), as well as the PEG ratio. After addition, the functionalized AuNP solution was allowed to stir for 2h, and then filtered three times using a 10,000 MCWO filter (Millipore) to remove unreacted molecule and adjust the volume to concentration the solution.

Characterization of nanoparticulate CTB

The concentration of functionalized AUNPs and confirmation of conjugation was characterized by UV-visible spectroscopy (Varian Cary 5000 UV Vis-NIR Spectrophotometer). Dynamic Light Scattering (Malvern Zetasizer Nano-ZS90) was utilized to measure the hydrodynamic size and surface charge of the functionalized AuNPs.

Cell culture of HeLa cells

HeLa cells were cultured in Dulbecco's modified Eagle medium with high glucose (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum, 1% Glutamax (Invitrogen), and 1% streptomycin-penicillin antibiotic in a 37°C humidified incubator with 5% CO_2. Prior to transfection, 25,000 HeLa cells were seeded in a well of a 24-well plate. After 24 h, the cells were incubated with a mixture of the 1 nM NanoScript solution. After 4 h, the cells were washed twice with PBS and fresh culture medium was added.

HAT activity assay

HAT activity of the cells treated with the modified CTB and the different formulations of nanoparticulate CTB were tested at different time points (12, 24, 48 h) using HAT Activity Fluorometric Assay Kit (BioVision) from their nuclear extracts according to the manufacturer protocol. Nuclear/Cytosol Fractionation Kit (BioVision) was used to separate the nuclear extracts from the cells following the manufacturer protocol.

Cell viability assay

Cell viability was determined by an MTS assay (Promega) at 24 h after incubating the cells with the functional AuNPs, using the standard protocol described by the manufacturer.

Chapter 3

Conclusion and Perspectives

An advance in understanding epigenetic mechanisms and the links of epigenetics to development and diseases significantly change the view of scientists toward understandings of fundamental biological processes as well as development of therapeutic inventions. Molecules that can modulate epigenetic state, epigenetic modulators thereby became a powerful and meaningful tool for studying chemical biology and developing epigenetic therapy. In the past decades, a small molecule activator of HAT was discovered. After that the utilizations of HAT activators were demonstrated and showed a promise as an epigenetic drug candidate. Like HDAC inhibitors, HAT activator can induce hyperacetylation, which, in part, promote the chromatin structure to ?open? and allow accessibility to DNA. This feature is attractive for combing the modulator with DNA targeting molecules, such as transcription factors or anticancer drugs that intercalate to DNA. Our on-going projects utilize this feature of hyperactyation induced by HAT activator for controlled differentiation and treatment of cancer. Specifically, we combine Au-CTB with Nanoscript - a nanoparticle-based artificial transcription factor - possessing DNA-binding domain that target *SOX9* sequence to induce expression of SOX9 protein. SOX9 protein is a well-known key regulator of chondrogenic differentiation of mesenchymal stem cells (MSCs). [73–75] Our hypothesis is that CTB can induce 'open' chromatin structure that facilitates binding of Nanoscript to DNA leading to enhance transcription of *SOX9*, and thereby enhance chondrogenic differentiation. For cancer application, the use of HAT activator can sensitize the cancer cells by controlling the chromatin to stay open, and then delivering drugs that intercalate to DNA, such as doxorubicin, may potentially enhance apoptosis.

These applications make possible due to the achievement in developing nanoparticle-based HAT activator CTB (Au-CTB) described in this thesis. We first presented a new CTB derivative that bears functionality that allow conjugation to AuNPs through a facile surface chemistry. The solubility issue was addressed by incorporation of PEG moiety. Importantly, the integration of nuclear localization signal peptide overhauls the lack of membrane permeability of the parent CTB analog. The use of AuNPs allows the facile surface functionalization via thiol-gold chemistry. We successfully demonstrated the combination of the functional ligands (CTB, NLS, PEG) on to the same nanoparticles with different ratios. This feature has the advantage in providing facile method to conjugate to different ligands onto the same nanoparticle permitting an effective mean for co-delivery. Lastly, we verified that HAT activity was preserved throughout these modifications. Overall, this nanoparticle-based CTB platform can further utilize to a wide ranges of applications ranging from fundamental biological studies to develop new therapy.

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