Nanomaterials in theranostics: therapeutics and diagnosis against infectious diseases

Unnatiben S. Patel

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Unnatiben S. Patel

04/22/2022

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DISSERTATION APPROVAL FORM

Submitted by Unnatiben Shaileshkumar Patel in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology Science and Engineering and accepted on behalf of the Faculty of the School of Graduate Studies by the dissertation committee.

We, the undersigned members of the Graduate Faculty of The University of Alabama in Huntsville, certify that we have advised and/or supervised the candidate on the work described in this dissertation. We further certify that we have reviewed the dissertation manuscript and approve it in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology Science and Engineering.

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ABSTRACT

The School of Graduate Studies

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Degree  Doctor of Philosophy  College/Program Science/Biotechnology Science and Engineering

Name of Candidate  Unnatiben Shaileshkumar Patel

Title  Nanomaterials in Theranostics – Therapeutics and Diagnosis Against Infectious Diseases

Nanomaterials-based hybrid nano therapy is gaining attraction as a promising way to treat intracellular bacterial infections. Gold-based nanomaterials have been widely used for biomedical applications such as photothermal therapy (PTT). This thesis discusses the development of a combination therapeutic approach that kills intracellular bacteria in conjunction with photothermal and antibiotic therapy using gold nanorod (GNR) based nano-assembly. This NIR laser-activated nano-assembly delivers antibiotics to the site of infection and offers PTT. The synergistic application of both therapies increases the efficacy of treatment. The protected delivery of antibiotics and their release in the proximity of the bacterial surface decreases off-target toxicity and drug dosage. The core of the nano-assembly is composed of GNRs coated with a mesoporous silica shell (MS). The MS shell serves as a carrier for the anti-tuberculosis drug bedaquiline. The core-shell nanoparticle is encapsulated within a thermo-sensitive liposome (TSL). The TSL layer is further conjugated to the mycobacteria-targeting peptide NZX. NZX mediates the adhesion of the final nano-assembly onto the mycobacterial surface. Upon NIR laser irradiation
GNRs convert the photon energy of the laser to localized heat, which melts the TSL, triggering the release of bedaquiline. The antibacterial activity of the final nano-assembly against *Mycobacterium smegmatis* (*Msmeg*) was 20 folds more efficacious than the free drug equivalent. *Mtbc* can alter immune defense mechanisms exerted by the host macrophage. Hence, host-targeted nano-assemblies (HTNs) were fabricated by conjugating host targeting ligands (β-Glucan) onto the nano-assembly. The binding of β-Glucan conjugated HTNs to the dectin-1 receptor present on macrophages increases the free radical production and cellular uptake of HTNs. An NIR laser triggers the photothermally induced structural disruption of the nano-assembly, releasing the drug at the targeted sites. The released bedaquiline within the macrophage promotes phagosome acidification and phagolysosome formation, effectively killing 99% of intracellular bacteria. Similar nano-assemblies were developed for dual-targeted drug delivery against lung carcinoma and proved to be 20 fold more effective than the anticancer drug alone. Finally, a simple and rapid diagnostic test was developed for detecting mycobacteria within a minute using lectin conjugated multi-core silica-coated magnetic nanoparticles.
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<tr>
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<tr>
<td>DSPE</td>
<td>1,2-Distearoyl-sn-glycero-3-phosphoethanolamine</td>
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<td>EE</td>
<td>encapsulation efficiency</td>
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<td>MPPC</td>
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<td>MSNPs</td>
<td>mesoporous silica nanoparticles</td>
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<td>$Mtb$</td>
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\begin{itemize}
  \item NaBH\textsubscript{4} sodium borohydride
  \item NIR near-infrared region
  \item NPs nanoparticles
  \item PAMPs pathogen-associated molecular patterns
  \item PTPA protein tyrosine phosphatase
  \item PTT photothermal therapy
  \item QDs quantum dots
  \item RIF rifampicin
  \item RNS reactive nitrogen species
  \item ROS reactive oxygen species
  \item SPR surface plasmon resonance
  \item T\textsubscript{c} phase transition temperature
  \item TRIDENT thermo-responsive-inspired drug-delivery nano-transporter
  \item TSL thermo-sensitive liposome
  \item TSPR transverse surface plasmon resonance
  \item TB tuberculosis
  \item WHO World Health Organization
\end{itemize}
CHAPTER 1. BIBLIOGRAPHIC STUDY OF NANOMATERIALS IN THERANOOSTICS

1.1 Introduction

Theranostics is the combination of therapy and diagnosis. Diagnosis is the ability to detect disease state, and therapy is defined as treating the diagnosed disease. Nanotheranostics is applied when nanomaterials are used for theranostics. Nanomaterials can be well-defined as a material with sizes ranging between 1 and 1000 nm, which employs as curative agents or carriers at the nanoscale level to develop nanomedicines. The field of biomedicine is an interdisciplinary area comprising nanobiotechnology, drug delivery, biosensors, and tissue engineering, which has been powered by nanomaterials. In recent years, the use of nanomaterials in medicine and, more specifically, in drug delivery has expanded into a broad range of clinical applications. Nanomaterials offer many benefits, such as improving the stability and solubility of encapsulated cargos, promoting transport across membranes, site-specificity, and targeted delivery of precise medicine. Nanoscale-sized particle have unique structural, chemical, physical, optical, magnetic, electrical, and biological properties. Based on their physical and chemical properties, a variety of nanomaterials have been developed up to date, such as inorganic nanoparticles (NPs) including metal NPs, gold-based, magnetic, mesoporous silica NPs, and
quantum dots, lipids nano-system, - liposomes, micelles, dendrimer, and polymeric NPs are increasingly being utilized as vehicles for antibiotics delivery. Selected organic nanomaterials such as liposomes, polymer NPs/micelles, and metallic NPs such as gold NPs have been approved by Food and Drug Administration (FDA) for theranostics purposes. Liposomes, polymeric NPs, micelles, hydrogels are considered “soft matter.” In recent years hybrid nano-systems, a co-formulation of metallic NPs with the soft matter have been synthesized. These hybrid nano-systems harvest the properties of both metallic and soft matter counterparts. The incorporation of metallic NPs into soft-matter systems offers unique advantages in that it combines the highly desirable drug-delivery properties of traditional drug-delivery vehicles such as excellent colloidal stability, ease of fabrication, biocompatibility, and high drug-loading capacities with novel stimuli-responsive antibiotics release mechanisms imparted by the inorganic NPs.

1.2 Gold-based nanomaterials

Based on the morphology and properties of gold nanoparticles (AuNPs), they can be classified into two main categories: isotropic (spherical) and anisotropic (non-spherical). The synthesis of isotropic AuNPs is performed in several ways, and the most common route is the reduction of Hydrogen tetrachloroaurate(III) (HAuCl$_4$) to gold ions. In the early 1950s, Turkevitch produced AuNPs using sodium citrate as a reducing agent, and numerous researchers have adapted this method to synthesize AuNPs of various sizes. It was first utilized to make isotropic NPs with a diameter of 15 nm. The reduction of gold salt be utilized to synthesize AuNPs from 10-150 nm. AuNPs exhibit unique optical properties, and the main properties that govern their theragnostic applications are surface plasmon resonance (SPR). When AuNPs are exposed to light, the light's oscillating
electromagnetic field causes the gold's free electrons (conduction band electrons, 6s) to oscillate collectively. This electron oscillation across the particle surface induces charge separation in the ionic lattice, resulting in a dipole oscillation in the direction of the light's electric field. The oscillation amplitude reaches its maximum at a specific frequency, known as a localized surface plasmon resonance (LSPR).

Because the SPR causes strong absorption of the incident light, it can be measured with a UV–Vis absorption spectrometer. The intensity and wavelength of the LSPR band are affected by parameters such as the metal type, particle size, shape, structure, and composition. In gold nanospheres (isotropic or spherical), this electron oscillation induces strong light absorption and an LSPR peak around 520 nm. In contrast, anisotropic (non-spherical) nanoparticles can exhibit electron oscillation along multiple axes. Anisotropic or non-spherical AuNPs include gold nano - rods, prisms, stars, triangles, cubes, pyramids, cages, wires. These varied shapes can be synthesized by varying the concentration of reducing agents surfactants and controlling reaction conditions. According to perspective application, it becomes important to control the physical parameters of the AuNPs, such as size and shape. Shape variation of anisotropic NPs provides surface plasmon absorbance over wider wavelengths in the electromagnetic spectrum than isotropic NPs. Anisotropic AuNPs can absorb in the near-infrared (NIR) range, making them useful as photothermal agents in biological applications where tissue absorption in this region is minimal, thanks to gold's recognized biocompatibility. In this thesis, we focus mainly on gold nanorods (GNRs).
1.2.1 Gold nanorods (GNRs) synthesis

GNRs are one type of anisotropic gold nanostructures that have received much attention for their optical properties in theranostics applications. Mie's theory cannot explain the optical behavior of nanorods because Mie’s theory only applies to isotropic particles. Gustav Mie discovered that the optical properties of AuNPs are affected by several factors, including nanoparticle size, medium refractive index, and gold dielectric constant. The extinction maximum redshifted slightly as the particle size of AuNPs increased from 20 to 100 nm. Later on, Richard Gans refined Mie's boundary conditions for spheroids to predict the existence of two absorption bands: a transverse surface plasmon resonance peak (TSPR) linked to the rod's width and a longitudinal surface plasmon resonance peak (LSPR) linked to the rod's length.

Figure 1.1. The absorption spectrum of an aqueous solution of GNRs.
resonance peak (LSPR) band related to the rod's length.\textsuperscript{19, 34, 35} In Figure 1.1, the transverse band can be seen at 520 nm, and the longitudinal band is seen around 790 nm. The absorbance wavelength of the LSPR band can be changed to the NIR region by changing the aspect ratio of the nanorod. The aspect ratio of nanorods is defined as the ratio of the length of the major axis to the width of the minor axis. Researchers have developed several synthesis protocols for GNRs, including a seed-mediated method,\textsuperscript{36} seedless methods,\textsuperscript{37} photochemical methods,\textsuperscript{38} and electrochemical methods.\textsuperscript{39} Out of all listed methods, the seed-mediated method by Murphy \textit{et al.} has been widely accepted to generate a high aspect ratio of nanorods.\textsuperscript{40} In Murphy \textit{et al.} used sodium citrate to reduce gold salt to prepare aqueous solutions monodispersed spherical gold seeds (2 – 3 nm). The citrate stabilized gold seeds were then added to the growth solution, which contained cetyltrimethylammonium bromide (CTAB), ascorbic acid, gold salt, and silver nitrate ($\text{AgNO}_3$). CTAB is used to stabilize GNRs, ascorbic acid act as a mild reducing agent, and $\text{AgNO}_3$ controls the aspect ratio of nanorods. Three different mechanisms for the longitudinal growth of GNRs have been proposed: (1) silver underpotential deposition, in which a monolayer quantity of Ag(0) is proposed to preferentially deposit onto the growing longitudinal facets to favor anisotropic growth,\textsuperscript{41} (2) surfactant templating, in which elongated CTAB micelles form a soft template for the growth of GNRs, potentially with the synergistic involvement of silver in the micelles,\textsuperscript{42, 43} (3) facet-specific capping, where CTA–Br–Ag$^+$ complexes act as capping agents to block specific facets for anisotropic growth.\textsuperscript{44, 45} The experimental data support all three hypotheses to some extent, but none of them can entirely explain all experimental findings, and there is still a lot of speculations in the literature about the complete mechanism.\textsuperscript{46, 47}
However, the GNRs synthesized with this approach contained many spherical AuNPs. El-Sayed et al. improved this technique by replacing citrate with CTAB during Au seed formation. They also showed that the nanorod aspect ratio could be controlled by varying the concentration of Ag$^{+}$ ions in the growth solution. In this thesis, the seed-mediated method was employed to synthesize GNRs. In a typical seed-mediated method, as shown in Scheme 1.1, the gold seed solution (~ 2 nm) is prepared by reduction of HAuCl$_4$ in the presence of CTAB and cold sodium borohydride (NaBH$_4$), reducing Au$^{3+}$ to Au$^{0}$ and forming CTAB capped gold seeds. When sodium borohydride is added to the solution, the color changes from pale yellow to pale brown, indicating complete reduction. These as-prepared gold seeds were subsequently added to the growth solution consisting of 0.1 M CTAB, 0.50 mM HAuCl$_4$, 10 mM AgNO$_3$, and 0.1 M ascorbic acid to form CTAB-stabilized GNR. The exact mechanism is still debated; however, the well-known hypothesis is that the surfactant CTAB is employed as a stabilizer and a capping agent to adhere to attach to the \{100\} facets of the seeds. This slows growth in that direction while allowing elongation on the \{111\} facets at the ends of the nanorods. The
crystallography of GNRs has been studied very well, previously published.\textsuperscript{50,52} The typical crystallography of GNRs showed in Scheme 1.2. The longitudinal axis is \{110\} which is the preferential binding site for the CTAB bilayer, forcing anisotropic growth on the \{111\} ends while the end facets are \{111\}.\textsuperscript{48,53} Ascorbic acid acts as a mild reducing agent added to the growth solution to reduce \(\text{Au}^{3+}\) to \(\text{Au}^{+}\), and the solution turns light yellow to colorless, followed by the gold seeds added to the growth solution. Because ascorbic acid is a weaker reducing agent, growth is slower, allowing for the formation of CTAB-capped GNRs with length 42 ± 3.8 nm and width from 11.2 ± 1.1 nm, and the calculated average aspect ratio is 3.8:1. \(\text{AgNO}_3\) controls the aspect ratio of nanorods. As the amount of silver increases, different colors (red wine color - aspect ratio 3.8 by adding 4.8 mL of 10 mM \(\text{AgNO}_3\)) of GNRs solutions can be produced with red-shifted LSPR maxima. GNRs are purified by centrifugation, then the pellets are resuspended in deionized water, and can be stored at room temperature for several months.

GNRs synthesis must be carried out carefully, as each reagent plays a significant role in the growth of GNRs. The function of the surfactant in GNRs synthesis has been well studied, and the surfactant's purity is also essential in the application. During GNRs synthesis, we observed that using CTAB from different commercial vendors and different
lot numbers gave inconsistent GNRs aspect ratios. Smith et al. documented the variability in the aspect ratio of GNRs using CTAB from 10 different vendors.\textsuperscript{54} They suspected the variability was due to differences in iodide concentration in CTAB between suppliers. They found that adding potassium iodide at 50 ppm completely prevented rod growth due to iodide adsorption on the Au (111) face.\textsuperscript{54} Rayavarapu et al. also examined different lot numbers of CTAB from some of these sources, and they observed that iodide impurities varied lot-to-lot and not just by a supplier.\textsuperscript{55} Hence, researchers should test the CTAB reagent for contaminant iodine concentration to ensure successful GNRs synthesis.

1.2.2 Photothermal activity of GNRs

As previously stated, AuNPs absorb light very strongly due to SPR, and SPR absorption is affected by the shape and size of the AuNPs.\textsuperscript{34} When the shape of AuNPs changes from sphere to the rod, the SPR absorption splits into two bands: LSPR, a stronger long-wavelength band in the NIR region due to longitudinal electron oscillation, and TSPR, a weak short wavelength band in the visible region due to transverse electronic oscillation.\textsuperscript{56} The LSPR peak is extremely sensitive to the aspect ratio (length/width) of GNRs, whereas the TSPR peak is not.\textsuperscript{57} This strong SPR absorption, followed by rapid energy conversion and dissipation, can be easily used to heat the local environment using NIR light radiation with a frequency that strongly overlaps with the SPR absorption band of GNRs.\textsuperscript{57} Anisotropic geometries are thought to aid GNRs in exhibiting intense LSPR bands, resulting in highly efficient and localized light-to-heat conversion after suitable NIR irradiation making them very useful for photothermal therapy (PTT) of cancers and other diseases. GNRs are a promising candidate for use as a photothermal agent because of (1) their ability to convert light energy to thermal energy and (2) their tunable absorption in
the NIR region of the biological window (700–1000 nm), where biological tissue has a high transmissivity.\textsuperscript{58} Huang \textit{et al.} were among the first to use GNRs to demonstrate photothermal killing of cancer cells.\textsuperscript{59} GNRs were coupled with anti-epidermal growth factor receptor monoclonal antibodies, which are overexpressed in cancer cells. Upon illumination of laser irradiation on GNRs resulted in more effective destruction of malignant oral epithelial cancer cells than nonmalignant cells.\textsuperscript{59} To target the surface of the \textit{Pseudomonas aeruginosa}, Norman \textit{et al.} employed antibody-functionalized GNRs.\textsuperscript{60} Laser irradiation of bacteria cultured with functionalized GNRs resulted in 75% cell death and irreversible cell wall damage.\textsuperscript{60} Using targeted GNRs, photothermal death can be highly successful, as seen in these two examples. Another study by Ocsoy \textit{et al.} prepared two types of gold NPs, spherical gold nanoparticles (AuNPs) and gold nanorods (GNRs).\textsuperscript{61} These nanomaterials were further modified with DNA aptamers that can specifically bind with MRSA. Based on their results, both the Apt@AuNPs and the Apt@GNRs successfully bound with MRSA. However, only the Apt@GNRs effectively inactivated the bacteria through hyperthermia. According to the authors, one possible explanation is their longitudinal absorption of NIR radiation and photothermal conversion. This could confirm that the shapes of AuNPs have a large influence on their photothermal effects. There are several reports where GNRs used as a photothermal agent in antibacterial mechanism.\textsuperscript{62, 63}

1.2.3 Encapsulation strategies of GNRs

Despite the benefits of CTAB in GNR synthesis, CTAB's high cytotoxicity is a significant concern that threatens to undermine any potential use of GNRs in a biological setting. The CTAB layer must be replaced or completely encapsulated by a more biocompatible coating for biological application. The interactions of CTAB with the cell's
phospholipid bilayer and the inhibition of the enzyme adenosine triphosphate (ATP) synthase by the cetrimonium cation are the causes of cell toxicity, both of which result in cell death.\textsuperscript{64} However, several methods are reported to tailor the surface chemistry of GNRs to avoid these issues, which could prevent GNR’s bio applications.\textsuperscript{65} Here, we focus on a few standard techniques which are utilized in this thesis that includes the methods of inorganic mesoporous silica coating and liposome encapsulation as shown in Scheme 1.3.

Scheme 1.3. Encapsulation strategies of CTAB-coated GNR.

1.2.3.1 Mesoporous silica coating of GNRs

Silica coating has been shown to improve GNRs colloidal stability in organic solvents while also improving thermal stability by preserving the optical characteristics of GNRs. Silica coating is a helpful technique for GNRs surface modification. Silica can increase the colloidal stability of GNRs in organic solvents while enhancing the thermal stability to preserve GNRs’ optical properties.\textsuperscript{66} On the silica surface, the reactive silanol
(-Si-OH) groups are present, which can be modified with other silane-functionalized ligands such as polyethylene glycol (PEG) or targeting ligands peptide, antibodies, aptamers, and small molecules. Furthermore, silica nanoparticles with a porous structure known as mesoporous silica nanoparticles (MSNs) have grown in popularity in recent years. Its uniform and controllable pore size, straightforward independent functionalization of the surface, internal and exterior pores, and pore opening gating mechanism distinguish it as a unique and potential drug carrier. MSNs are those with pore sizes ranging from 2 to 50 nm with an ordered arrangement of pores, resulting in a porous ordered structure. The pore size of MSNs can be adjusted and fine-tuned using different surfactants and reaction conditions. In terms of synthesis, Stöber was the pioneer in synthesizing silica nanoparticles and MSNs. Since then, several changes have been made to Stöber synthesis to produce monodisperse, ordered, nanosized MSNs. Grun et. al., were the first to modify the Stöber synthesis method by using a cationic surfactant as a template to produce a spherical structure. To be an excellent carrier for drug delivery, MSNs must have homogeneous particle size and a large pore volume to increase loading capacity. These parameters can be fine-tuned by variations in the pH of the reaction mixture, surfactant concentration, silica source, and temperature of the reaction.

In this thesis, we have coated GNRs with mesoporous silica shells where we have loaded both hydrophilic and hydrophobic drugs as cargo in different experiments. We have synthesized MSNs by the surfactant-template method developed by the Matsuura group. Tetraethyl orthosilicate (TEOS) was used as a hydrolytic inorganic silica precursor and 3-aminopropyltriethoxysilane (APTES) co-condenses with TEOS to form the silica shell layer in addition to acting as a morphological stabilizer. The surfactant CTAB acts both as
a stabilizer and mesostructure-directing agent. CTAB forms micelles, which act as a framework for the growth of the TEOS/APTES based silica shell. Base catalyst NaOH hydrolyzes TEOS/APTES to silicate. The electrostatic interactions between negatively charged silicate oligomers hydrolyzed from TEOS/APTES and positively charged CTAB-stabilized gold nanorods lead to the formation of core-shell structured GNRs coated mesoporous silica shell. Gorelikov et. al., have developed surfactant-based templet method in which they have directly coated mesoporous silica onto CTAB gold nanorods.\textsuperscript{74} NaOH is added to GNRs to adjust the pH to 11 and is followed by injections of TEOS which polymerizes to form silica in a base-catalyzed reaction.\textsuperscript{74} Other researchers have modified reaction parameters such as temperature, TEOS, APTES, and CTAB concentration to control mesoporous silica shell thickness on GNRs.\textsuperscript{75, 76}

Researchers have successfully used MSN carriers to transport a wide range of cargo, including drugs and macromolecules such as proteins, DNA, and RNA.\textsuperscript{77} Numerous studies have reported various loading mechanisms of MSNs such as pore filling,\textsuperscript{78} adsorptions,\textsuperscript{79} and grafting.\textsuperscript{72} The loading strategies of MSNs are highly dependent on the carried biomolecules. For example, small drug molecules or short peptides can be easily loaded within pores or grafted onto surfaces, whereas larger and more charged molecules may be rejected if the mesopores are not properly conditioned.\textsuperscript{80} Both hydrophilic and hydrophobic drugs can be incorporated into the pores of MSNs by adsorption mechanism, which is a straightforward method.\textsuperscript{72} In the adsorption method, the mesoporous silica is immersed in the concentrated drug solution, and drug molecules are then adsorbed on the MSNs’ pores. Centrifugation is then used to separate the drug-loaded MSNs from the solution. After that, the particles are dried to remove any remaining solvent. MSNs have a
higher loading capacity than other carriers because of their large pore volume. In terms of the release profile of drugs from MSNs, the profile is primarily determined by the drug diffusion from pores. This can be tailored by modifying the surface of the MSNs to meet biological requirements.\textsuperscript{81} Recently, there has been much interest in studying the drug release from MSN or hybrid nano-assembly in response to external specific stimuli such as magnetic, ultrasound, and NIR light.\textsuperscript{82} The mechanism is based on the nanocarriers' conformational changes or destruction of MSNs in response to light. Li \textit{et al.} reported a NIR laser-responsive MSNs nanocarriers composed of GNRs capped with reversible single-strand DNA.\textsuperscript{83} This DNA strand was controlled by a laser switcher, which controlled the drug release from the nanocarrier. These nanocarriers were able to deliver drugs precisely at a specific time and to a specified location.\textsuperscript{83}

1.2.3.2 Liposome encapsulation

Liposomes are the most widely researched nanoscale drug delivery platform. Liposomes were discovered in the 1960s by Bangham and colleagues at the Babraham Institute, University of Cambridge, and were published in 1964.\textsuperscript{84} Liposomes are colloidal spherical structures formed by the self-assembly of amphiphilic lipids (lipids having polar end and non-polar end) molecules such as phospholipids and cholesterol. One or more lipid bilayers arranged around an internal aqueous core, with the polar head groups orientated to the inner and non-polar head groups towards the outer, make up a liposome membrane.\textsuperscript{85} Liposomes have the unique capacity to load and deliver molecules of variable solubility thanks to their well-organized structure.\textsuperscript{86} Hydrophilic molecules can be loaded into the aqueous core, hydrophobic molecules into the lipid bilayer, and amphiphilic molecules into the water/lipid bilayer interface.\textsuperscript{86} A suitable liposomal formulation can be achieved
according to the application by selecting an appropriate liposome composition, functionalization, and even a targeting method. The choice of phospholipids, head group and chain length, and the ratio of liposome components are all critical factors in determining liposome safety, stability, and efficiency. Liposomes are mostly made up of phospholipids and are classified into two types: natural and synthetic. Phosphatidylcholine (PC), and phosphatidylethanolamine (PE), which are abundant phosphatides in plants and animals, are the most natural phospholipids used to make liposomes. Natural phospholipids are primarily found in egg yolk and soya beans.

Natural lipids are used to make synthetic phospholipids. Natural phospholipids are modified in the head groups, aliphatic chains, and alcohols to produce a variety of synthetic phospholipids that have proven to be more stable. The synthetic lipids includes 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine, (DOPE), 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) and monopalmitoyl phosphocholine (MPPC). Because of their amphipathic nature, phospholipids have a strong ability to form stable bilayers in aqueous environments. Liposomes are formed due to hydrophilic interactions between polar head groups, van der waals forces between hydrocarbon chains (which hold the long hydrocarbon tails together), and hydrogen bonds with water molecules. Water molecules repel hydrophobic chains, resulting in spontaneous liposome self-assembly in a closed bilayer. Liposomes can acquire positive, negative, or neutral charges depending on the head group of the phospholipids. Incorporation of cholesterol into the lipid bilayer of liposomes can affect bilayer fluidity and rigidity, reducing permeability and increasing in vitro and in vivo stability.
Cholesterol, as a hydrophobic molecule, inhibits lipid chain interactions by intercalating between them, enhancing liposome membrane stabilization.\textsuperscript{90} Using various PEGs in the liposome composition or on the surface of liposomes can be an excellent way to extend the half-life of blood circulation from a few minutes (standard liposomes) to many hours.\textsuperscript{91} It was reported that longer-chain PEGs improved blood circulation time than short chains.\textsuperscript{91} To prepare liposome, there are various techniques have been reported such as thin-film hydration,\textsuperscript{92} reverse-phase evaporation,\textsuperscript{93} solvent injection techniques,\textsuperscript{94} size reduction methods including probe sonication and extrusion.\textsuperscript{93}

Liposomes have a distinct phase transition temperature ($T_c$) at which they transition ('melt') from a gel to a liquid phase. The phase transition temperature of phospholipids is a crucial parameter that can alter the fluidity of the lipids within the bilayer.\textsuperscript{95} At a temperature below $T_c$, the phospholipids are in the gel phase, and present low fluidity and low permeability as individual molecules within the bilayer move gently.\textsuperscript{96} In the gel phase, lipid molecules are organized and condensed with fully extended hydrocarbon chains, which are restricted to the membrane's two-dimensional plane.\textsuperscript{96} At temperatures greater than the $T_c$, the phospholipids bilayer exists entirely in the liquid phase. Individual lipid molecules are still limited to the two-dimensional plane of the membrane, but they can move freely and quickly (millions of exchanges per second) within it.\textsuperscript{97} As a result, the membrane becomes fully fluidized and permeable throughout.\textsuperscript{97} Taking advantage of this liposome property, temperature-sensitive (thermo-sensitive) liposomes have recently emerged as an appealing option for treating cancer and infectious disease in a controlled and predictable manner using external energy sources.\textsuperscript{98} When thermosensitive liposomes are exposed to heating, the lipid bilayer undergoes a melting phase transition from a gel to
a liquid-crystalline phase, allowing a rapid increase in bilayer permeability and thus the rapid release of small molecules or drugs. The exact temperature and broadness of the phase transition depend on the lipid composition and can be adjusted to clinically relevant mild-hyperthermia temperatures (39–43 °C). DPPC and MPPC are widely used to prepare thermo-sensitive liposomes as their $T_c$ values are around 43 °C.

In this thesis, we have prepared a thermo-sensitive liposome, as the amount of DPPC used in liposome preparation was the highest (85%) in molar ratio compared to cholesterol (10%) and DSPE-PEG(2000) amine (5%). As previously studied in our recent paper, these liposomes become permeable at temperature ~ 50 °C. Thermo-sensitive liposomes are the widely researched nanoscale anti-cancer and antimicrobial delivery system primarily due to their ability to provide thermal stability, colloidal stability, controlled drug release, which also act as a biocompatible carrier and provide a surface for attaching targeting biomolecules such as antibodies, peptides, aptamer and small molecules.

Regarding GNRs, Hua et al. reported that docetaxel drug encapsulated multifunctional GNRs in liposomes for cancer treatment. Cui et. al. reported lipid bilayer modified GNRs coated MSNs for NIR controlled drug delivery for cancer treatment. Thermo-responsive-inspired drug-delivery nano-transporter (TRIDENT) nano-system was developed by Quing et al. in which nano-system formulated from natural fatty acids with a tunable melting point around 43 °C was chosen as the hydrophobic vehicle for encapsulation of antibiotic imipenem and photosensitizer IR780 dye. The drug-loaded nano-system was wrapped with lecithin and DSPE-PEG2000 to increase the biocompatibility of the resulting TRIDENT. TRIDENT's 100% bactericidal capacity against antibiotic-resistant and clinical MDR bacteria using chemo-photothermal therapy was demonstrated in vitro and in vivo.
1.2.4 Nanoparticles (NPs)-mediated targeted drug delivery

Targeted drug delivery can be accomplished either passively or actively. Passive targeting is achieved by incorporating the therapeutic agent into a macromolecule or NPs that passively reaches the target organ. Active targeting necessitates the conjugation of targeting ligands such as peptides, antibodies, proteins, aptamers, carbohydrates, and small molecules onto the NPs’ surface.\(^{106}\) These targeting ligand conjugated NPs selectively bind to specific cell surface receptors, resulting in cell internalization via receptor-mediated endocytosis and increasing host immune response in some applications.\(^{107}\)

The properties of nanomaterials make them attractive candidates for targeted drug delivery platforms. Due to the nanoscale size, they can easily penetrate the tissue system, allowing for easy drug uptake by cells and efficient drug delivery at the desired location.\(^{108}\) Surface area of NPs is larger, providing more space to accommodate bulky targeting ligand on its surface, which helps design a targeted drug delivery platform.\(^{109}\) Furthermore, surface functionality is the principal parameter dominating NPs interactions with cells and subsequent cell uptake.\(^{110}\) When using NPs for targeted delivery with any of the ligands, it is necessary to chemically modify the surface of the NPs with appropriate chemistry to introduce reactive moieties, providing functional groups that can be conjugated to a targeting ligand of choice. The selective ligand must contain a functional group that can also be used for conjugation. The majority of the conjugation chemistries used to modify NPs are covalent. Some of the most common covalent reactions used in conjugating NPs to targeting ligands include chemical reactions that use reactive carbonyl groups (carbonyl reacts with hydrazide or alkxyoxyamine to form hydrazone or oxime bond), amine-reactive groups (amine reacts with activated carboxylate or imidoeester to form amide or amidine
bond), and reactive sulfhydryl groups (thiol reacts with maleimide, gold surface, to form thioester, and gold-thiol bond, respectively). These NPs conjugated targeting ligands could guide the NPs to the intended target and specifically recognize the target site. Xiao et al. reported conjugation of targeting peptide on GNRs via a two-step process; thiolated linkers was anchored onto the GNRs through the thiol moiety. Other end of thiol linkers were used to conjugate the heterobifunctional PEG derivative of NHS ester, which is then used for the targeting ligand conjugation. Doxorubicin (Dox) was also covalently conjugated on GNR via PEGylation. They have developed this nano system for combined anticancer targeted drug delivery and PET imaging.

Multivalency is yet another important factor in designing a ligand-receptor-mediated targeted drug delivery platform. Multivalent interactions defined as multiple ligands with proper ligand presentation binds to specific receptors having two or more binding sites. In this regard, NPs can be used as a scaffold to present multiple copies of ligands on the NPs. Depending on the size of the NPs and the size of ligands, hundreds and thousands of ligands can be presented on the NPs. Once targeting ligands are conjugated to NP surface, they can bind to cell surface receptors in a multivalent manner. While free ligands only bind to receptors in a monovalent fashion, conjugating them to NP surface increases valency to be multivalent. Multivalent binding increases the strength of binding. For example, free carbohydrates interact weakly with cell surface lectins with a dissociation constant ($K_D$) in the millimolar range. But functionalizing carbohydrates on NPs (to create glyconanoparticles), binding interactions can be increased to nanomolar range. Cells also utilize multivalency to increase binding affinity towards weak ligands. Wang et al. studied the binding affinity of glyconanoparticle (GNP)-protein multivalent interactions
based on a fluorescent competition binding assay.\textsuperscript{114} Wang et al., synthesized carbohydrate conjugated AuNPs, to create GNPs. They measured the binding affinities of these GNPs with lectins and found a 1000-fold enhancement in $K_D$ in the nanomolar range compared to the micromolar range for free carbohydrate-lectin interaction.\textsuperscript{114}

When we discuss active targeting between ligand conjugated nanomaterials and cell surface receptors, it is essential to study the ligand-receptor binding interaction. Targeting ligand conjugated nanomaterials interact with receptors present on the cell surface or bacterial cell wall, and the strength of interaction directly impacts the outcome, such as the uptake, toxicity, and clearance of the nanomaterials.\textsuperscript{115} The strength of such interactions can be quantified by measuring the binding affinity ($K_D$). There are several techniques by which binding affinity between targeting ligand conjugated nanomaterials and receptors can be measured, such as fluorescence/absorption-based assay,\textsuperscript{116} Surface Plasmon Resonance Spectroscopy (SPR),\textsuperscript{117} Isothermal Titration Calorimetry (ITC),\textsuperscript{118} and enzyme-linked assay.\textsuperscript{119, 120} Out of all, fluorescence-based techniques have been widely used to determine binding affinities between ligands and receptors. In this assay, either the nanomaterial or the receptor needs to be inherently fluorescent or is conjugated with a fluorophore. The fluorescence-based techniques have been used widely to determine binding affinities of surface-bound ligands on nanomaterials, including microarrays, fluorescence quenching, and fluorescence anisotropy. Yan group has developed the fluorescence competition assay to determine the $K_D$ between concanavalin A (Con A) and carbohydrate-functionalized AuNPs.\textsuperscript{114} They have used varying concentrations of ligand conjugated fluorescent NPs, together with a fixed concentration of free carbohydrate (mannose) ligands incubated and the fluorescence of the supernatant was measured. Then
from the dose-response curve, IC\textsubscript{50} values were obtained.\textsuperscript{114} Then, after using the Cheng-Prusoff equation, the K\textsubscript{D} was calculated. Using a similar method, they studied how the ligand presentation impacted the binding affinity of ligand conjugated fluorescent NPs including the ligand density, linker length, and size.\textsuperscript{114} Their results showed that the more ligands present on the NPs, the longer the dwelling time of the lectin on the ligands and the slower the lectin would dissociate.\textsuperscript{114} Hence, binding affinity increased with the ligand density. Regarding the spacer, their results clearly demonstrate that longer spacers led to the enhanced binding affinity of GNPs with lectins.\textsuperscript{114} This is due to curved surfaces of NPs, reducing the steric hindrance when the lectin approaches the ligands, making the ligands more accessible for interacting with the lectin.\textsuperscript{114} Smaller NPs yielded the highest affinity enhancement, likely due to their large surface-to-volume ratio and higher mobility in solution.\textsuperscript{114}

In this thesis, we have conjugated various targeting ligands (peptides, small molecules) on a liposomal surface via an amide linkage. These functionalized nano-assemblies were used for treating intracellular mycobacterial infections. We have also systematically studied binding interaction between ligand conjugated nano-assemblies and cell surface receptors. Also, the impact of ligand presentation in terms of size of nano-assembly and ligand density on binding affinity was studied. In the cancer project, we have conjugated peptide GE-11 and folic acid on nano-assemblies and investigated targeted therapy for lung cancer treatment.

1.3 Drug delivery platforms for infectious disease

Globally, infectious diseases are a significant cause of morbidity and mortality.\textsuperscript{121} Infectious diseases mainly caused by pathogenic organisms such as bacteria, viruses, fungi,
and parasites continue to be the leading cause of hospitalization and death worldwide.\textsuperscript{122, 123} Infectious diseases are complicated to treat, as their continued transmission and high fatality rates are seen.\textsuperscript{124} Although significant progress has been made using small-molecule antibiotics, peptides, and nucleic acids, there are still significant challenges in the effective treatment of infectious diseases.\textsuperscript{125} Overuse of antibiotics leads to antibiotic resistance, which is a concerning situation that can weaken an individual's immune system. The rapid emergence of drug resistance is now a serious global health issue.\textsuperscript{126} Bacteria resist antibiotics through two main mechanisms: (1) preventing antibiotics from reaching their target at high concentrations and (2) modifying or bypassing the target that antibiotics act on.\textsuperscript{127} Bacteria eliminate antibiotics by using efflux pumps in their cell walls that remove antibiotic drugs that enter the cell.\textsuperscript{128} These efflux pumps are ubiquitous in bacteria and can transport signal molecules and nutrients. Some of these pumps can also transport antibiotics out of the bacterium, lowering antibiotic concentrations within the bacterial cell.\textsuperscript{128} In some cases, mutations in the bacterial DNA can cause the bacteria to produce more of a specific pump, increasing resistance.\textsuperscript{128} For example, \textit{Pseudomonas aeruginosa} bacteria can produce pumps to eliminate several antibiotic drugs, including fluoroquinolones, beta-lactams, chloramphenicol, and trimethoprim.\textsuperscript{129} Bacteria restrict access by modifying or restricting the number of entryways available.\textsuperscript{130} The gram-negative bacteria have a protective outer coat (membrane) that keeps them safe from the environment.\textsuperscript{131} These bacteria use this membrane to prevent antibiotics from entering selectively.\textsuperscript{131} Bacteria can modify or destroy the antibiotics with their enzymes, proteins which break down the drug.\textsuperscript{132} For example, Carbapenemases are enzymes produced by \textit{Klebsiella pneumoniae} bacteria that break down carbapenem drugs and most other beta-
lactam antibiotics. Antibiotics are often designed to target and kill specific bacteria components (or targets). However, bacteria alter the antibiotic's target, causing the drug to no longer fit and function properly. For example, the mcr-1 gene in *Escherichia coli* bacteria can add a chemical to the exterior of the cell wall that prevents the antibiotic colistin from latching onto it. By all of these mechanisms, bacteria develop antibiotic resistance. Hence, innovative strategies for maximizing the efficacy of currently available drugs must be developed.

In this regard, nanotechnology is emerging as a potential strategy for overcoming antibiotics' many drawbacks while also enhancing their therapeutic benefits. NPs could be a promising solution because they can fight against bacteria and act as carriers for antibiotics and natural antimicrobial compounds. The use of NPs as a carrier in antibiotic delivery suggests a promising way to design effective therapeutics against many pathogenic bacteria. Nanocarriers have the potential to overcome drug solubility and stability issues and reduce drug-induced side effects. It is possible to achieve co-delivery of two or more drugs or therapeutic modalities for combination therapy using nanocarriers. The administration of antimicrobial agents via nanocarriers can advance therapeutic index, extend drug circulation, reduce drug dosage and achieve controlled drug release, thereby improving overall pharmacokinetics. NPs’ surface characteristics can be altered for targeted drug delivery by targeting ligands such as small molecules, proteins, peptides, and nucleic acids. The immune system is unaware of the loaded NPs that are efficiently targeted to specific sites. A drug-loaded nanocarriers can be used for various administration routes, including oral, nasal, parenteral, intra-ocular, and so on. Hence, NPs-based drug delivery offers tremendous benefits over free antibiotics. Various
nanocarriers have been studied extensively, including polymeric NPs, hydrogels, micelles, liposomes, solid lipid NPs, quantum dots, silica NPs, and inorganic metal NPs, including gold and silver, and magnetic NPs. This thesis focused on drug delivery platforms for intracellular bacterial infections such as TB.

1.3.1 Tuberculosis (TB)

Globally, an estimated 10 million people had TB in 2020. *Mycobacterium tuberculosis* (*Mtb*) is the primary cause of tuberculosis, a chronic communicable disease that primarily affects the lungs (pulmonary tuberculosis) but can also affect other organs (extrapulmonary tuberculosis). *Mtb* is a gram-positive aerobic, rod-shaped acid-fast bacillus that is highly contagious. The primary host for *Mtb* is humans. It is primarily an intracellular pathogen and survives mainly in the host macrophage. Tubercle bacilli can survive in host macrophages because the bacterium possesses an innate ability to suppress the antimicrobial response of the macrophage. TB infection is spread by infected patients with aerosolized sputum. These aerosols contain viable tubercle bacilli that can infect an uninfected person. An aerosol droplet carrying viable tubercle bacilli is inhaled and travels to the alveoli in the lungs. Tubercle bacilli are phagocytosed and multiply within alveolar macrophages. The immune cell (macrophage) evading mechanism by *Mtb* is shown in Scheme 1.4., where *Mtb* manipulates host immune response in two ways; (1) inhibition of phagosome maturation (2) phagolysosome inhibition.

The interaction between *Mtb* and macrophage occurs by recognizing pathogen-associated molecular patterns (PAMPs) present on *Mtb*. Some of the pattern recognition receptors present on the surface of macrophages include C-type lectin receptors, toll-like receptors, folate receptors, and CD44 receptors. After entering the macrophage, *Mtb* is
taken up by the phagosome. Under normal conditions, the V-ATPase pump present on the cell membrane of the phagosome pumps protons into the phagosome. This increases the acidity of the phagosome and is called the maturation of phagosome or acidification of phagosome. In the case of *Mtb* infection, *Mtb* releases the protein tyrosine phosphatase (PTPA) protein. Thus, inhibition of phagosome acidification is one of the survival strategies of *Mtb* inside the macrophage. In a common bacterial infection, phagosome fusion with lysosome leads to phagolysosome formation. Upon initiation of this process – free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) production increases. This also increases the formation of bactericidal enzymes such as peptidase. All these processes together act conjunctly to kill intracellular bacteria. However, in *Mtb* infection, phagolysosome formation is inhibited by *Mtb*. Also, intracellular *Mtb* can inhibit ROS/RNS generation and prevent the decrease in intracellular pH. Finally, these *Mtb* infected macrophages form a hard shell called a granuloma. The bacilli replicate within the granuloma and then burst out and infect other healthy cells and

Scheme 1.4. Survival mechanism of mycobacteria inside host macrophage cells.
tissue to develop active TB. Therefore, it is paramount to kill intracellular \textit{Mtb} to prevent the formation of active TB.

Antibiotics have significantly reduced the prevalence of TB. The treatment of TB is intensive and requires daily intake of ‘first-line’ antibiotics isoniazid (INH) and rifampicin (RIF) for at least 6 months to achieve sufficient suppression of \textit{Mtb}. If \textit{Mtb} becomes resistant to first-line antibiotics, the \textit{Mtb} is then identified as multi-drug resistant (MDR). If infected with MDR-TB, second-line drugs such as levofloxacin, moxifloxacin, and linezolid, which are more cytotoxic, are given for 18 months. Long treatment (6-24 months) periods of antibiotic treatment raise extremely drug-resistant TB (XDR-TB). Due to the rising global incidence of MDR-TB, more emphasis is being placed on developing new drugs with novel antibacterial mechanisms. In 2012, FDA approved two drugs against MDR-TB: bedaquiline (BQ) and delamanid. According to Giraud-Gatineau \textit{et al.} BQ inhibits the mycobacterial adenosine triphosphate (ATP) synthesis and has been shown to alter the human immune response to bacterial infection independently of its direct antimicrobial actions. Anti-tuberculosis drugs face a significant hurdle to eradicate intracellular \textit{Mtb} that resides in lung macrophages. Even upon entering macrophages, antibiotics are quickly cleared from the cell before its action on intracellular \textit{Mtb}. As a result of these factors, there is an increasing need for more effective therapies that offer higher efficiency in killing intracellular \textit{Mtb} in a shorter treatment time frame. Based on all the benefits that nanotechnology offer, it has gained attention as a tool for improving current treatment methods, especially as drug delivery platforms for delivering drugs with high cytotoxicity and low solubility.
1.3.2 Drug delivery platform for TB

Drug delivery using nanocarriers is an emerging strategy to fight against various diseases. Nanoscale size of nanomaterials offers several benefits to overcome the challenges of current TB treatment. Due to their size and surface chemistry, antibiotics-loaded NPs (nanocarriers) are avidly taken up by macrophages. The key advantages of nanocarrier systems over free drugs include increased bioavailability, prevention of premature drug release, protection from inactivation of the entrapped drug, sustained and controlled drug release, and the potential to reduce antibiotic doses, and hence adverse effects and administration frequency. The variety of nanocarriers have been developed, including inorganic nanocarriers, liposomes, lipid NPs, polymeric NPs, nano micelles, dendrimers, nanogels, etc. There are various methods by which antibiotics can be loaded into nanocarriers such as chemical conjugation, physical encapsulation, and physical adsorption. Overall, nanocarriers can target host cells in two ways: passive targeting and active targeting, as shown in Scheme 1.5. Passive targeting refers to the drug delivery of nanocarriers without requiring a specific targeting ligand. In the case of tuberculosis, we can use the fact that immune cells like macrophages, monocytes, and dendritic cells phagocytose and endocytose nanoparticles readily. Immune cells are constantly being recruited to infection sites, notably granulomas.

Furthermore, nanocarriers can be customized to improve macrophage uptakes by altering their properties (size, charge, rigidness, shape). Soria-Carrera et al. developed polypeptide micelles coated with alginates where they encapsulated BQ drug and showed enhanced antimicrobial activity by 3x fold (with MIC 0.01-0.03 µg/mL) against Mtb H37Rv compared to free drug BQ and enhanced stability in gastric and intestinal simulated
Another study by El-Ridy et al. fabricated niosomes (Niosomes are delivery vesicles made up of surfactants with or without cholesterol and can transport the drug.) and loaded drug ethambutol and showed enhanced antimicrobial activity in vivo. Antimycobacterial peptides such as NZX also gained interest in TB therapeutics. Tenhald et al. reported delivery of NZX antimycobacterial peptide using mesoporous silica nanoparticles and showed inhibition of intracellular mycobacteria in macrophage. Several studies reported using liposomes, polymers, micelles, inorganic NPs, and NPs as a drug delivery vehicle for TB treatments. In contrast, active targeting involves the delivery of a drug to targeted sites based on recognizing specific ligands conjugated to the NPs, which bind on specific receptors expressed by cells at the targeted site. Active

Scheme 1.5. Drug delivery strategies for TB infection.
targeting offers many benefits compared to passive targeting, such as enabling the administration of drugs at a lower effective dose, reducing off-site toxicity of drug, receptor-mediated endocytosis enhances intracellular drug concentration at target sites, specifically targeting infectious sites without affecting healthy cells. For TB, to enhance the specificity of drug-loaded nanocarriers, we can take advantage of pattern recognition receptors of macrophage cells. Shrivastava et al. reported mannosylated liposomes loaded with dual anti-TB drugs RIF and INH to treat pulmonary TB. Many studies have reported using mannose as a targeting ligand conjugated on nanocarriers to target intracellular Mtb. For example, mannose conjugated on solid lipid NPs, polymeric NPs, gelatin NPs, and liposomes. Hydrophilic or hydrophobic drug-loaded mannose conjugated NPs have been used for antibiotic delivery with some success. Targeting folate receptors that are overexpressed on macrophages has been used as a strategy to target Mtb infected macrophages. Recently, Shah et al. developed nanocapsules, in which RIF was loaded into folic acid conjugated chitosan NPs. Shan and co., demonstrated promising results for targeted delivery of drug; enhanced uptake of chitosan NPs, and higher lung drug concentration.

1.3.3 Gold NPs-based drug delivery platform for TB

In recent years, the development of stimuli-responsive hybrid nano-systems co-formulated with inorganic (magnetic, silver, gold NPs) and organic liposomes, polymers, and micelles have become a fast-emerging field. Stimuli-responsive release of antibiotics in response to a magnetic field, ultrasounds, and light has been developed. Light or electromagnetic radiation has been widely used as a trigger to design responsive drug delivery systems. Electromagnetic radiation employing ultraviolet (<400 nm), visible, or
near-infrared – I (NIR- I) regions (700 – 1000 nm) frequencies have been used as stimuli.\textsuperscript{169} Among them, the NIR region of the electromagnetic spectrum has received much attention.\textsuperscript{169-176} NIR responsive materials are usually called photothermal agents.\textsuperscript{177} Photothermal heating occurs due to the absorption of light by an ensemble of electrons on the surface of conductive materials (\textit{e.g.}, gold) and the subsequent dissipation of that energy as heat. Plasmonically active metal NPs (The plasmonically active metal NPs have free electrons in metal NPs and when they interact with light, it is called plasmonic effect), such as gold nanorods (GNRs) are particularly effective photothermal agents because of their large absorbance cross-sections, tunable optical properties, and highly efficient conversion of light into heat.\textsuperscript{178} These properties make them an appealing PT agent in biomedical applications.

Photothermal heating can either be used to deliver thermal energy to a localized area as reported by the Liu group in their research work,\textsuperscript{179, 180} or throughout the drug carrier, thereby causing the destruction of the encapsulating material and triggering the release of the loaded therapeutics. Some early demonstrations of photothermal effects to release antibacterial agents from drug carriers have been reported in several research studies.\textsuperscript{181-183} Specifically for TB treatment, so far in the literature, none of the studies has reported the use of GNRs as the photothermal agent, which is then coated with mesoporous structure, into which hydrophilic and hydrophobic drugs can be loaded, followed by thermo-sensitive liposome encapsulation, and finally conjugating targeting antimycobacterial peptide or host-targeting carbohydrate ligand. Hybrid nano-assemblies that was developed for this thesis offer (a) colloidal stability, (b) prevent premature drug release, (c) increased internalization into infected host cells, (d) control release of drug
through melting of thermo-sensitive liposome (e) photothermal activity (f) enhanced intracellular drug concentration due to targeting efficiency (g) modify host immune responses (h) low toxicity.

1.4 Drug delivery platforms for cancer

Globally, cancer is one of the leading causes of death, and based on Global Cancer Observatory (GCO), by 2030, approximately 30 million cancer patients will die from cancer each year.\(^{184}\) Cancer pathology is distinguished by inherent genetic alterations and cellular disorders, indicating abnormal and uncontrollable cellular growth that eventually leads to the patient's death.\(^{185, 186}\) Chemotherapy is the most commonly used systemic treatment for cancer cell proliferation, disease progression, and metastasis. On the other hand, chemotherapeutic drugs kill cancer cells and inevitably attack healthy cells, causing side effects.\(^{187}\) Drug carriers that preserve or improve chemotherapeutic efficacy while decreasing the severity of side effects are therefore critically required.

In the past decades, nanoparticles-mediated drug delivery platforms have been extensively studied by researchers. Nanoparticle-based drug delivery has systems area already being used in cancer treatments. For example, the FDA approved a liposomal version of doxorubicin (Doxil) in 1995 to treat AIDS-related Kaposi sarcoma.\(^{188}\) The FDA approved protein-bound paclitaxel (Abraxane), an albumin-based nanoparticle, for clinical use in the treatment of breast cancer, non-small cell lung cancer, and pancreatic cancer in 2005.\(^{189}\) Nanomaterials offers many benefits when designing a drug delivery system, for example (1) they can improve the pharmacokinetic and pharmacological properties of drugs by increasing their water solubility and protecting them from being dissolved in the bloodstream, (2) offer targeted drug delivery to specific tissues or cells (3) limiting drug
accumulation in non-targeted organs such the kidneys, liver, and spleen, and improving therapeutic efficacy, (4) offer real-time monitoring of treatment efficacy with a combination of imaging and therapeutic agents. In this thesis we focus mainly on drug delivery towards lung cancer.

1.4.1 Drug delivery platforms for lung cancer

In developed countries, lung cancer is the most common cancer in men and women.\textsuperscript{190} It is also the most significant cause of cancer death globally, accounting for 18.4\% of all cancer deaths.\textsuperscript{190} The top three cancer types in terms of mortality are lung, breast, and colorectum cancer.\textsuperscript{191} Due to treatment difficulties and late prognosis on a global scale, lung cancer is responsible for the most significant number of deaths among all cancer types, and it is also the leading cause of cancer death in men. Currently, the World Health Organization (WHO) differentiates several types of lung cancer, mainly classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) based on the cell morphology. NSCLC is considered an aggressive type of cancer; it affects a much broader population and accounts for 80\% of all lung cancer cases.\textsuperscript{192} Current cancer treatment approaches include surgery, chemotherapy, radiotherapy, and immunotherapy, which can be used alone or in combination, and the decision will be based primarily on the stage of cancer and the patient's overall health. All of these techniques have their limitations. For example, surgery does not always result in complete tumor removal, whereas radiation therapy may reduce tumor size but never result in complete eradication.

In contrast, photodynamic therapy or chemotherapy are two different methods used for advanced-stage lung cancer. Furthermore, radiation therapy and chemotherapy have an indiscriminate effect on cancerous and healthy cells.\textsuperscript{193, 194} Hence, there is an urgent need
to improve current treatment options. Nanotechnology offers more precise and effective treatments.\textsuperscript{195} Various types of drug delivery nano systems, including lipid-based, polymeric, and inorganic NPs have been widely used for lung cancer treatment.\textsuperscript{196} Chemotherapeutic agents are usually either encapsulated or conjugated or adsorbed on these nanomaterials to improve existing chemotherapy.

1.4.2 Gold NPs-based drug delivery platforms for lung cancer

Although there are many different types of inorganic NPs only gold NPs have been approved by the FDA to treat lung cancer.\textsuperscript{197} PEG-coated silica gold-nanoshell was used for NIR facilitated thermal ablation of solid primary and metastatic lung tumors.\textsuperscript{197} Due to its nanoscale size, electrons at the gold surface can interact with light, resulting in a phenomenon called surface plasmon resonance (SPR – see section 1.2). As per biomedical application, it is vital to tune SPR absorption wavelength, so the wavelength falls within the NIR region of the electromagnetic spectrum (650–1300 nm).\textsuperscript{198} This is called the ‘biological window’ as there are no absorptions by other biological matrices in that window. Therefore NIR light can penetrate deeper into the tissue.\textsuperscript{198} Photodynamic therapy (PDT) and Photothermal therapy (PTT) are two emerging cancer treatment approaches with a lot of promise, and the materials employed in these two therapies are related to optical interference. In PDT, a photosensitizer accumulates in cancerous sites, and when exposed to a specific wavelength of light, cytotoxic free radicals are produced, resulting in apoptosis and/or necrosis of cancerous cells.

While PTT employs nanomaterials with high photothermal conversion efficiency to raise the temperature of cancerous areas, resulting in cancer cell death. Various light-responsive nanomaterials have been investigated for PTT-against lung cancer. For
example, gold-based nanomaterials, carbon-based nanomaterials (CNMs), black phosphorous, and polydopamine-based nanomaterials.\textsuperscript{198} Hauck \textit{et al.} reported that the heat produced by GNRs in conjunction with the chemotherapeutic drug cisplatin killed 78\% more cancer cells than cisplatin alone.\textsuperscript{199} Combination therapy can reduce the toxicity of chemotherapeutics by lowering the effective drug dosage.\textsuperscript{199} Xie and collaborators fabricated hollow carbon nanospheres loaded with Dox and conjugated mitochondria targeting peptide and hyaluronic acid moieties on the surface and observed PT activity induced damage in lung cancer cells.\textsuperscript{200} Prasad and colleagues reported a synergistic combination strategy of black phosphorus (BP)-based photothermal therapy and anti-CD47 antibody (aCD47)-based immunotherapy to improve cancer treatment.\textsuperscript{201} Cao \textit{et al.} synthesized polydopamine NPs that carry photosensitizers and act as PT agents. Hence, combining PTT and PDT showed enhanced antitumor therapy.\textsuperscript{202} A phase II clinical trial that investigated the effects of two well-known anticancer drugs under mild hyperthermia conditions using poly(lactic-co-glycolic acid) (PLGA) coated Fe\textsubscript{3}O\textsubscript{4} NPs, which revealed the promise of using PTT in conjunction with chemotherapeutics (NCT00178763).\textsuperscript{203} The use of gold nanomaterials in PTT has gained attention as it enters clinical trials for lung carcinomas. AuroShells\textsuperscript{®}, gold-silica nanoshells, have entered an open-label, single-center, single-dose efficacy pilot study for the treatment of primary and metastatic lung tumors. (NCT01679470).\textsuperscript{204} Participants in this trial received an intravenous infusion of PEG-coated AuroShells, followed by laser irradiation of lung tumors via fiber optic bronchoscopy. The trial's primary outcomes were a reduction in tumor volume and the absence of thermal lesions in individuals over six months.\textsuperscript{204} Another study reported by El-Sayed group demonstrated the feasibility of GNR based PTT in squamous cell carcinoma.
They used thiolated PEG that was covalently bound to the surface of the GNR and injected it into squamous cell carcinoma and found a significant decrease in tumor volume. Other studies Chiang et al. showed conductive polymeric nanomaterials exhibits PTT and PDT, however high laser intensity have such as 2 W has been used to generate photothermal activity.

We have developed hybrid nano-assemblies composed of dual photothermal agents: GNR and IR 780. Our hybrid nano-assemblies are composed of GNRs coated with mesoporous silica shells that offer a surface for drug loading. Anticancer drug doxorubicin (Dox) was loaded into a mesoporous silica shell. A near-infrared dye, IR 780, was used as a photosensitizer to enhance the photothermal activity. The increased photothermal activity offers ROS generation under less intense laser irradiation. Cationic peptide GE-11 and folic acid were conjugated to the surface of the nano-assemblies to specifically target the epidermal growth factor receptor (EGFR) and folate receptor overexpressed on NSCLC cells. This dual ligand-targeting approach enhances the cancer-targeting, increases specificity, minimizes non-specific attacks on surrounding tissues, and improves the therapeutic efficacy of anticancer drugs by enhancing cellular uptake.

1.5 Nano diagnostics for bacterial infections

Even though enormous efforts have been made to prevent epidemics and death from infectious diseases, it remains one of the primary healthcare challenges that significantly influence humanity. As a result, developing specific, sensitive, accurate, quick, low-cost, and simple-to-use diagnostic tools is in urgent demand. Nano diagnostics is defined as utilizing nanotechnology in diagnostic applications. This technology has been extensively explored to develop diagnostic tests with great sensitivity and the ability to detect infection
before it occurs. Nano diagnostic platforms benefit from the unique features of nanomaterials or nanostructures, which allow for quick and real-time detection utilizing extremely small amounts of patient samples. Hence, nano diagnostic techniques hold much promise as low-cost, user-friendly, and reliable devices. Most nano diagnostic applications are currently focused on pathogen identification in infectious disorders and cancer biomarkers in cancer therapy.\textsuperscript{211} For infectious diseases, nano diagnostics can achieve simple, reliable, and fast conclusions by using blood, sputum, or urine samples from patients. Furthermore, the sensitive nano diagnostics platforms, which have much promise to be robust, inexpensive, and reproducible, could be useful for diagnosing infectious diseases, especially in developing countries.\textsuperscript{211}

1.5.1 Nanoparticle-based diagnosis for infectious disease

The distinct properties of NPs dictate nano diagnostic platforms and the ability to detect clinical samples in very small volumes. Various NPs have been effectively used to diagnose infectious disorders, including metallic NPs,\textsuperscript{212} fluorescent NPs,\textsuperscript{213} and magnetic NPs.\textsuperscript{214} Because of their high surface area-to-volume ratio and reactive surface atoms, NPs can be functionalized with affinity ligands capable of binding to pathogen-associated analyte targets.\textsuperscript{215} These functionalized NPs transduce the binding events into optical,\textsuperscript{216} electric,\textsuperscript{217} mechanical,\textsuperscript{218} or magnetic output signals.\textsuperscript{215}

1.5.1.1 Gold nanoparticles (AuNPs)

Gold nanoparticles (AuNPs) have unique physiochemical (inert and nontoxic) and optical properties, making them ideal for clinical diagnosis, therapies, and other multidisciplinary research.\textsuperscript{214} Upon light irradiation on AuNPs, it causes a surface plasmon resonance (SPR) which can be used to induce a color shift or localized heating.\textsuperscript{219} AuNPs
have the following distinguishing features: (1) a large surface area allowing functionalization with biomolecules,\(^{220}\) (2) intense bright colors allowing easy colorimetric bio-recognition,\(^{221}\) (3) high degree of control over shape and size,\(^{222}\) (4) long term stability in a wide range of solvents and PH conditions,\(^{223}\) (5) excellent optical performance due to a strong SPR.\(^{224}\) The most common application of SPR to infectious disease diagnostics is the lateral flow test (LFT), a well-known assay technique exemplified by the home pregnancy test.\(^{225}\) When functionalized AuNPs attach to their target analytes and aggregate on the LFT test strip, their optical signals are apparent as red lines; in this case, the AuNPs absorb light in the green region of the visible light spectrum and emit light in the red region.\(^{226}\) Recently, Moitra et al. have developed a simple, quick, and specific visual colorimetric SARS-CoV-2 detection test using AuNPs.\(^{227}\) Specific antisense oligonucleotides (ASOs) for the N phosphoprotein of SARS-CoV-2 were modified by thiol ligand and conjugated on AuNPs. When the target RNA sequence SARS-CoV-2 was present, thiol-modified ASOs conjugated AuNPs aggregate, resulting in a change in SPR with a detection time of 10 minutes.\(^{227}\)

### 1.5.1.2 Fluorescent nanoparticles

Quantum dots (QDs) are semiconductor-based fluorescent nanostructures with a diameter of 1–10 nm that exhibit many physicochemical properties, including photostability and high quantum yield.\(^{228}\) QDs are brighter and more stable than organic fluorophores, and they have broad absorption and narrow emission spectra, which is beneficial for simultaneous excitation and detection.\(^{229}\) The detection of bacteria such as *Escherichia coli* (*E. coli*),\(^{230}\) *Salmonella typhimurium*,\(^{231}\) *Mycobacterium bovis*,\(^{232}\) and oral bacteria has been studied extensively using ligand-conjugated QDs.\(^{233}\) In another example,
Zhang et al. have diagnosed Hepatitis B through the immunoassay using the QDs nanobeads with detection limit as low as 78 pg of hepatitis B surface antigen, indicating the diagnosis of infectious diseases.\textsuperscript{234}

1.5.1.3 Magnetic nanoparticles (MNPs)

MNPs have been used to detect bacteria in the past few decades and have been viewed as extremely promising nanomaterials due to their distinctive physical features, which include magnetic properties, structural qualities, and the ability to be easily modified.\textsuperscript{235} The magnetic properties of nanosized MNPs differ from conventional magnetic materials. Fe atom has a strong magnetic moment due to four unpaired electrons in its 3d orbitals. As shown in Scheme 1.6, there are four different types of magnetism exhibited in crystals; (1) In paramagnetic state, the magnetic moments are randomly oriented and when an external magnetic field is applied to the crystal, it has a small net magnetic moment, and when the field is removed, the magnetic moment is zero.\textsuperscript{236} (2) Even in the absence of an external magnetic field, all magnetic moments are aligned in a ferromagnetic state.\textsuperscript{237} (3) In antiferromagnetic state, the crystal shows no net magnetic moment if the antiparallel magnetic moments are of the same magnitude.\textsuperscript{237} (4) In a ferrimagnetic crystal are like antiferromagnetic materials, two types of atoms with different magnetic moments are aligned in an antiparallel fashion, resulting in ferrimagnetic material with a net magnetic moment.\textsuperscript{237} Both magnetite [Fe$_3$O$_4$] and maghemite [Fe$_2$O$_3$] are ferrimagnetic.\textsuperscript{237} Superparamagnetism is one type of magnetism that occurs in ferromagnetic and ferrimagnetic nanostructures. This feature is size-dependent, and it
usually occurs when NPs are as small as 10–20 nm. Because these NPs are so small, they do not have several domains like large magnets do; instead, they form a single magnetic domain and behave as a "single spin" with great magnetic susceptibility. Thus, when a magnetic field is applied, these NPs provide a stronger and faster magnetic response than bulk magnets. By taking advantage of the magnetic properties of MNPs, MNPs-mediated diagnosis carry out based on separation and detection of MNPs coupled with target biomarkers under an applied magnetic field. For example, superparamagnetic MNPs have been used to develop a magnetic immunoassay. The target analyte permits MNPs to form a sandwich conformation, resulting in a local magnetic field that the sensor can detect after an external field is utilized to induce a magnetic moment in the MNPs.
1.5.2 Nanodevice-based diagnosis for infectious disease

Many novel diagnostic methods are under investigation using nanotechnology to develop nanodevice-based diagnostic systems for the diagnosis of infectious diseases.\textsuperscript{240} Multiple assays can be incorporated into a single nanodevice.\textsuperscript{241} This would result in a reduction in the sample volume, material consumption, and processing time.\textsuperscript{241} For example, using microfluidics, high-throughput and multiplexed assays have been developed to detect blood-borne infectious agents such as Human immunodeficiency virus (HIV) in serum samples.\textsuperscript{242} Another example is a developed lab-on-a-chip to detect MDR-TB.\textsuperscript{243} This lab-on-chip consists of a single disposable device and microfluidic PCR and microarray modules. The platform includes a temperature control system and an optical reader, which allow for automatic microarray analysis and the generation of a user-friendly diagnostic report of MDR-TB.\textsuperscript{243} For specific detection of \textit{Mtb} using SPR, Prabhakar et al., employed cysteine modified NH\textsubscript{2}-end peptide nucleic acid probes and 5'-thiol end labeled DNA probes mounted onto BK-7 gold-coated glass plates.\textsuperscript{244}

Furthermore, nanotechnology-based systems have the potential to develop portable, robust, and affordable point-of-care testing (POCT) platforms to detect infectious diseases in developing countries.\textsuperscript{245} For example, researchers have created a smartphone dongle using gold and silver nanoparticles that can be used as a POCT system to test infectious diseases such as HIV and syphilis.\textsuperscript{246} They demonstrated that a full immunoassay, which is usually performed in the laboratory, can be integrated into a smartphone accessory to achieve nanotechnology-based POCT.\textsuperscript{246} The variety of POCT nano diagnostics systems has been developed by researchers, including magnetic barcode assay using magnetic nanoprobe and paper-based POCT (lateral flow system) using silver NPs.\textsuperscript{211} The magnetic
barcode system has also been used successfully for multiplex detection of the most common infectious bacteria, including *Mtb*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, and *Klebsiella pneumoniae*. Geissler et al. reported the paper-based POCT that used to detect yellow fever virus, Ebola virus, and dengue virus. More improved nano diagnostics focusing on the development of the challenging diagnosis of infectious diseases are considerably required if the patient is infected with more than one pathogen, such as HIV.

1.5.3 Diagnostics for TB

TB diagnostics methods are divided into four categories: traditional methods, immunological methods, molecular methods, and novel nano diagnostics methods. The traditional methods include conventional microscopy with acid-fast bacilli (AFB) staining culture methods that are time-consuming and labor-intensive. Immunological approaches, such as the Enzyme-linked immunosorbent assay (ELISA), Tuberculin Skin Test (TST), and IFN-Gamma Release Assay (IGRA), have been developed for the detection of TB/latent TB infection (LTBI). However, these methods fail to distinguish between latent TB and active TB infection. The molecular methods include Xpert MTB/Rif assay, nucleic acid amplification-based tests (NAAT), that is, polymerase chain reaction (PCR), line probe assay (LPA), Loop-mediated isothermal amplification (LAMP) and can detect *Mtb* within a few hours to days in suspected TB patients. Despite all advances in TB diagnosis, there is no accurate, rapid, inexpensive, point-of-care test available for *Mtb* detection particularly for developing nations where resources are very limited. Taking all of these factors into account, a nano diagnostic for TB detection is urgently required.
1.5.4 Nanoparticle-based diagnosis for TB

In past decades various nano diagnostics systems have been developed for the molecular diagnostics of TB. NPs such as gold, silica, and MNPs have been widely used for TB diagnostics. As mentioned earlier, AuNPs hold promise in developing colorimetric-based diagnostic tests due to having excellent optical properties. Baptista et al. were the first to use AuNPs in TB diagnosis by conjugating DNA probes with AuNPs for colorimetric detection of \textit{Mtb}.\textsuperscript{254} When complementary DNA is present, the nanoprobe solution remains pink (no DNA probe aggregation) at wavelength 526 nm, however, when complementary DNA is absent, the solution turns purple (due to high NaCl concentration causing nanoprobe aggregation).\textsuperscript{254} Compared to other diagnostic procedures, such as InnoLiPA-Rif-TB, the method developed by Baptista et al. is more accurate.\textsuperscript{254} The test was more sensitive than smear microscopy and easily observed for detection. The main advantage of this method is that it has a low risk of contamination (since it is performed in a single tube), and it is rapid, detection time is 15 min per sample.\textsuperscript{254}

Indirect immunofluorescence microscopy has been developed to detect \textit{Mtb} using silica NPs coupled with fluorescent dye.\textsuperscript{255} In this test, SYBR Green I-mediated assay stained only bio-conjugated fluorescent silica NPs, yielding fluorescent signals five times stronger than the traditional fluorescence isothiocyanate (FITC)-based detection approach. The detection time is less than 2 hours, and it is regarded as a promising method for the rapid detection of \textit{Mtb}.\textsuperscript{255} Various biosensors have also been developed by researchers for \textit{Mtb} detection.\textsuperscript{251} Moreover, MNPs have been shown to have a wide range of exciting and promising applications in medical diagnostics and therapy. Superparamagnetic iron oxide nanoparticles SPIONs, composed of magnetite [Fe$_3$O$_4$] or maghemite [Fe$_2$O$_3$] NPs are
widely used in cell tracking, drug therapy, and drug delivery. Lee et al. developed a technique in which mycobacteria were targeted by MNPs with a large Fe core and a thin ferrite shell, then concentrated in a microfluidic chamber and identified using nuclear magnetic resonance. The diagnostic platform's clinical efficacy was tested by detecting TB using the bacillus Calmette-Guérin (BCG) surrogate for Mtb. Several studies have reported the use of diagnostic magnetic resonance (DMR) in conjunction with iron oxide nanoparticles to detect Mtb. A nanoparticle-based colorimetric biosensing assay (NCBA) to identify acid-fast bacilli (AFB) in decontaminated sputum samples was described by Bhusal et. al., in 2019. Using glycan-coated magnetic nanoparticles (GMNP), the researchers were able to isolate AFB from decontaminated sputum samples using a magnet. Followed by the complex was stained and observed under a light microscope to check GMNP-Mtb interaction. Because the Mtb cell envelope is rich in complex carbohydrates and glycoproteins, which can bind with the GMNP, the GMNP captures Mtb cells via glycan-glycoprotein interaction. The method detected 10^2 CFU/mL with 100% sensitivity.

To summarize, NPs have been used in various studies to improve the diagnosis of TB. The results were promising in many cases, indicating that the use of nanotechnology to improve the sensitivity of infectious disease diagnosis is effective. However, many Mtb biomarkers have not yet been evaluated with NPs. In this thesis, we have synthesized multi-core silica-coated magnetic nanoparticles (SMNPs), these SMNPs are then conjugated to plant lectins Concanavalin A (Con A) and Aleuria aurantia lectin (AAL). The lectins Con A and AAL were chosen of their strong binding affinity for mannose (dissociation constant K_d 2.8 μM) and arabinose (dissociation constant K_d 16 μM), respectively. These lectins
conjugated SMNPs exhibited excellent magnetic properties. Following incubation of these SMNPs with mycobacteria, large visible agglomerates form due to cross-linking of SMNPs with mycobacteria. Furthermore, these large agglomerates could be easily separated using a magnet. This is discussed in detail in chapter 5.
CHAPTER 2. NEAR-INFRARED RESPONSIVE TARGETED DRUG DELIVERY SYSTEM THAT OFFERS THE CHEMO-PHOTOTHERMAL THERAPY AGAINST BACTERIAL INFECTION

2.1 Introduction

Antibiotic remedies have been used to date to treat bacterial infections. Chemotherapeutic antibiotics have since revolutionized medicine, with their high treatment efficiency and ease of manufacture leading to widespread usage. However, overuse has driven the development of multi-drug resistance (MDR) in bacteria, dramatically decreasing antibiotic efficacy and increasing the risks of infection-related mortalities.\textsuperscript{261} MDR bacterial infection has caused 2.8 million illnesses and 35,000 deaths in the United States in the past few years.\textsuperscript{261} Therefore, newer therapeutic options are required to combat this increasing problem.\textsuperscript{262, 263} Targeted drug delivery is an attractive alternative to traditional oral broad-spectrum non-targeted small molecule antibiotics in capsules.\textsuperscript{105, 264} But even with cutting-edge antibiotic delivery strategies, antibacterial activity still relies upon the therapeutic efficacy of the encapsulated drug.

Combination nano-therapy combining an antibiotic with a different modality of treatment such as photothermal (PT), magnetothermal therapies have recently emerged to the surface of antibacterial therapies.\textsuperscript{182, 265-267} Recently, Wang \textit{et al.} have developed a new strategy for effectively killing Gram-positive bacteria based on vancomycin (Van)-
modified gold nanostars (AuNSs). The Van-modified AuNSs (AuNSs@Van) can not only selectively recognize methicillin-resistant *Staphylococcus aureus* (MRSA) but also kill 99% MRSA under near-infrared laser irradiation (808 nm for 10 min, 2.5 W/cm²) in vitro and in vivo as compared to free drug. Additionally, AuNSs@Van shows satisfactory biocompatibility and antibacterial activity in treating a bacterial infection in vivo. In terms of magnetothermal therapies, magnetic nanoparticles (MNPs) have recently been used as hyperthermia agents to treat bacterial infections. MNPs would absorb electromagnetic radiation and then convert the magnetic energy to localized heat when placed in an alternating magnetic field with a high frequency and amplitude. A powerful nanocarrier based MNPs for antibiotics and silver nanoparticles (Ag NPs) delivery designed by Xing group. This nanocarrier penetrates *Staphylococcus aureus* biofilm and significantly enhances the biofilm disruption in an external magnetic field. Studies have shown that combination therapy (combitherapy) approaches combining PTT and antibiotic therapy to combat bacterial infections works better. Another work done by Quing *et al.* reported combined chemo-PTT offering engineered nano-structure against methicillin-resistant *Staphylococcus aureus* (MRSA) bacterial infection. Ma *et al.* also reported strategies for chemo-PTT therapy against *Escherichia coli* and *Staphylococcus aureus*. Both reported combinational therapies against bacteria lack nanomaterials' targeting ability towards the bacteria surface. Our approach allows targeted antibiotic delivery to the bacteria cell surface, which increases the drug's efficacy and reduces off-target toxicity. Among available nanoplatforms, gold nanoplatforms are used most extensively in PTT. These include gold nanospheres, nanorods (GNRs), nanocages, nanoshells, nanostars, and they all been successfully used both in vitro and in vivo. GNRs exhibits
strong longitudinal absorbance at the NIR I window of 700–1000 nm with excellent application in the photo-induced therapies.\textsuperscript{274} Additionally, by adjusting aspect ratios, GNRs can easily attenuate a strong absorption at a particular wavelength.\textsuperscript{275} Yang \textit{et al.} have recently reported the immobilization of gold nanorods (GNRs) on the surface of titanium via electrostatic interaction.\textsuperscript{276} This GNR-modified surface was resistant to the gram-positive and gram-negative bacteria, and with NIR irradiation enhancing the photothermal impact and high antibacterial activities.\textsuperscript{276} Based on the literature and application of GNRs as a photothermal agent in drug delivery, we used GNRs in this thesis that absorbs NIR light and generates PT activity. GNRs were coated in a mesoporous silica shell (GNR@MSNP) to improve drug loading capability. Porous inorganic shells are widely used in drug delivery applications due to their large pore volume, pore size and surface area.\textsuperscript{174, 277-279} Therefore, drug (bedaquiline, BDQ) loaded into GNR@MSNP assembly and then GNR@MSNP@BDQ was encapsulated in a thermo-sensitive liposome (TSL, GNR@MSNP@BDQ@TSL) which gives PT activity of GNRs. Liposome encapsulation offers controlled drug release and stops premature release before the nano-assembly approaches the target.\textsuperscript{280, 281} Drug selected was BDQ, a novel antibacterial therapeutic against tuberculosis (TB). The heat generated from the GNRs upon NIR exposure causes the TSL to undergo a phase transition and become permeable, releasing encapsulated drug BDQ. Exposure to NIR laser generates hyperthermia, leading to permeability in the bacterial cell membrane, causing leakage of bacterial cell content and subsequent cell death.\textsuperscript{282} To guide the liposome to the mycobacteria surface, mycobacteria recognizing antimicrobial peptide (AMPs) NZX was used.\textsuperscript{283} AMPs are small oligopeptides that have lately shown promise in combating antibiotic resistance
mechanisms due to their capacity to lyse bacterial membranes, resulting in broad-spectrum actions that target microorganisms ranging from viruses to parasites. The key feature of AMPs is their capacity to selectively disrupt bacterial membranes while causing no harm to mammalian cells, making them safe to use. NZX is an antimycobacterial peptide with a peptide sequence “GFGCNGPWSEDDIQCHNKSIKGYKGYCARGGFVCKCY”. Tenhald et al. investigated the impact of the NZX peptide on Mtb in vitro and in vivo. In both in vitro and in vivo infection models, they discovered that NZX is proteolytically stable and nontoxic to mammalian cells, but it targets and kills Mtb at concentrations comparable to conventional antibiotics. Since, AMPs can also exert a degree of antibacterial activity, and are therefore far more attractive than other biomarker targeting ligands. As a result, NZX is an appealing targeting peptide to use in our nano-assemblies. With the addition of NZX peptide, the final nano-assembly abbreviates as GNR@MSNP@BDQ@TSL@NZX.

TB is a life-threatening infectious disease. Pulmonary TB is caused mainly by Mycobacterium tuberculosis (Mtb) bacteria, an intracellular pathogen. Active TB is symptomatic, while latent TB is asymptomatic and difficult to treat. In latent pulmonary TB, Mtb can lie dormant in lung tissue, unaware of the immune system for years. Immune cells such as macrophages cannot eradicate the Mtb bacteria while within lung tissue cells. Antibiotics are ineffective when accessing macrophages and eradicating intracellular Mtb. Antibiotics are quickly cleared from macrophages before they act on intracellular Mtb. Hence treating intracellular Mtb is very challenging. Our nano-assembly is designed to target and kill these mycobacteria that survive within the lung epithelial cells. Previous nanotechnology-based drug delivery systems designed to target
Tenland and co-workers used mesoporous silica nanoparticles as a porous drug delivery nanocarrier loaded with antibacterial peptide NZX, which also can target intracellular Mtb residents in lung macrophage. They demonstrated an 88% reduction in Mtb in in-vivo studies. Ali and co-workers reported GNRs as a delivery vehicle delivering antibiotic rifampicin, GNRs
also act as a source of inherent bactericidal activity against \textit{Mtb}.\textsuperscript{10} Li \textit{et al.} reported combined antimycobacterial and PTT using core-shell upconversion nanoparticles carrying anti-tuberculotic drug rifampin.\textsuperscript{286} All these drug delivery systems suffer from a lack of targeting ability towards intracellular \textit{Mtb}.

Our engineered nano-assembly also displayed (a) stability in medium (b) no premature drug release (c) increased internalization into infected host cells due to having TSL and NZX peptide (d) control release of drug through melting of TSL. The final nano-assembly GNR@MSNP@BDQ@TSL@NZX (Scheme 2.1) was observed to have the best antibacterial activity against \textit{Mycobacterium smegmatis} (\textit{Msmeg}) in a series of in-vitro experiments. \textit{Msmeg} is a biosafety level 1 (BSL-1) mimic of \textit{Mtb}. We also assessed the ability of the nano-assembly to target and kill 99.9\% \textit{Msmeg} internalized within lung epithelial cells.

2.2 \textbf{Experimental}

2.2.1 \textbf{Materials and Instruments}

Hexadecyltrimethylammonium bromide (CTAB, 99\%), L-ascorbic acid (AA, 98\%), silver nitrate (AgNO\textsubscript{3}, 99\%), hydrogen tetrachloroauric (III) acid trihydrate (HAuCl\textsubscript{4}·3H\textsubscript{2}O, 99.9\%), sodium borohydride (NaBH\textsubscript{4}, 98\%), were used for gold nanorod synthesis and purchased from Sigma-Aldrich (St. Louis, USA). Tetraethyl orthosilicate (TEOS, 98\%), 3-aminopropyl-triethoxysilane (APTES, 97\%), 3-(aminopropyl)trimethoxysilane (APTMS) (97\%), rhodamine B isothiocyanate isomer (RITC), were used for mesoporous silica shell synthesis and purchased from Sigma-Aldrich (St. Louis, USA). \textit{N}-hydroxysulfosuccinimide sodium salt (sulfo-NHS), 1-ethyl-3-(3-dimethylaminopropyl) - carbodiimide (EDC, 98\%) were used for conjugation of
peptides and purchased from Alfa Aesar (Massachusetts, USA). Sodium hydroxide (NaOH, 98.5%) was purchased from Lab Chem (Pennsylvania, USA). Ammonium nitrate (NH₄NO₃, 99%) was purchased from ACROS Organics. Water was used from a Milli-Q water ultrapure water purification system (Millipore. Absolute ethanol (200 proof), sodium lauryl sulfate (SLS), chloroform (CHCl₃), HPLC grade acetonitrile (ACN), water with 1% trifluoroacetic acid (TFA, 1% v/v) HPLC grade were purchased from Fisher Scientific Inc. (Massachusetts, USA).

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG 2000 amine) and mini extruder kit were used for liposome preparation and purchased from Avanti Polar Lipids Inc., (Alabama, USA). N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES solution), MES buffer, 2’,7’-Dichlorofluorescin diacetate (DCFH-DA) reactive oxygen species kit (ROS) assay kit and cell counting kit WST-8 were purchased from Sigma-Aldrich (St. Louis, USA). Msmeg strain mc² 155 (ATCC® 700084) was purchased from American Type Culture Collection (Vancouver, USA). Mycobacteria media Difco™ Middlebrook 7H9 broth, Difco™ Middlebrook 7H10 agar, BBL™ Middlebrook ADC enrichment, and BBL™ Middlebrook OADC enrichment were purchased from Becton Dickinson (New Jersey, USA). A549 (ATCC® CCL-185) lung cancer cells were also purchased from ATCC. Synthesized NZX peptide was purchased from ProteoGenix (Schiltigheim, France). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation kit, live/dead® BacLight™ bacterial viability kit, Hoechst dye, 4’,6-diamidino-2-phenylindole (DAPI) and calcein-AM were purchased from ThermoFisher Scientific (Massachusetts, USA). Ham’s F-12K nutrient
mixture with L-glutamine (F-12K, 1X), fetal bovine serum (FBS, 10%), trypsin EDTA (2.21 mM), and Penicillin Streptomycin (Pen-Strep, 1X) was purchased from Corning (New York, USA).

The particle size was determined using a Dynamic Light Scattering (DLS) Malvern Zetasizer, Malvern Panalytical Inc., (Massachusetts, USA). Transmission electron microscopy (TEM) images were obtained on an FEI Tecnai Osiris operating at an accelerating voltage of 200 kV. Nitrogen adsorption-desorption was performed using a surface area and porosity analyzer (Autosorb iQ-C-MP/XR, USA). Absorbance spectra of particles and optical density measurements were performed with a Spectramax M2 Molecular Devices Ltd., plate reader (Silicon Valley, USA). Fourier-Transform Infrared (FTIR) spectroscopic analysis was done using Nicolet is50 ATR FTIR ThermoFisher Scientific (New Jersey, USA). Thermogravimetric analysis (TGA) was performed on a Discovery Q500, TGA from TA Instruments (Massachusetts, USA). High-Performance Liquid Chromatography (HPLC) analysis was performed using a Hitachi Primaide Separation module and a 1430 diode array detector equipped with a reverse-phase SunFire C18 column.

The 808 nm laser diodes (G pin code) of 200 mW and 500 mW power intensities, laser diode controller (LDC210C), laser diode temperature controller (TED200C), laser diode mount (LDM56), digital thermometer, and 25 mm objective lens (LA1560) were all purchased from Thor Labs Inc., (New Jersey, USA). The fluorescence microscopy images were taken from the CKX53 inverted fluorescence microscope, Olympus (USA). Mammalian cells were quantified using an automatic cell counter (Corning, USA). All aseptic bacteria techniques and sterile mammalian cell culture were conducted in a separate
1300 Series A2 biosafety cabinet (ThermoFisher Scientific). Shaking incubator (Corning, USA) and CO₂ incubator (ThermoFisher Scientific) was used for bacteria and mammalian cell culture incubation. Sterile disposables, sterilized glassware, and medium were used for all experiments. Unless specifically mentioned, all liquid media were sterilized using a benchtop BioClave liquid sterilizer (Ward’s Science, New York, USA).

2.2.2 Synthesis of gold nanorods (GNRs)

A modified seed-mediated method by El-Sayed et al. was used for GNRs synthesis.⁴⁸ CTAB coated seeds were prepared by chemical reduction of gold salt with NaBH₄. Briefly, a HAuCl₄ solution (0.25 mL, 0.01 M) was mixed with a CTAB solution (7.5 mL, 0.1 M) in a 25 mL round bottom flask under stirring. An ice-cold NaBH₄ solution (0.6 mL, 0.01 M) was injected quickly under vigorous stirring. The solution turned immediately brownish yellow color, suggesting the formation of the gold seeds. The mixture was kept undisturbed at 30 °C for another 3-4 h to ensure the complete degradation of unreacted NaBH₄. All the solutions were prepared from a stock solution to get reproducible results. The growth solution consisting of CTAB (600 mL, 0.1M), HAuCl₄ (30 mL, 0.01M), AgNO₃ (4.8 mL, 10 mM) and H₂SO₄ (12 mL, 0.5 M) was stirred together in a 1000 mL round-bottom flask. The growth solution was equilibrated at 30 °C while stirring for 10 min. Ascorbic acid (4.8 mL, 0.1 M) was quickly injected into the growth solution with vigorous stirring. The solution turned colorless immediately. Gold seeds (1.4 mL) were added and stirred for 5 min and then left to age overnight. The prepared GNRs were purified via two cycles of centrifugation at 12000 rpm for 15 min and then re-dispersed into 150 mL of water for further modification.
2.2.3 Synthesis of mesoporous silica shell coated gold nanorods (GNR@MSNPs)

GNR@MSNPs were synthesized according to the protocol developed by Matsuura et al. with some minor modifications. Firstly, 100 mL of purified GNRs were transferred into an aqueous solution of CTAB (500 mL, 0.8 mM) then the basicity of the solution was adjusted to pH 10-11 with NaOH (0.1 M). After equilibration at 30 °C for 10 min, a solution of TEOS/APTES/ethanol (400 µL, 20 µL, and 1380 µL respectively, total 1.8 mL) was added into the mixture by three times injecting 0.6 mL with gentle stirring. The reaction mixture was allowed to react at 30 °C overnight. The synthesized GNR@MSNPs were centrifuged at 12000 rpm for 15 min once and then re-dispersed in 120 mL of ethanolic NH₄NO₃ (0.75 mM) at 45 °C and kept overnight to remove CTAB. The final GNR@MSNPs were purified by centrifugation (10000 rpm for 10 min) and re-dispersed in ethanol.

2.2.4 RITC-labelled GNR@MSNPs synthesis

The RITC precursor was prepared in the following manner. RITC (8.0 mg, 0.015 mM in ethanol) was stirred overnight with APTMS (5.2 mL, 0.03 mM) in anhydrous ethanol (15.0 mL) at 42 °C to obtain the RITC-APTMS precursor. RITC-labeled GNR@MSNPs were synthesized via the same procedure as GNR@MSNPs except that the TEOS/APTES/ethanol solution was replaced by TEOS/RITC-APTMS/ethanol solution (400 mL, 20 mL, and 1380 mL respectively, total 1.8 mL) while the injected volume at each 30-minute interval stayed the same. This reaction was performed in the dark.

2.2.5 Surface and pore volume determination of GNR@MSNPs

The surface area was determined using the Brunauer–Emmett–Teller (BET) model, Autosorb iQ-C-MP/XR (Quantachrome, USA), and the cumulative pore volume was
calculated from the adsorption branch of the isotherm using the Barrette–Joyner–Halenda (BJH) model.

2.2.6 Drug loading in GNR@MSNPs

GNR@MSNPs (21 mg) was mixed with BDQ (7 mg) in 10 mL of DMSO while stirring overnight at 37 °C. The ratio of GNR@MSNPs and BDQ used in the entire study was 3:1 w/w (GNR@MSNPs 21 mg: BDQ 7 mg). The BDQ loaded GNR@MSNPs samples were collected by centrifugation at 10000 rpm for 10 min and vacuum dried to obtain a dry powder. The encapsulation efficiency and drug loading of BDQ in GNR@MSNPs were determined by using a standard calibration curve. To construct a standard calibration curve for BDQ, BDQ (0.1 mg) was resuspended in 1 mL of HEPES and then diluted to concentrations of 1-10 μg/mL. Drug (BDQ) encapsulation efficiency and drug loading were determined by HPLC.287-289

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Encapsulation\ efficiency = \frac{\text{Initial amount of drug added} - \text{Amount of drug in supernatant}}{\text{Initial amount of drug added}} \times 100\% \tag{2.1}
\]

\[
Drug\ loading\ content = \frac{\text{Initial amount of drug added} - \text{Amount of drug in supernatant}}{\text{Nanoparticles weight}} \times 100\% \tag{2.2}
\]

2.2.7 Encapsulation of GNR@MSNPs@BDQ in TSL

TSLs were prepared by a lipid film-based method previously reported in the literature.104 Briefly, DPPC (85% molar ratio), cholesterol (10% molar ratio), and DSPE-
PEG2000 amine (5% molar ratio) were dissolved in 2 mL of CHCl₃ and evaporated in a rotary evaporator, yielding a thin lipid film. This thin lipid film was rehydrated in 10 mM HEPES buffer containing GNR@MSNP@BDQ (2 mg/mL) before being extruded 15 times through two stacks of Nuclepore, Whatman polycarbonate membranes with a pore size of 800 nm (GE Healthcare, New York, USA) using a mini extruder set (Avanti Polar Lipids, Alabama, USA). The resultant nano-assembly (GNR@MSNP@BDQ@TSL) was stored at 4 °C until use.

2.2.8 Conjugation of bacteria targeting NZX peptide on TSL

GNR@MSNP@BDQ@TSL were conjugated with NZX via the EDC activation method, activating the NZX peptide's carboxyl group to react with the primary amines of DSPE-PEG2000 amine in the liposomes. NZX 0.1 mg/mL was dissolved in 0.1 M MES, 0.5 M NaCl, pH 6.0 (reaction buffer) and then activated with 10 fold molar excess of EDC (pH = 4.0) and 25 fold molar excess of sulfo-NHS for 30 min. Liposomes (GNR@MSNP@BDQ@TSL) were added to the reaction mixture and reacted 24 h at 37 °C. The molar ratio of NZX: liposome was maintained at 10:1. After 24 h, the reaction mixture was purified by centrifugation at 10000 rpm for 15 min at 25 °C to remove excess unreacted EDC.

2.2.9 TGA analysis of various nano-assemblies

TGA was carried out under argon gas (99.999%) where dried GNR@MSNP@BDQ (~ 1 mg) was heated at a rate of 5 °C /min to 100 °C and then kept at isothermal at 100 °C for 15 min followed by 5 °C /min ramp to 700 °C. The BDQ loaded in GNR@MSNPs was calculated by analyzing the weight loss difference between GNR@MSNPs and GNR@MSNP@BDQ. Similarly, the TSL content was analyzed by measuring the weight
loss difference between GNR@MSNP@BDQ and GNR@MSNP@BDQ@TSL. Finally, the amount of peptide was calculated by measuring the weight loss difference between GNR@MSNP@BDQ@TSL and GNR@MSNP@BDQ@TSL@NZX.

2.2.10 Colloidal stability study of nano-assemblies

The stability of GNR@MSNP and liposome coated GNR@MSNP (GNR@MSNP@TSL) was conducted at 37 °C to mimic the static condition in an *in vitro* experiment. Nano-assemblies were dissolved in mammalian cell culture medium to a 1 mg/mL concentration and incubated at 37 °C for five days. 290 Kah *et al.* reported calculating an aggregation index (AI) as a stability parameter to determine the stability of GNRs. 291 AI is a measure of the LSPR peak broadening. AI is calculated by dividing the total area under the curve (AUC) of the absorbance spectra of the GNRs LSPR band from 600 to 900 nm by the LSPR peak intensity (equation 2.3). 291 AI calculates an equivalent bandwidth of longitudinal peak for a spectrum normalized to the LSPR peak intensity. A similar analysis was performed for GNR@MSNP and GNR@MSNP@TSL stability studies. The higher AI indicates a higher degree of aggregation, hence lower colloidal systems stability. 291, 292

\[
AI (nm) = \frac{AUC \text{ of LSPR from } 600 \text{ to } 900 \text{ nm}}{LSPR \text{ peak intensity}}
\]

(2.3)

2.2.11 Photothermal studies

The thermo-optical setup used for this purpose is shown in Scheme 2.2. The setup was used with two separate NIR diode lasers with 500 and 200 mW laser intensities (L808P500MM and L808P200) (Thor Labs, USA). The laser diode was fixed in a temperature-controlled mount (LDM56) (Thor Labs, USA), which is connected to the laser
diode controller (LDC210C, Thor Labs, USA). A laser diode controller was used to control the intensity of the laser output power. The laser diode temperature controller (TED200C, Thor Labs, USA) helped maintain a stable output power wavelength and prevent heating that can damage the laser diodes. The laser beam was focused to a spot size of ~ 2 mm diameter, with a 25 mm focal length objective lens to the middle of a single well in a 96 well plate. A thermocouple monitor was used to monitor the temperature of the solution upon exposure to the laser. The entire study used a power intensity of 500 mW as 200 mW was insufficient to induce required hyperthermia effects. Various weight concentrations of the final nano-assemblies ranged from 50, 100, 500, and 1000 µg/mL (calculated with respect to the weight of GNR@MSNPs) GNR@MSNP@BDQ@TSL@NZX were irradiated with the 808 nm NIR diode laser (500 mW) for a time period of 1 min –15 min. A digital thermocouple monitored the temperature change.

2.2.12 NIR laser triggered drug release from nano-assemblies

Scheme 2.2. Schematic illustration of the laser setup used for photothermal studies.
Nano-assemblies such as GNR@MSNP@BDQ, GNR@MSNP@BDQ@TSL, and GNR@MSNP@BDQ@TSL@NZX were dispersed in 3 mL of release media (0.5 % w/w SLS in 10 mM HEPES buffer, pH 7.2), then incubated at 37 °C with constant agitation. At predetermined time intervals (2, 4, 6, 8, 12, 18, 24, 36, 48, and 72 h), the suspension was exposed to the NIR laser (808 nm, 500 mW) for 15 min and then centrifuged at 15000 rpm for 15 min, the supernatant collected and replaced with the same volume of fresh prewarmed (37 °C) release media. The pellet was resuspended in the new release media and further incubated. Each sampled aliquot of the supernatant was passed through a 0.22 μm nylon filter. The drug release was quantified by HPLC (equipped with UV detector): HPLC Hitachi Primaide separation module equipped with a 1430 diode array detector and a reverse phase SunFire C18 column. Elution was performed using a mobile phase composed of a linear gradient of ACN and TFA at 0.1% (v/v) in water. BDQ was eluted using linear-gradient mode with 50:50 TFA: ACN for 2 min, 30:70 TFA: ACN 2.1 – 15.1 min, and final column wash using 50:50 TFA: ACN ratio from 15.2 to 18 min. The injection volume was 20 μL, and the absorbance was recorded at 230 nm. The operating temperature was 40 °C, and the flow rate was kept at 1.2 mL/min. Calibration curve for BDQ was generated using 1.0, 2.5, 5.0, 7.5 and 10.0 μg/mL of BDQ diluted in the release media release media: 0.5% w/v SLS containing 10 mM HEPES, pH 7.2. To quantify the BDQ release without laser exposure, a similar experiment was conducted in the absence of the NIR laser light exposure. HPLC quantified the BDQ release from the final nano-assembly.
2.2.13 *Msmeg* culture and viability assays

*Msmeg* were grown at 37 °C in Middlebrook 7H9 medium supplemented with 10% ADC and 0.05% glycerol. WST-8 cell viability assays were conducted using assay instructions. Briefly, in a flat-bottom 96-well plate, 10 µL of *Msmeg* (10⁸ CFU/mL), 170 µL of Middlebrook broth, and an aliquot of 20 µL of various nano-assemblies added and shaken (2000 rpm) at 37 °C on a shaker bed for 24 h. After 24 h incubation, the bacterial viability was determined by a WST-8 assay. The absorbance was recorded at 450 nm using a plate reader, and high absorbance at 450 nm was interpreted as higher viability of the bacterial cells. The viable count of bacteria in each well was quantified using standard spread plate methods followed by the colony counts. From each well, a volume of 10 µL was serially diluted and spread onto the nutrient agar plates and incubated at 37 °C for 24 h. Agar plates with 30 to 300 colonies were counted, and the viable bacteria was reported as CFU/mL.

2.2.14 NIR responsive antibacterial activity

The viability of bacteria treated with nano-assemblies with and without NIR laser (808 nm, 500 mW) exposure was determined by WST-8 cell viability assay and verified by the colony counting. Antibacterial activity of (a) GNR@MSNPs (b) GNR@MSNP@BDQ (c) final nano-assembly GNR@MSNP@BDQ@TSL@NZX (e) BDQ (f) NZX was determined by WST-8 assay and colony counts. Various sample concentrations of a-f from 0.01-1000 µg/mL were used, and MIC of each material was determined. MIC is the minimum inhibitory concentration, *i.e.*, the lowest concentration of drug that effectively inhibits the growth of bacteria. Furthermore, the antibacterial activity of the nano-assemblies with or without NIR laser exposure was analyzed by colony counts.
The viable percentage count of bacteria treated with the material in presence and absence of laser was calculated using the following:

\[
% \text{Reduction} = \frac{\text{Initial } \left( \frac{\text{CFU}}{\text{mL}} \right) - \text{Test } \left( \frac{\text{CFU}}{\text{mL}} \right)}{\text{Initial } \left( \frac{\text{CFU}}{\text{mL}} \right)} \times 100\%
\]  

(2.4)

The initial colony count was kept at \(10^8\) CFU/mL, and the test count (CFU/mL) was obtained after treatment.

2.2.15 ROS quantification

DCFH-DA was used as a fluorescent probe to assess ROS generation in bacteria. Briefly, 50 μL of \(Ms\)\text{meg} \((10^8\) CFU/mL) was incubated with 150 μL (1mg/mL concentration) of GNR@MSNP@BDQ and final nano-assembly GNR@MSNP@BDQ@TSL@NZX for 4 h. Bacteria alone is used as a negative control. After incubation, all samples were exposed to NIR laser (808 nm, 500 mW) for 15 min. This was followed by adding 100 μL of ROS reagent (DCFH-DA) and incubating for 1 h. Fluorescent intensity was quantified at 525 nm, with excitation at 490 nm. Samples were also observed by fluorescence microscopy.

2.2.16 Live-dead assay

To further investigate the antibacterial, PT effect, and targeting capacity of the nano-assemblies, a live/dead assay was conducted. In a flat bottom 96-well plate, \(Ms\)\text{meg} \((10^8\) CFU/mL, 10 μL) was treated with BDQ, GNR@MSNP@BDQ, and final nano-assembly GNR@MSNP@BDQ@TSL@NZX (1 mg/mL, 20 μL) and diluted with 170 μL of growth medium. After incubation for 3 h (37 °C), the samples were irradiated with the NIR laser (808 nm, 500 mW) for 15 min. The supernatant
was removed by centrifugation at 5000 rpm for 10 min, and the pellet was washed 3x with HEPES. The bacteria treated with nano-assemblies were stained with SYTO 9 and propidium iodide (PI) for 30 min at 37°C. Finally, the live and dead cells were examined under an inverted fluorescence microscope.

2.2.17 A549 cell proliferation

A549 cells were grown in T75 flasks in a complete growth medium. Complete growth medium was prepared by mixing Ham’s F-12K nutrient mixture with L-glutamine (F-12K) (1X, 445 mL), FBS (10%, 50 mL), and Pen-Strep (1X, 5 mL) followed by sterile filtration. The cells were grown in 20 mL of the fresh and prewarmed medium at 37°C in a humidified atmosphere of 5% CO₂. Cells viability was assessed in trypan blue assay, and cells were counted through an automatic cell counter.

2.2.18 Cellular uptake by nano-assemblies

In order to image the cellular uptake of the final nano-assembly, GNR@MSNP@BDQ@TSL@NZX was labeled with the red fluorescent dye RITC. A549 cells (10³ cells/mL) were cultured on a 24 well plate for 24 h until complete adhesion. After adhesion and confluence (~ 90%), A549 cells were incubated at 37 °C with RITC-GNR@MSNP@BDQ@TSL@NZX (50 µg/mL) for 0.5, 1.0, or 3.0 h. The cells were washed 3x with cold PBS and followed by calcein-AM (10 µM) and Hoechst dye (10 µM), staining for 30 min at 37 °C. The samples were analyzed through an inverted fluorescent microscope.

2.2.19 Cellular uptake mechanism by nano-assemblies

A549 cells (10³ cells/mL) were cultured on a 24 well plate for 24 h until complete adhesion occurred and then pre-treated with different endocytosis inhibitors:
chlorpromazine (20 μg/mL), nystatin 20 μg/mL, and wortmannin 1 μg/mL for 30 min. A549 cells were treated with 50 μg/mL GNR@MSNP@BDQ, and GNR@MSNP@BDQ@TSL@NZX then incubated for 2 h at 37 °C. An inverted fluorescent microscope was used to qualitatively evaluate the uptake of the nanoassemblies by lung cells. An increase in red fluorescence intensity within the lung cells is related to the high uptake of nano-assemblies. To quantitatively determine cellular uptake of nano-assemblies, the lung cells (A549) were lysed with 0.5% Triton X-100 in the presence of 0.2 M NaOH. A fluorescence microplate reader was used to measure the fluorescence intensity at excitation and emission wavelengths of 544 and 576 nm, corresponding to the excitation and emission of RITC. The fluorescence intensity of the lysed samples was measured. The fluorescence intensity of the controls (without inhibitor) was set as 100%. The reduction fluorescence intensity of the samples (with inhibitor) was measured in comparison to the control.

2.2.20 Intracellular antibacterial and targeting capacity of nano-assemblies

A549 cells (10^3 cells/mL) were seeded in a 24 well plate and incubated overnight in a 5% CO2 at 37 °C. An aliquot of 5 μL Msmeg (17 x 10^4 CFU/mL) was added per well of A549 cells and incubated for 30 min at 37 °C. After incubation, the supernatant was discarded, and the infected cells were washed twice with PBS (1X). Then the mammalian cell culture medium was replaced with a medium supplemented with 30 μg/mL BDQ to kill the remaining extracellular bacteria without affecting the intracellular bacteria. The infected A549 cells were treated with 50 μg/mL of GNR@MSNP@BDQ and GNR@MSNP@BDQ@TSL@NZX, then exposed to NIR laser (808 nm, 500 mW, 15 min) and incubated for an additional 24 h. After overnight incubation, the cells were stained
with calcein-AM (10 μM) and Hoechst dye (10 μM) for 30 min at 37 °C. The samples were analyzed through confocal fluorescent microscopy. Finally, A549 cells were lysed in distilled water for 3 h. The cell lysate was plated on agar, and the bacterial colonies were quantified. The number of colonies reflects the viable intracellular *Msmeg* present after treatment.

### 2.2.21 Mammalian cell viability assay

Cell viability was evaluated by using an MTT assay. A549 cells were seeded onto 24 well plates at a $1 \times 10^3$ cells/well (1 mL per well). After reaching 90% confluence, they were treated with various concentrations (1.0, 0.5, and 0.1 mg/mL) of GNR@MSNPs, GNR@MSNP@BDQ, and final nano-assembly GNR@MSNP@BDQ@TSL@NZX. Treated cells were exposed to NIR laser 808 nm, 500 mW for 15 min. Finally, 10 μL of the MTT solution (1 mg/mL) was added to each well, and the cells were further incubated for 4 h. After incubation, the supernatant was discarded, and 150 μL of DMSO was added to each well. The absorbance intensity was determined at 590 nm by a microplate reader. Results were presented as the percentage (%) with respect to untreated control A549 cells.

### 2.3 Results and Discussion

#### 2.3.1 Morphology characterization of nano-assemblies

The morphological characterization of nanomaterials was done mainly by electron microscopy such as Transmission Electron Microscopy (TEM). It is a powerful imaging tool for characterizing nanomaterials as it provides visualization of the shape, size, homogeneity, and lattice structure of nanomaterials at the atomic level. Here, the morphologies of nano-assemblies were examined on TEM. The fabrication of the final nano-assembly GNR@MSNP@BDQ@TSL@NZX was achieved in a stepwise manner, as
illustrated in Scheme 2.1a. GNRs were synthesized via a seed method described by El-Sayed et al. with some minor modifications. As shown in Figure 2.1a, the average length and width of GNRs were $42 \pm 3.8 \text{ nm}$ $11.2 \pm 1.1 \text{ nm}$, and the calculated average aspect ratio is 3.8:1. The GNRs displayed a uniform and well-disposed rod-like morphology (Figure 2.1a). GNRs coated with a porous silica shell were synthesized by a modified protocol from Matsuura et al. CTAB was used as a soft template, which creates the mesoporous silica shell structure during the polymerization of silane around the GNRs (to
give GNR@MSNP). TEM images (Figure 2.1b) show GNRs encapsulated in a mesoporous silica shell (GNR@MSNP). The silica shell has an average thickness of 15 ± 3 nm, and its porous structure can be clearly seen by TEM (Figure 2.1b). BDQ, an anti-tuberculosis drug, was encapsulated into the porous structure of the GNR@MSNP to create GNR@MSNP@BDQ. The GNR loaded mesoporous structure was wrapped by TSL to give GNR@MSNP@BDQ@TSL. The size of GNR@MSNP@BDQ@TSL was found to be 80 ± 3 nm (n = 20, n particle number) by TEM analysis and was calculated by Image J software. The liposomal layer was visible through TEM as shown with red double headed arrow (Figure 2.1c). The shape of GNR@MSNP dictates the oblong shape of the TSL layer. High-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) images (Figure 2.1e-i) show the presence of the elements of gold (Au), silica (Si), oxygen (O), and carbon (C) present in the layers of GNR@MSNP@BDQ@TSL. The merged image in Figure 2.1i clearly shows the individual layers overlaid on each other to show the final construct of GNR@MSNP@BDQ@TSL. HAADF images reveal the presence of carbons from the long hydrocarbons chains of lipids used to form the TSL. This proves the presence of TSL surrounding GNR@MSNP@BDQ as indicated by the red double-headed arrow in the merged image of Figure 2.1i. The combined TEM (Figure 2.1c) and HAADF-STEM (Figure 2.1i) images clearly show the TSL coating surrounding GNR@MSNP@BDQ.

2.3.2 Elemental analysis of nano-assemblies

Energy-dispersive X-ray spectroscopy (EDS) is an analytical method for the chemical characterization of materials. Generally, EDS systems are attached to an electron microscopy instrument like TEM. EDS captures and analyzes the characteristic x-rays
generated from the material when a high-energy electron beam focuses on the material. Excitation and relaxation of the electrons in the sample generate x-rays, which have discrete energies determined by the orbital structure of each element present in the sample. This allows for a fast, effective, and precise analysis of the elemental composition of materials. EDS analyzed the chemical composition of GNR@MSNPs and GNR@MSNP@TSL. The EDS spectra of GNR@MSNPs (Figure 2.2a) displayed the peaks of gold (Au), oxygen (O), and silica (Si). Copper (Cu) peak was present in the EDS spectra because a Cu mesh TEM grid was used as a substrate. EDS spectrum of GNR@MSNP@BDQ@TSL (Figure 2.2b) shows the peaks for elements Au, Si, O, and C. Phospholipids in the TSL layer can be indicated using a phosphorous peak (P) present in the EDS spectrum (Figure 2.2b). However, the peaks for P and Au overlap with one another (see Figure 2.2b). Therefore, to avoid confusion of using overlapping P peaks to indicate the presence of TSL, we have used the presence of carbons (C) to indicate the presence of the TSL layer. TSL is composed of lipids with a long chain hydrocarbon; for example, DPPC lipid has a 40-carbon alkyl chain. HAADF-STEM image in Figure 2.1h shows the clear presence of C in the outermost TSL layer. The merged image in Figure 2.1i clearly

Figure 2.2. EDS profile mapping of (a) GNR@MSNP@BDQ and (b) GNR@MSNP@BDQ@TSL.
shows the presence of C in the exterior of the nano-assembly.

2.3.3 Surface charge

The zeta potential was measured after the addition of each layer to monitor the change in surface charge. The altered surface zeta potential is shown in Figure 2.3a. The zeta potential of GNRs was 28 ± 0.59 mV, possibly due to the presence of positively charged CTAB on the surface. After silica coating, the surface charge was reduced to -23.54 ± 0.8 mV due to abundant Si-OH groups. BDQ is hydrophobic and shows high solubility in DMSO and little or no solubility in water. Therefore, zeta potential for BDQ was not obtained. After loading BDQ to MSNP, the zeta potential increased to +2.27 ± 0.76 mV. We hypothesize this is possibly due to the reduction in the available surface OH groups after the loading of BDQ. TSL coating increased the zeta potential to +7.25 ± 0.6 mV due to amines groups from DSPE-PEG 2000 amine on the surface. The cationic peptide NZX conjugated to the to the abundant -NH₂ groups present on the surface of the liposome through EDC coupling chemistry, which is responsible for the final increase the zeta potential up to +11.6 ± 0.88 mV.

Figure 2.3. (a) zeta potential (b) UV-vis absorbance spectra of nano-assemblies.
2.3.4 Optical properties of nano-assemblies

The optical properties of nano-assemblies were analyzed by UV-Vis spectroscopy. The UV-vis spectra indicated two characteristic absorption peaks of GNRs: transverse surface plasmon resonance (TSPR) peak at 514 nm and longitudinal surface plasmon resonance (LSPR) peak at 790 nm. With the mesoporous silica coating, the LSPR peak underwent a red shift towards 805 nm due to the change in the local refractive index of the surrounding medium and changes in geometry. The laser was selected to match the new absorbance at 805 nm. There was no observable shift in the LSPR peak with the loading of BDQ drug and TSL wrapping (Figure 2.3b).

2.3.5 Functional group characterization of nano-assemblies

The FT-IR spectra of the different components of the fabrication step are presented in Figure 2.4a. GNRs show -CH\textsubscript{2} symmetric and asymmetric vibrations at 2847 cm\textsuperscript{-1} and 2870 cm\textsuperscript{-1}, respectively. The presence of a peak at 1473 cm\textsuperscript{-1} corresponds to the -CH\textsubscript{2} scissoring modes of vibrations. In the fingerprint region, the bands at 960 and 817 cm\textsuperscript{-1} are consistent with C-O stretching and in-plane C-H bending vibrations. The appearance of a
peak at 1064 cm\(^{-1}\) (Si-O-Si stretching) and the disappearance of symmetric (2847 cm\(^{-1}\)) and asymmetric (2870 cm\(^{-1}\)) vibrations which were seen in GNR, proves the mesoporous silica coating on the surface of GNRs. After BDQ loading, the characteristic peaks from benzene rings, C- H stretching: 2922 cm\(^{-1}\) and 2914 cm\(^{-1}\) and C-C stretching: 1407 cm\(^{-1}\), were observed. The presence of N-H stretching at 1662 cm\(^{-1}\) and C-N stretching at 1230 cm\(^{-1}\) shows the primary amine group of BDQ, which confirms the successful loading of BDQ to the nano-assembly. The symmetric and anti-symmetric -CH\(_2\) stretching 2848 cm\(^{-1}\) and 2918 cm\(^{-1}\) confirms the presence of the lipid layer on the surface of GNR@MSNP@BDQ. NZX was conjugated to the abundant -NH\(_2\) groups present on the surface of TSL through EDC coupling chemistry. The -COOH groups of the peptide were activated with EDC and reacted with sulfo-NHS. The sulfo-NHS of the peptide reacts with the –NH\(_2\) groups on the TSL. The conjugation of NZX peptide on TSL introduces amide I and II bands at 1650 and 1540 cm\(^{-1}\) and C-O stretching vibration at 1241 cm\(^{-1}\).

2.3.6 Porosity analysis of mesoporous silica shell

The Brunauer–Emmett–Teller (BET) technique is based on the principle of physisorption of gas on a solid surface, where the adsorption and desorption isotherms of nitrogen gas are used to quantify the surface area, pore size, and pore volume of a nanomaterial. The N\(_2\) adsorption-desorption isotherm and pore size distribution curves of GNR@MSNP are given in Figure 2.4b. This exhibits a typical type IV isotherm with a narrow pore size distribution with a mean of 19.13 ± 1.2 Å. This verifies the mesoporous character of the MSNP shell. Through BET, the total surface area and pore volume of GNR@MSNP were quantified to be 53.852 m\(^2\)g\(^{-1}\) and 0.23 mLg\(^{-1}\), respectively.
2.3.7 TGA of various nano-assemblies

Thermogravimetric analysis (TGA) monitors the mass change while the sample is heated, from which a thermogram is recorded as the percent weight loss versus the temperature.\textsuperscript{119} The quantitative analysis of nano-assemblies after each synthesis step was done through TGA. The results are shown in Figure 2.5. Percentage (\%) weight loss between the GNR@MSNP@BDQ and GNR@MSNP was calculated to determine the amount of BDQ loaded onto GNR@MSNP. The BDQ loading was found to be 16 ± 0.5 \% in GNR@MSNP. Additionally, the percentage weight loss between GNR@MSNP@BDQ@TSL and GNR@MSNP@BDQ displayed that the coated lipid layer was 10 ± 1.6\% of GNR@MSNP@BDQ@TSL. Finally, NZX peptide loading on GNR@MSNP@BDQ@TSL was quantified by TGA analysis between the GNR@MSNP@BDQ@TSL@NZX and GNR@MSNP@BDQ@TSL. The total peptide NZX loading was 12 ± 1.5 \% on GNR@MSNP@BDQ@TSL.
2.3.8 Drug loading and encapsulation efficiency

The encapsulation efficiency (EE) and loading content of BDQ were calculated using the HPLC. First of all, the standard calibration curve of BDQ was obtained using an HPLC, as shown in Appendix Figure A.1. The encapsulation efficiency and drug loading efficiency of BDQ were analyzed to be 65.0 ± 0.5 % and 14.6 ± 0.9%, respectively.

2.3.9 Colloidal stability

Colloidal stability of nanomaterials is crucial when it comes to drug delivery applications. Thus, we wanted to check if our nano-assemblies were stable in physiological conditions and could be used in-vitro or not. The colloidal stability of GNR@MSNP and GNR@MSNP@TSL were monitored using UV-vis spectroscopy and DLS analysis. The localized surface plasmon resonance (LSPR) of gold nanoparticles (NPs) is highly affected by nanoparticle aggregation.\(^{294}\) When plasmonic NPs are aggregate individual plasmon oscillation can couple with each other through near-field interactions, resulting in coupled LSPR modes.\(^{295}\) This impacts the distribution of the electric field around the nano-structure.\(^{294-296}\) As a consequence, particle aggregation results in a UV-vis spectral red shift, broadening and reducing LSPR peak intensity.\(^{297}\) This was confirmed by a color shift of the colloid gold NPs from deep red to purple, which is characteristic of unstable plasmonic colloidal systems.\(^{298}\) The stability of GNR@MSNP and GNR@MSNP@TSL) was conducted at 37 °C in mammalian cell culture media for 5 days. As shown in Figure 2.6a, the LSPR peak intensity for GNR@MSNP is reduced in cell culture media after day
1 as compared to GNR@MSNP@TSL. It is seen that GNR@MSNP is not stable in mammalian cell culture media after day 1 at 37 °C. However, with the additional TSL coating (Figure 2.6b) GNR@MSNP@TSL can be seen to be stable in cell culture media at 37 °C until day 5. Aggregation index (AI) was calculated for GNR@MSNP and GNR@MSNP@TSL, seen in Figure 2.6c. The higher AI indicates a higher degree of aggregation, i.e., lower colloidal systems stability.\textsuperscript{291, 292} AI for GNR@MSNP was higher and increased with time in media at 37 °C. In GNR@MSNP@TSL, the AI values were low and constant with time in media at 37 °C. Therefore GNR@MSNP@TSL shows better
colloidal stability than GNR@MSNP. Zeta potential was measured to check the stability of the nano-assemblies. As shown in Figure 2.6d, the zeta potential of GNR@MSNP changed from day 0 to day 5 from -23.5 ± 0.7 mV up to +5.0 ± 0.9 mV. This continuous change in zeta potential in media suggests that GNR@MSNP is not stable. In contrast, GNR@MSNP@TSL has shown a constant zeta potential value +10.6 ± 0.7 mV up to 5 days. These observations reveal that GNR@MSNP@TSL is stable in cell culture media for up to 5 days. The UV-vis spectral analysis of LSPR peak intensities, AI results, and zeta potential values indicates that GNR@MSNP@TSL has more colloidal stability than GNR@MSNP. Photographic evidence of the comparative stability of the two solutions, GNR@MSNP@TSL and GNR@MSNP, is shown in Appendix Figure A.2. All the above stability parameters indicate that liposomal coating improves the nano-assembly's colloidal stability, promoting their suitability for antibacterial applications both in-vitro and in-vivo.

Figure 2.7. Photothermal properties of nano-assemblies (a) Photothermal effect of final nano-assembly GNR@MSNP@BDQ@TSL@NZX at different concentrations using an 808 nm, 500 mW, laser for 15 min. (b) The temperature profile of GNR@MSNP@BDQ@TSL@NZX (1 mg/mL) with different laser power intensities.
2.3.10 Photothermal performance of GNRs

The photo/thermo-responsive properties of nano-assembly were evaluated via a series of experiments. An 808 nm laser diode was selected to match the LSPR peak associated with the final nano-assembly GNR@MSNP@BDQ@TSL@NZX. The photothermal efficiencies of the final nano-assemblies were evaluated in-vitro, after irradiation with 808 nm laser at 500 mW for 15 min. A 25 mm objective lens was used to tightly focus the laser light onto the sample (spot size ~ 2 mm, diameter). Figure 2.7a shows the photothermal capacity of the final nano-assembly GNR@MSNP@BDQ@TSL@NZX at different concentrations. Under these conditions, the temperature of the final nano-assembly GNR@MSNP@BDQ@TSL@NZX increased to 58 ± 3.0 °C at its highest concentration (1000 μg/mL) and 45 ± 2.0 °C for the lowest concentration (50 μg/mL). The control containing 10 mM HEPES solution increased by only 2 °C, indicating that the temperature elevation was caused primarily by the PT effect of GNRs. The temperature increase of the final nano-assembly GNR@MSNP@BDQ@TSL@NZX was positively correlated to the concentration, laser power intensity, and irradiation time (Figure 2.7a and Figure 2.7b).

2.3.11 NIR laser triggered drug release

NIR-triggered drug release study of nano-assemblies were performed in 0.5% w/w SLS in 10 mM HEPES buffer with and without NIR laser irradiation. As shown in Figure 2.8a GNR@MSNP@BDQ without laser irradiation, cumulatively released 65.1 ± 1.4% of BDQ within 10 hours. With laser irradiation, GNR@MSNP@BDQ cumulatively released 78.9 ± 1.8% in the same span of 10 hours. This indicates that the subsequent temperature increase with laser irradiation has increased the non-specific release of BDQ.
GNR@MSNP@BDQ@TSL demonstrated only a 48.32 ± 1.5% BDQ release in a similar time frame, without laser irradiation. The presence of TSL has helped to reduce the non-specific release of the encapsulated drug compared to that of GNR@MSNP@BDQ with similar conditions (without laser). However, triggered with laser irradiation, GNR@MSNP@BDQ@TSL released 90.2 ± 1.4% of BDQ within 10 h. Final nano-assembly...
assembly GNR@MSNP@BDQ@TSL@NZX demonstrated a similar behavior with 92.7 ± 1.5% of BDQ release upon laser irradiation, while only 50.1 ± 0.9% of BDQ was released in the absence of laser (Figure 2.8a). It is apparent from the above results that TSL plays a role in reducing premature BDQ release. Laser-irradiation generates localized heat from GNR, which subsequently weakens the interactions between the mesoporous silica matrix and enhances the permeability of the TSL (Figure 2.8b). TSL is composed of a thermo-sensitive lipid DPCC (85% molar ratio); melting of DPCC increases the permeability of TSL. The drug, BDQ, release profile upon laser irradiation suggests that the melting of the TSL resulted in the release of the drug from final nano-assemblies (Figure 2.8a). The melting of TSL (48 ± 3 °C) was further evaluated by calcein dye release assay that proved that upon laser irradiation, the calcein dye release with respect to laser irradiation time and temperature is explained in Appendix A.1 (Appendix Figure A.3.).

2.3.12 Antibacterial phototherapy of Msmeg

The in vitro antibacterial activity of the nano-assemblies against Msmeg was determined using WST-8, a colorimetric bacterial viability assay, and a bacterial colony counting assay. WST-8 produces a yellow color formazan dye (absorb at 460 nm) upon reduction of tetrazolium by dehydrogenase enzymes in live bacteria. The absorbance of produced formazan was measured at 460 nm, which is proportional to the number of live bacteria. The MIC was reported with respect to the encapsulated BDQ in the nano-assemblies at their MIC concentrations. The MIC established by colorimetric assay and verified by colony counting, The MIC of the free drug BDQ was 40 ng/mL. The MIC of the final nano-assembly GNR@MSNP@BDQ@TSL@NZX (with laser) was 2 ng/mL with respect to the encapsulated BDQ (Table 2.3.1).
Table 2.3.1. MIC of the materials (MIC was reported with respect to encapsulated BDQ in the nano-assemblies). *

<table>
<thead>
<tr>
<th>Nano-assemblies</th>
<th>MIC (ng/mL) of BDQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDQ</td>
<td>40 ± 1.5</td>
</tr>
<tr>
<td>NZX</td>
<td>400 ± 1.8</td>
</tr>
<tr>
<td>GNR@MSNP@BDQ</td>
<td>25 ± 1.8*</td>
</tr>
<tr>
<td>GNR@MSNP@BDQ + Laser</td>
<td>10 ± 1.5*</td>
</tr>
<tr>
<td>GNR@MSNP@BDQ@TSL@NZX</td>
<td>30 ± 1.9*</td>
</tr>
<tr>
<td>GNR@MSNP@BDQ@TSL@NZX + Laser</td>
<td>2 ± 0.8*</td>
</tr>
<tr>
<td>GNR@MSNP</td>
<td>NA</td>
</tr>
</tbody>
</table>

This shows that BDQ encapsulated in the final nano-assembly is 20 -fold efficacious than the free drug BDQ. The higher efficacy is due to the combitherapy of PTT working together with an antibiotic.

The quantitative antibacterial efficacy of nano-assemblies was further analyzed using colony counts and expressed as a percentage of viable bacteria (Figure 2.9a). The antibacterial activity of the nano-assemblies was compared to one another using percentage of viable bacteria present after treatment with various nano-assemblies with and without laser (Figure 2.9a). The percentage of viable bacteria cells obtained from bacteria colony counts from agar plates as seen in the photographs of Figure 2.9b. Msmeg exposed to GNR@MSNP@BDQ@TSL@NZX (with laser) exhibited 5 ± 1.2 % bacterial viability, and GNR@MSNP@BDQ@TSL@NZX (without laser) had 78 ± 1.9 % bacterial viability. Therefore, it is clear that laser irradiation is vital to the antibacterial effect of the final nano-assembly. Msmeg exposed to GNR@MSNP@BDQ with and without laser exhibited 33 ±
2.1 % and 68 ± 1.5 % bacterial viability, respectively. Because of the lack of TSL, GNR@MSNP@BDQ with laser exposure cause a high release of BDQ. *Msmeg* treated with the only laser and GNR@MSNP (without laser) had similar bacterial viability (98 ±
1.7); therefore, little or no antibacterial activity was seen with GNR@MSNP. However, when *Msmeg* was treated with GNR@MSNP with laser had 78.5 ± 2.5 bacterial viability. This is due to PT activity, the viability of bacteria decreased compared to in the absence of laser (GNR@MSNP without laser). Photographs of selected bacterial plates after treatment with nano-assemblies can be seen in Figure 2.9b, and they can be clearly related to the MIC results. Figure 2.9c shows a hypothetical illustration of GNR@MSNP@BDQ@TSL@NZX effectively target and kill bacteria upon laser irradiation.

It has been established previously that bacteria can produce ROS in response to heat stress during PT treatment.\textsuperscript{301, 302} Therefore establishing the presence of ROS and quantification of ROS is useful when the antibacterial efficacy of the material is related to PT activity.\textsuperscript{302} ROS produced was measured quantitatively by a fluorescent ROS assay, and fluorescence produced was imaged through fluorescence microscopy (Figure 2.10). *Msmeg* + laser displayed slight green fluorescence (Figure 2.10a-b), suggesting that *Msmeg* produces a small quantity of ROS when exposed to a laser. In contrast, a comparatively more significant amount of ROS was generated when *Msmeg* was treated GNR@MSNP@BDQ + laser (Figure 2.10a-d). *Msmeg* treated with GNR@MSNP@BDQ@TSL@NZX + laser displayed further green fluorescence (Figure 2.10a-f), indicating higher ROS production. Quantitative analysis of ROS production, measured as fluorescence intensity, is shown in Figure 2.10b. These results are consistent with the fluorescent images (Figure 2.10b).
Live/dead assay was also done to complement the other antibacterial assays. In the live/dead assay, SYTO9 and PI fluorescent stains were used to stain intact and damaged bacterial cells (Figure 2.11). SYTO9 green fluorescent dye penetrates the cell membrane of both live and dead bacterial cells, while PI red fluorescent dye penetrates bacterial cells with damaged membranes. In the presence of both stains, bacteria with intact cell membranes appear fluorescent green, whereas cell membrane-compromised bacteria appear in red fluorescent. BDQ, GNR@MSNP@BDQ + laser, and GNR@MSNP@BDQ@TSL@NZX + laser were incubated with Msmeg and stained with SYTO9/PI, and results were observed through fluorescence microscopy. Msmeg treated with BDQ showed more green and less red fluorescence, indicating more live bacteria than...
dead bacteria (Figure 2.11a). GNR@MSNP@BDQ + laser increased red-fluorescent intensity, suggesting some bacteria cells had compromised cell membranes (Figure 2.11b). Msmeg treated with final nano-assembly GNR@MSNP@BDQ@TSL@NZX + laser had high bacterial aggregation with a high amount of red fluorescence, compared to free BDQ and GNR@MSNP@BDQ + laser. Aggregation can also be related to NZX mediated targeting and subsequent cross-linking of bacteria (Figure 2.11c).

### 2.3.13 Cellular uptake mechanism of nano-assemblies

Fluorescent microscopy was used to visualize the intracellular uptake of the nano-assemblies by A549 lung cells. As shown in Figure 2.12a cytoplasm of lung cells was stained with calcein, a green fluorescent dye (Figure 2.12a-a). Nucleus stained with Hoechst blue, fluorescent dye (Figure 2.12a-b), and nano-assemblies with RITC red
fluorescent dye (Figure 2.12a-c). Figure 2.12a-d shows red fluorescent nano-assemblies were distributed in the lung cell close to the nucleus. Figure 2.12a-e clearly shows the yellow/orange color, resulting from calcein's green color merging with the red fluorescence from the RITC-stained nano-assemblies. This proves GNR@MSNP@BDQ@TSL@NZX was effectively taken up by the A549 cells and distributed throughout the cells after 3 h of incubation.

The mechanism for the internalization of nano-assemblies into the lung cells was monitored by fluorescence microscopy (Figure 2.12b). The cellular uptake mechanism of RITC labeled GNR@MSNP@BDQ and GNR@MSNP@BDQ@TSL@NZX in lung cells was determined under different endocytosis inhibited conditions. Specific endocytosis inhibitors: chlormpromazine - an inhibitor of clathrin-mediated endocytosis, nystatin – inhibitor for caveolin-dependent endocytosis, and wortmannin- macropinocytosis inhibitor were investigated to study the mechanism of cellular uptake. We have also quantified the cellular uptake of nano-assemblies using fluorescent assays. The fluorescence microscopy images reveal little or no visible red fluorescent GNR@MSNP@BDQ@TSL@NZX in chlormpromazine treated lung cells (Figure 2.12b). This indicates chlormpromazine-treated lung cells show inhibited uptake of GNR@MSNP@BDQ@TSL@NZX. However, uptake of GNR@MSNP@BDQ@TSL@NZX into lung cells was not hindered when treated with wortmannin and nystatin (Figure 2.12b). These observations suggested that nano-assemblies trafficking occurred preferably through clathrin-mediated endocytosis.

To investigate the role of TSL in cellular uptake, we quantified the cellular uptake of nano- assemblies nano- assemblies GNR@MSNP@BDQ@TSL@NZX and
Figure 2.12. Selective cellular uptake of GNR@MSNP@BDQ@TSL@NZX by A549 cells. (a) Intracellular uptake of the final nano-assembly GNR@MSNP@BDQ@TSL@NZX by A549 cells. A549 cells are stained with calcein dye (green), cell nucleus stained with Hoechst dye (blue), nano-assemblies were labelled with red fluorescent RITC dye (red). Scale bar: 10 μm. (b) Evaluation of cell uptake mechanism of nano-assemblies by A549 cells under different endocytosis inhibitory conditions - fluorescence microscopy - A549 cells shown in bright field and nano-assemblies were tagged with RITC (Scale bar = 10 mm). (c) Shows quantitative intracellular uptake of GNR@MSNP@BDQ and GNR@MSNP@BDQ@TSL@NZX by A549 cells after inhibitor treatment.

GNR@MSNP@BDQ by measuring intracellular fluorescent intensity using a fluorescent microplate reader. As shown in Figure 2.12c, chlorpromazine - an inhibitor of clathrin-mediated endocytosis- significantly inhibited GNR@MSNP@BDQ and GNR@MSNP@BDQ@TSL@NZX delivery into lung cells. The quantified intracellular uptake of GNR@MSNP@BDQ and GNR@MSNP@BDQ@TSL@NZX was 3.5 ± 1.2 % and 15.34 ± 2.5 %, respectively. Even in the presence of an inhibitor, a higher amount of...
GNR@MSNP@BDQ@TSL@NZX uptake was found in lung cells, which points out that the inclusion of TSL and NZX increases the ability to internalize. GNR@MSNP@BDQ has consistently shown lesser uptake throughout all inhibitory assays (Figure 2.12c).

These studies collectively suggested that the nano-assemblies mediated drug delivery occurred preferably through clathrin-mediated endocytosis, and nano-assemblies internalization enhanced due to having liposome and NZX cationic peptide. Liposome coating enhances cellular uptake of nano-assemblies due to interaction with the lipophilic cell membrane, allowing cellular entry of nanoparticles by various endocytosis pathways. In addition, TSL was modified with cationic peptide NZX, so due to electrostatic interaction with the negatively charged cell membrane, the cellular uptake was higher for GNR@MSNP@BDQ@TSL@NZX.

2.3.14 Intracellular antibacterial activity of nano-assemblies

The intracellular antibacterial activity of GNR@MSNP@BDQ@TSL@NZX was assessed by quantifying their ability to target and kill intracellular *Msmeg* bacteria. A schematic illustration of *Msmeg* infection studies with A549 cells is shown in Figure 2.13a. A549 lung cells were infected with a concentration of $17 \times 10^4$ CFU/mL *Msmeg*. From the starting concentration, $8 \times 10^4$ CFU/mL of *Msmeg* was internalized into the lung cells. *Msmeg* internalized into lung cells were quantified using bacterial colony count. The *Msmeg* infected lung cells were treated with a 50 μg/mL concentration of GNR@MSNP@BDQ and the GNR@MSNP@BDQ@TSL@NZX in the presence of laser. After treating with nano-assemblies, lung cells were lysed, and lysates were plated on agar plates. After treatment, lung cells treated with GNR@MSNP@BDQ@TSL@NZX + laser
Figure 2.13. Intracellular antibacterial activities in \textit{Msmeq} infected A549 cells. (a) Schematic illustration of the bacterial infection and treatment studies with A549 cells: A- infection of A549 cell with \textit{Msmeq}, B- treatment with nano-assemblies C- NZX mediated adhesion of GNR@MSNP@BDQ@TSL@NZX to \textit{Msmeq}, D- the disintegration of TSL and BDQ release after NIR laser exposure, E- complete degradation of \textit{Msmeq}. (b) Photographs of agar plates of cultured intracellular \textit{Msmeq remained} after treatment with GNR@MSNP@BDQ and GNR@MSNP@BDQ@TSL@NZX. (c) Intracellular bacteria were targeting the capacity of nano-assemblies by A549 cells analyzed through fluorescent microscopy. A549 cells are stained with calcein dye (green), \textit{Msmeq} bacteria and A549 cell nucleus stained with Hoechst dye (blue), nano-assemblies were labeled with red fluorescent RITC dye (red). Scale bars represent 5 \textmu m.
had 22 CFU/mL of intracellular *Msmeg* (Figure 2.13b). This is a significant decrease of over 99.9% of intracellular *Msmeg*. In contrast, treatment with GNR@MSNP@BDQ (with laser) reduced intracellular *Msmeg* only up to $3 \times 10^4$ CFU/mL (Figure 2.13b), which is a 37.5% reduction in intracellular *Msmeg*. These results demonstrate the targeting ability and high antibacterial efficacy of the laser-irradiated GNR@MSNP@BDQ@TSL@NZX, which inhibits 99.9% intracellular *Msmeg* (Figure 2.13b). The ability of nano-assemblies to target *Msmeg* within lung cells was also evaluated by confocal fluorescence microscopy. A549 cell cytoplasm was stained with Calcein green, fluorescent dye, *Msmeg*, and A549 cell nucleus stained with Hoechst blue fluorescent dye. GNR@MSNP@BDQ@TSL@NZX and GNR@MSNP@BDQ were conjugated with RITC - red fluorescent dye. The fluorescence merge image (Figure 2.13c-d) clearly shows

![Figure 2.14. Percentage cell viability of A549 cells after incubation with different concentrations of irradiated nano-assemblies: GNR@MSNP, GNR@MSNP@BDQ and GNR@MSNP@BDQ@TSL@NZX after 24 h.](image-url)
that GNR@MSNP@BDQ@TSL@NZX surrounding the intracellular *Msmeg* bacteria resident in lung cells. While little or no GNR@MSNP@BDQ are seen within lung cells infected with *Msmeg* (Figure 2.13c-h). These results agree with TEM analysis where morphological changes on bacteria was observed upon treatment with laser irradiated nano-assemblies (Appendix A.2 and Figure A.4.). Furthermore, MTT assay, a mammalian cell viability assay, was conducted to evaluate any cytotoxic effect of laser and nano-assemblies treatment on lung cells. Lung cells were treated with GNR@MSNP, GNR@MSNP@BDQ, and GNR@MSNP@BDQ@TSL@NZX with laser irradiation. As shown in Figure 2.14, A549 lung cells did not show significant cytotoxicity, and lung cell viability was close to 100%. A live-dead assay was also performed to evaluate cytotoxicity towards lung cells. The methods and results are explained in Appendix A.3 and Figure A.5. After laser treatment, it is clear that both the MTT assay and the live-dead assay have negligible cytotoxic effects on lung cells.

The overall results indicate that the final nano-assembly GNR@MSNP@BDQ@TSL@NZX with laser treatment serves as an effective drug delivery platform that combines the antimicrobial efficacy of the antibiotic with PTT to target and inhibit even intracellular mycobacteria.

### 2.4 Conclusion

With the rise in antibiotic-resistant bacteria, novel therapeutic approaches have to be engineered to reduce the dependency on small-molecule antibiotics. The GNR@MSNP@BDQ@TSL@NZX is a complex nano-assembly that is a drug delivery platform and a conduit for PTT. Our multifaceted platform offers (1) synergistic therapy of small molecule antibiotics and PTT (2) targeted antibiotic delivery (3) NIR triggers the
release of antibiotics. The GNR@MSNP@BDQ@TSL@NZX were prepared by BDQ loading to GNR@MSNP and encapsulating within NZX tagged TSL. Upon irradiation with NIR laser light, GNRs convert light into heat, causing melting of TSL, which induces the release of antibiotics. Targeting peptide NZX guides the GNR@MSNP@BDQ@TSL@NZX to approach the bacterial cell surface. BDQ encapsulated in GNR@MSNP@BDQ@TSL@NZX was 20-fold more efficacious than free drug equivalent. The final nano-assembly was capable of targeting and inhibiting 99.9% intracellular mycobacteria residing within A549 cells. This work provides a proof-of-concept unique therapeutic approach to treat pathogenic infections and reduce the dependency on conventional antibiotics.
CHAPTER 3. HOST-TARGETED MULTIFUNCTIONAL NANO-THERAPY FOR ERADICATION OF INTRACELLULAR BACTERIAL INFECTION

3.1 Introduction

One-third of the world’s population is infected with *Mtb* (latent TB), while about 10% suffer from clinical symptoms of active TB. In 2020, WHO reported almost 10 million new cases and 1.5 million deaths caused by TB, making it one of the most virulent diseases. During the early infection stages, macrophages are known to phagocytose mycobacteria. However, several strategies for survival and replication within macrophages have been developed by *Mtb*. Normally, in case of any bacterial infection, phagosome fuse with lysosome where acid-secreting enzymes such as acid hydrolyse and protease destroy any encapsulated pathogens. However, this step does not occur in *Mtb* infected host cells. After internalization in host macrophages, *Mtb* is encapsulated within the phagosome but escapes the fusion with lysosomes. This prevents phagolysosome formation, suppressing the production of intracellular reactive oxygen species (ROS), reactive nitrogen species (RNS), and pro-inflammatory cytokines, hence creating a path for survival and replication for *Mtb*.
Traditional antituberculosis treatment includes first-line therapy for 6 to 9 month involving four antibiotics in a sequential combination (isoniazid, rifampin, pyrazinamide, and ethambutol).\textsuperscript{308} Recently, two drugs have been approved against multi-drug resistant \textit{Mtb}: bedaquiline (BQ) and delamanid.\textsuperscript{152} Anti-tuberculotic drugs have a significant challenge for entering macrophages to eradicate the \textit{Mtb}.\textsuperscript{154} Even after entering the macrophage, the drug is rapidly cleared from the macrophage before the drug acts on intracellular \textit{Mtb}. Moreover, poor patient compliance during lengthy TB treatment plays a role in generating drug-resistant strains. The sub-optimal period of antibiotic exposure allows \textit{Mtb} to mutate and become resistant. Therefore, it is necessary to develop host-directed therapies targeting intracellular \textit{Mtb} by reactivating the host's defense mechanisms.

In recent years, host-directed therapy has become an emerging method to target infected host cells to increase host defense mechanisms to treat diseases.\textsuperscript{309, 310} Immunotherapeutic nanoparticles (NPs) have been used previously for TB infection, in which researchers have used polymeric NPs functionalized with an immune-modulating compound such as β-Glucan and rifampicin drug was also loaded into the NPs core, which could be released in a sustained manner.\textsuperscript{311} Choi’s group have developed β-Glucan NPs, which can be used for genetic material delivery and immune response enhancement.\textsuperscript{312} Recently, Hwang \textit{et al.} conjugated single-stranded β-glucan onto silica NPs. These NPs also encapsulated isoniazid drug, which could be released in a sustained manner. However, these nanoparticles were observed to minimally activate peripheral blood mononuclear cells (PBMCs) as both the silica NPs and INH-loaded silica/glucan NPs stimulated the PBMCs at similar levels to control.\textsuperscript{313} All these combinational therapies still lack a specific
targeting ability towards immune cells. Therefore, incorporating host-targeting molecules provides opportunities in the design of targeted nanocarriers to enhance efficiency and reduce adverse effects.

In our work, we proposed fabricating host-targeting nano-assemblies using β-Glucan and FA as a host-targeting ligand that specifically binds to dectin-1 and folate receptors present on the macrophage, respectively. We have screened both ligands for their free ligand-receptor binding affinity. The project has carried forward free ligands that show higher binding interaction. We have selected β-Glucan as it showed a higher binding affinity toward dectin-1 receptors. The binding affinity between ligands and receptors plays a vital role in determining the therapeutic potential of any nanocarriers. Hence, we have systematically tested the impact of varying sizes of β-Glucan conjugated nano-assemblies and varying β-Glucan ligand density on binding affinity. β-Glucan conjugated nano-assemblies increased host response against intracellular *Msmeg* by increasing host defense mechanisms such as enhancing ROS/RNS generation. The composition of fabricated nano-assemblies was similar to our previously designed nano-assemblies. Briefly, these nano-assemblies consisted of a gold nanorod (GNR) core (act as a photosensitizer) and mesoporous silica (MS) shell containing TB antibiotic bedaquiline (BQ), GM@BQ. This assembly would be wrapped within a thermo-sensitive liposome (L), (GM@BQ)L shell that has tagged with host-targeting ligand β-Glucan. The final host-targeting nano-assemblies (HTNs) are abbreviated as (GM@BQ)L-Glu. Upon NIR laser exposure, the BQ released from nano-assemblies and thereby activated macrophage by enhancing phagosome maturation and inducing phagolysosome formation. Tailleux and co-workers reported that antibiotic BQ activates host immune response by promoting phagosome
Therefore, we hypothesize that our (GM@BQ)L-Glu can also induce phagosome acidification and phagolysosome formation upon the release of BQ. Guided by our previous work, we hypothesized that the host-targeting nano-assemblies (HTNs) would be effective against Msmeg through a combined approach of (1) binding to specific receptors present on Msmeg infected host (macrophages) (2) upon binding, would enhance internalization of HTNs to the macrophage (3) upon internalizing would subsequently elicit an increase in ROS, and RNS (4) upon exposure to NIR laser irradiation would trigger the release of anti-tuberculotic drug BQ from HTNs, and released BQ activates macrophage thereby killing intracellular bacteria (5) induce PT activity that would trigger apoptosis mechanisms in Msmeg infected macrophage which preventing further spread of the bacteria. The designed host-targeted nano-assemblies (GM@BQ)L-Glu was found to be 10-fold more efficacious compared to free drug equivalent. (GM@BQ)L-Glu was capable of targeting and inhibiting 99.9% of intracellular Msmeg residing within macrophages. This research will contribute to how nanoplastforms can be designed to modulate hosts’ responses against intracellular bacterial infections. The work proposed here will help evaluate complementary techniques and gain knowledge extending to future studies against fighting intracellular infections.

3.2 Experimental

3.2.1 Materials and Instruments

Chemicals used for gold nanorod, and mesoporous silica shell synthesis were similar as described in Section 2.2.1. Coumerine-6, 4’,6’-diamidino-2-phenylindole (DAPI, 98%), folic acid, β-Glucan, succinic anhydride, Phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, USA). Lipids used for liposome
preparation were similar as explained in section 2.2.1. *Msmeg* bacteria strain and its culture preparation were also similar as described in Section 2.2.1. THP-1 macrophage cells were purchased from American Type Culture Collection (ATCC, CCL-185) (Virginia, USA). The LysoTracker™ Red DND-99, ER-Tracker™ Red, CellEvent™ Caspase 3/7 Green Detection Reagent, Live/dead® viability kit, was purchased from ThermoFisher Scientific (New Jersey, USA). Fluorometric intercellular ROS kit, Nitrite assay kit, and MES buffer were purchased from Sigma-Aldrich (St. Louis, USA). RPMI 1640 media, fetal bovine serum (FBS, 10%), trypsin EDTA (2.21 mM), and penicillin-streptomycin (Pen-Strep, 1X) were obtained from Corning (New York, USA). The instruments were used for characterization of nano-assemblies were similar as described in Section 2.2.1.

### 3.2.2 THP-1 cell culture

A human monocyte cell line, THP-1, was grown in T75 flasks in a complete growth medium. The complete growth medium was prepared by mixing RPMI 1640 medium (445 mL), FBS (10%, 50 mL), and Pen-Strep (5 mL) followed by sterile filtration. The cells were grown in 20 mL of a fresh and prewarmed medium at 37 °C in a humidified atmosphere of 5% CO₂. To differentiate THP-1 cells into macrophages, phorbol 12-myristate 13-acetate (PMA) was used at a concentration of 100 nM. Cell viability was assessed by the Trypan blue assay and counted using an automatic cell counter.

### 3.2.3 Screening of ligand-receptor binding between free ligands and host (macrophage) receptors

We have screened two host-targeting ligand β-Glucan and FA to test their binding affinity toward dectin-1 and folate receptors, respectively. Firstly, the calibration curve for β-Glucan was carried out, the various concentration of free β-Glucan (5-50 nM ~ 1-10
ug/mL) interacted with Congo red 5 µg/mL (Congo red used to tag β-Glucan) for 30 minutes at room temperature with constant stirring. The calibration curve was prepared by recording the absorbance value at 546 nm using UV-Vis spectroscopy as shown in Appendix Figure B.1.a. Similarly, the various concentrations of free Glu (5-50 nM ~ 1-10 ug/mL) interacted with Congo red (5 ug/mL) for 30 min at room temperature with stirring. This tagged glucan was incubated with macrophage (10⁴ cells/well) for 3 h. The excess unbound ligand was washed off with HEPES buffer 3 times. Then, the absorbance of the ligand-receptor complex was recorded at 546 nm. The concentration of ligand-receptor complex and total receptor concentration was calculated from a calibration curve. The binding affinity (K_d) was calculated by running a saturation binding experiment on Prism 8 from GraphPad Software (San Diego, CA, USA).

Similarly, a calibration curve for folic acid (FA) was carried out, the various concentration of free FA 5-50 mM ~ 2-20 mg/mL was prepared. The calibration curve was prepared by recording the absorbance value at 310 nm using UV-Vis spectroscopy, as shown in Appendix Figure B.1.b. Similarly, various concentration of free FA (5-50 mM ~ 2-20 mg/mL) were incubated with macrophage (10⁴ cells/well) for 3 h. After that, the excess unbound ligand was washed off with HEPES buffer 3 times. Then, the absorbance of the ligand-receptor complex was recorded at 310 nm. The concentration of ligand-receptor complex and total receptor concentration was calculated from a calibration curve as shown in Appendix Figure B.1. The binding affinity (K_d) was calculated by saturation binding experiment and calculations done through Prism 8 from GraphPad Software (San Diego, CA, USA).
3.2.4 Synthesis of gold nanorods (GNRs)

GNRs were synthesized as described in chapter 2 (See Section 2.2.2).

3.2.5 Synthesis of mesoporous silica shell coated gold nanorods (GMs)

GMs were synthesized as described in chapter 2 (See Section 2.2.3).

3.2.6 BQ encapsulation in GMs

GMs (40 mg) were mixed with BQ (10 mg) in 20 mL of water while stirring overnight at 37 °C. The ratio of GMs and BQ used in the entire study was 4:1 w/w (GMs 40 mg: BQ 10 mg). The BQ-loaded GM samples (GM@BQ) were collected by centrifugation at 12,000 rpm for 10 min and vacuum dried to obtain a dry powder. The encapsulation efficiency and drug loading of BQ in GM@BQ were determined by using a standard calibration curve. Calibration curve for BQ was generated using 1.0, 2.5, 5.0, 7.5 and 10.0 µg/mL of BQ diluted in the release media (release media: 0.5% w/v SLS containing 10 mM HEPES, pH 7.2) using RP-HPLC (Appendix Figure B.3.).

\[
\text{Drug encapsulation efficiency} = \frac{\text{Initial amount of drug added} - \text{Amount of drug in suparnant}}{\text{Initial amount of drug added}} \times 100\%
\]

\[
\text{Drug loading content} = \frac{\text{Initial amount of drug added} - \text{Amount of drug in suparnant}}{\text{Nanoparticles weight}} \times 100\%
\]

3.2.7 Liposome preparation

Liposomes were prepared, as described by Patel et al. with minor modifications. Briefly, DPPC (85% molar ratio), cholesterol (10% molar ratio), DSPE-PEG(2000) amine (5% molar ratio), and green fluorescent Coumerine-6 (0.1 mg) were dissolved in 2 mL of
chloroform and evaporated in a rotary evaporator, yielding a thin lipid film. This lipid film was rehydrated in 2 mL of HEPES buffer and extruded 15 times through a polycarbonate membrane (pore size 800 nm) using a mini extruder. Here, the prepared liposome was a thermo-sensitive liposome, as the amount of DPPC used in liposome preparation was the highest in molar ratio compared to cholesterol and DSPE-PEG(2000) amine. As previously studied, these liposomes become permeable at temperatures ~ 50 °C. Coumerine-6 is widely used to make nano-assemblies green fluorescent.

3.2.8 Synthesis of COOH-β-Glucan

The synthesis was conducted following a modified protocol reported by Kim et al. The synthesis scheme was shown in Appendix Scheme B.1. β-Glucan (5 mg, 0.011 mM) was first dissolved in 5 mL of DMSO at 60 °C for 24 h with a continuous and mild stirring. The following day, succinic anhydride solution (10 mg, 0.1 mM in DMSO) was added to the solution drop by drop. The solution mixture was let stirring at 60 °C for 24 h, and the mixture was transferred to a dialysis tubing (MWCO: 6-8 kD). The dialysis against DI water went for 3 days. Finally, the mixture was freeze-dried, and the remaining product was a sponge-like fiber.

3.2.9 Conjugation of COOH-β-Glucan on liposome

To prepare, COOH-β-Glucan coupled on liposome (L-Glu), COOH-β-Glucan was conjugated to the liposomal shell by amide conjugation as shown in Appendix Scheme B.1. EDC was used to activate the carboxylic groups of β-Glucan. Once activated, these groups would react with the amine group of DSPR-PEG (2000) amine in the liposomal shell. COOH-β-Glucan (5 mg) was dissolved in 0.1 M MES containing 0.5 M NaCl at pH 6.0 (reaction buffer) and then incubated with a 10-fold molar excess of EDC (pH = 4.0) and
25-fold molar excess of sulfo-NHS for 30 min. Amine group-containing liposomes were added to the solution having carboxylic acid-activated β-Glucan and then reacted for 24 h at 37 °C. The molar ratio of COOH-β-Glucan: liposome was kept at 10:1. After 10 h, the reaction mixture was purified by centrifugation at 12,000 rpm for 15 min at 25 °C to remove excess unreacted EDC. This was lyophilized and stored at −20 °C until further use.

3.2.10 Congo red assay for β-Glucan structure modification

Congo red assay is widely used to confirm the triple helix structure of β-Glucan. This experiment was performed according to Palacios et al. with minor modifications. Briefly, the conformational structure of the β-Glucan was established by helix-coil transition analysis. Congo red was dissolved in different concentrations (10-100 µg/mL) in DMSO to optimize the concentrations of the dye. Congo red dye alone and dextran were used as control. The polysaccharide (β-glucan and dextran) were dissolved in DMSO at 1 mg/mL and were added to Congo red solutions. Spectra were recorded using a plate reader in intervals of 1 nm from 400 to 650 nm.

3.2.11 Conjugation of folic acid (FA) on liposome

To prepare, folic acid-coupled liposome (L-FA), folic acid (FA) was conjugated to the liposomal shell by amide conjugation as shown in Appendix Scheme B.2. EDC was used to activate the carboxylic groups of FA. Once activated, these groups would react with the amine group of DSPR-PEG (2000) amine in the TSL shell. FA (476 µg) were dissolved in 0.1 M MES containing 0.5 M NaCl at pH 6.0 (reaction buffer) and then incubated with a 10-fold molar excess of EDC (pH = 4.0) and 25-fold molar excess of sulfo-NHS for 30 min. Amine group-containing liposomes were added to the solution having carboxylic acid-activated FA and then reacted for 24 h at 37 °C. The molar ratio of
FA: liposome was kept at 10:1. After 10 h, the reaction mixture was purified by centrifugation at 12,000 rpm for 15 min at 25 °C to remove excess unreacted EDC. This was lyophilized and stored at −20 °C until further use.

3.2.12 Encapsulation of GM and GM@BQ into host-targeting ligand conjugated liposomes (L-Glu or L-FA)

To prepare (GM)L-Glu, (GM)L-FA, (GM@BQ)L-Glu, and (GM@BQ)L-FA, 1 mg of GM and GM@BQ was resuspended in a liposome (L-Glu or L-FA, 2 mL, 10 mg/mL) in HEPES buffer and mixed for 20 min in an ice bath. Nano-assemblies were separated from empty liposomes by centrifugation at 12,000 rpm for 5 min with repeated (3x) washing in HEPES. Similarly, (GM)L of 1000 nm size were prepared using filter pore size of 1000 nm filter. The resultant (GM)L-Glu, (GM)L-FA, (GM@BQ)L-Glu, and (GM@BQ)L-FA was lyophilized and dried at −20 °C until further use. The BQ loading and encapsulation efficiency were determined by a standard calibration curve of BQ, as shown in Appendix Figure B.3.

3.2.13 Determination of ligand density by TGA

For TGA analysis, measurements were carried out under argon (99.999%), where dried (GM)L-Glu and (GM)L-FA at a constant heating rate of 5 °C/min to 100 °C and then kept isothermal at 100 °C for 15 min followed by ramping 5 °C/min to 700 °C. Two sets of each analysis were carried out. The percent weight difference (%) before and after targeting ligand conjugation, together with the percent weight (%) after functionalization at 700 °C, was used for ligand density calculation. The ligand density $p_L$ (nmol/nm$^2$) of $\beta$-Glucan and FA on HTNs was calculated as shown in Appendix Section B.3.
3.2.14 Ligand density by phenol sulphuric acid assay

Calibration curves were obtained for β-Glucan where various concentrations (10-100 nM) of β-Glucan in 1 mL of DMSO were placed in boiling tubes. Add 1 mL of 2.5 N HCl and hydrolyzed glucan by keeping it in a boiling water bath for 1 h. The mixture was cooled at room temperature and neutralized with sodium carbonate until effervescence ceased. Then it was centrifuged at 15000 rpm for 10 min and collected the supernatant. 1 mL of supernatant was added in 24 well plates, following that 100 μL of 5% phenol solution and 500 μL of 96% H₂SO₄ were added. Then it was incubated at room temperature for 30 min. Finally, UV-Vis spectra of the resulting solutions were recorded at 490 nm, and the data were plotted against the β-Glucan concentration. This calibration curve is used to calculate the ligand density on HTNs, as shown in Appendix Figure B.4. β-Glucan coupled on HTNs were subjected to the same assay where freshly-prepared β-Glucan conjugated HTNs with varying size 100 and 200 nm and varying concentration (150-200 nM ~30–50 ug/mL) in 1 mL DMSO, and the solutions were treated with phenol/H₂SO₄ following the same protocol described above. Then un-functionalized HTNs were treated similarly with phenol/H₂SO₄, and the absorbance at 490 nm was used as the background deducted from the total signals measured from the β-Glucan conjugated HTNs. The density of β-Glucan immobilized was then determined using the calibration curve.

3.2.15 Fluorescent competitive binding assay

β-Glucan conjugated HTNs solutions, (GM)L-Glu of various concentrations (1-1000 nM) and FA conjugated HTNs, (GM)L-FA (1-1000 mM) were prepared in HEPES buffer. The HTNs were tagged with Coumerine-6 dye, a fluorescent molecule with an excitation peak at 457 nm and an emission peak at 501 nm. The fixed concentration of
targeting ligand β-Glucan (200 nM) and FA (200 mM) were prepared separately. For the competition assay, the various concentration of (GM)L-Glu (1-1000 nM) was mixed with a fixed concentration of β-Glucan (200 nM). Similarly, FA conjugated HTNs (1-1000 mM) were mixed with a fixed concentration of FA (200 mM). These resulting solutions were placed in macrophages (10^4 cells/well) followed by incubation for 3 h at 37 °C. After that, the excess unbound ligand was washed off with HEPES buffer 3 times. Then final fluorescence was recorded with an excitation peak at 457 nm and an emission peak at 501 nm. The dose-response curve will be obtained by plotting the normalized fluorescent intensity (%) against the log value of the varying concentration of HTNs. The IC_{50} value was obtained from the dose-response curve by running a competitive binding experiment on prism software. Three sets of data at each concentration were collected, and the mean intensity values were used. The apparent dissociation constant K_{D} was estimated by the Cheng-Prusoff equation:

\[
K_D = \frac{IC_{50}}{1 + \frac{[M]}{K_d}}
\]  

(3.3)

Where IC_{50} is the ligand concentration displaying 50% of specific binding, [M] is the concentration of free ligand, K_{d} is the dissociation constant of free β-Glucan with dectin-1, and free FA with folate. The K_{D} is the dissociation constant of β-Glucan conjugated HTNs with dectin-1 and FA conjugated HTNs with folate.
3.2.16 Surface area and pore volume determination of nano-assemblies

The surface area of GMs and GM@BQ were determined using the Brunauer–Emmett–Teller (BET) method. The cumulative pore volume was calculated from the adsorption branch of the isotherm using the Barret–Joyner–Halenda (BJH) model.

3.2.17 TGA analysis of nano-assemblies

TGA was carried out under argon (99.999%), where dried GM, GM@BQ, (GM@BQ)L, and GM@Dox)L-Glu (ca. 1 mg) were heated at a rate of 5 °C/min to 100 °C and then kept isothermal at 100 °C for 15 min followed by ramping 5 °C/min to 700 °C. The BQ loaded in GM was calculated by analyzing the weight loss difference between GM and GM@BQ. Similarly, the liposome content was analyzed by measuring the weight loss difference between GM@BQ and (GM@BQ)L. Finally, the amount of ligand was calculated by measuring the weight loss difference between (GM@BQ)L and (GM@BQ)L-Glu.

3.2.18 Stability analysis of nano-assemblies

The stability studies were carried out by following the protocol in our previously reported study. Briefly, GM and (GM@BQ)L-Glu solution were stored at 4, 25, and 37 °C in cell culture media for 5 days. The nano-assemblies were monitored daily, and their stability was characterized by UV absorbance to track the longitudinal surface plasmon resonance (LSPR) intensity, aggregation index (AI), and zeta potential to monitor the change in surface charge.
3.2.19 Intracellular uptake of nano-assemblies by macrophage

Intracellular uptake of nano-assemblies was checked by confocal microscopy. THP-1 (10^4 cells/mL) were seeded on 24-well plates with PMA stimulation for 24 h incubation to differentiate into a macrophage. Then the cells were infected with a volume of 10 μL Msmeq (17 × 10^3 CFU/mL) was added to each well and incubated for 2 h. After incubation, the supernatant was discarded, and the infected cells were washed twice with 0.1 M HEPES buffer. Then, the culture medium was replaced with a medium supplemented with 40 ng/mL BQ to kill the remaining extracellular bacteria without affecting the intracellular bacteria. Then, the cells were treated with Coumerine-6 stained (GM)L and (GM)L-Glu (50 μg/mL) for 6 h. Afterward, the cells were washed twice with HEPES and subsequently incubated with Hoechst dye for 30 min at 37 °C. After washing twice with HEPES, the cells were observed by a confocal microscope.

3.2.20 Mechanism for intracellular uptake of nano-assemblies

THP-1 cells (10^4 cells/mL) were seeded on 24-well plates with PMA stimulation for 24 h incubation that differentiated into a macrophage. Macrophages were pretreated with different endocytosis inhibitors: chlorpromazine (20 μg/mL), nystatin 20 μg/mL, and wortmannin 1 μg/mL for 30 min. Macrophages were treated with 50 μg/mL (GM)L-Glu, then incubated for 2 h at 37 °C. The confocal microscope was used to evaluate the nano assemblies' uptake by macrophage. Enhancement of green fluorescence intensity within the macrophage is related to the high uptake of nano-assemblies.

3.2.21 Intracellular ROS detection

ROS generation inside infected macrophages was measured with a cell-permeable dye 2, 7-dichlorofluorescein diacetate (DCFH-DA). THP-1 cells (10^4 cells/mL) were
seeded on 24-well plates with PMA stimulation for 24 h incubation to differentiate into a macrophage and then *Msmeg* infection for another 2 h. After 2h of infection, the medium was removed, and the samples were washed with 0.1 M HEPES buffer two times. Subsequently, the macrophage cultures were maintained in a growth media supplement with 40 ng/mL of BQ for 30 min and then infected macrophages were washed with 0.1 M HEPES to remove the extracellular bacteria. The infected macrophages were exposed to GM, β-Glucan, COOH-β-Glucan, (GM)L, and (GM)L-Glu (50 μg/mL) for 1 h. Following that, NIR laser treatment was given and incubated for 5 h. After exposure, cells were incubated with 100 μL of DCFH-DA for 1 h at 37 °C. Fluorescent intensity was quantified at 525 nm, with excitation at 490 nm. Samples were also observed by fluorescence microscopy.

### 3.2.22 Measurement of reactive nitrogen species.

According to the manufacturer's instructions, nitrite accumulation/release into culture’s supernatants was measured by Nitrite assay kit (Griess reagent). THP-1 cells (10^4 cells/mL) were seeded on 24-well plates with PMA stimulation for 24 h incubation to differentiate into a macrophage. Then infected with *Msmeg* and exposed to GM, β-Glucan, COOH-β-Glucan, (GM)L, and (GM)L-Glu (50 μg/mL) for 1 h. Following that, NIR laser treatment was given and incubated for 24 h. Culture supernatants of respective groups from various time points (0, 2, 4, 6, 8, and 24 h) were mixed with an equal volume of Griess reagent I and II incubated at room temperature for 10 min, and the absorbance was measured at 540 nm using a microplate reader. The standard series were prepared as per the manufacturer’s instructions, as shown in Appendix Figure B.5. Briefly, 1-10 nmol/well nitrite standards were prepared in a 96-well plate using nitrite assay buffer. Then after
Griess Reagent I (10 μL), Griess Reagent II (10 μL), and nitrite assay buffer (80 μL) were added to each well plate. For each background correction well, add 10 μL of Griess Reagent I and 90 μL of Nitrite Assay Buffer and mix well. The absorbance was taken at 540 nm in end-point mode at room temperature using a plate reader.

3.2.23 Photothermal study and NIR-triggered drug release

The laser setup is explained in chapter 2 (See Section 2.2.11). First, the photothermal effect due to the presence of GNR was evaluated by comparing various weight concentrations of (GM@BQ)L-Glu ranging from 50, 100, 500, and 1000 μg/mL (calculated with respect to the weight of GM) were irradiated with the 808 nm NIR laser (500 mW) for 15 minutes. Various nano-assemblies GM@BQ ± laser, (GM@BQ)L ± laser, and (GM@BQ)L-Glu ± laser were dispersed in 3 mL of release media (0.5 % w/w SLS in 10 mM HEPES buffer, pH 7.2), then incubated at 37 °C with constant agitation. At predetermined time intervals (2, 4, 6, 8, 10, and 12 h), the suspension was exposed to the NIR laser (808 nm, 500 mW) for 15 min and then centrifuged at 15000 rpm for 15 min. At the indicated time point, samples were taken out and then centrifuged, and the supernatant was collected and replaced with prewarmed release media. Each sampled aliquot of the supernatant was passed through a 0.22 μm nylon filter. Quantification of the drug release was performed by HPLC.

3.2.24 MIC determination of the nano-assemblies

*Msmeg* was cultured at 37 °C in Middlebrook 7H9 medium supplemented with 10% ADC and 0.05% glycerol on a shaking incubator (200 rpm) at 37 °C. Two hundred microliters of fresh Middlebrook broth and 20 μL of each sample (BQ, GM, GM@BQ, (GM@BQ)L, and (GM@BQ)L-Glu) having concentrations of 1–100 μg/mL were added
to a 96-well plate. Then, 10 μL of Msmeg (10^8 CFU/mL) was added to each well and finally treated with NIR laser (808 nm, 500 mW, 15 min), then shaken at 37 °C in a shaking incubator for 24 h. On the following day, the viability of bacteria was determined by the cell viability WST-8 colorimetric assay. The assay was performed by incubating 170 μL of sterile broth, 20 μL of each treated sample incubated with bacteria, and 10 μL of coloring agent, WST-8 at 37 °C on a shaker bed for 2 h. The absorbance was recorded at 460 nm.

3.2.25 In situ ablation of intracellular bacteria

To determine intracellular MIC, THP-1 cells (10^4 cells/mL) were seeded on 24-well plates with PMA stimulation for 24 h incubation to differentiate into a macrophage. Then the cells were infected with a volume of 10 μL Msmeg (17 × 10^3 CFU/mL) was added to each well and incubated for 2 h. After incubation, the supernatant was discarded, and the infected cells were washed twice with 0.1 M HEPES buffer. Then, the culture medium was replaced with a medium supplemented with 40 ng/mL BQ to kill the remaining extracellular bacteria without affecting the intracellular bacteria. The infected macrophages were cultured in a fresh medium in the presence of BQ and (GM@BQ)L-Glu having concentrations of 1–100 μg/mL. After 1 h of incubation, the NIR laser exposure was given and then incubated for an additional 24 h. The survival of intracellular Msmeg was assessed by lysing the macrophages with 0.5% Triton X-100, and serial dilutions of the lysate were plated on Middlebrook 7H11 agar plates. The survival rate of intracellular Msmeg was determined by the colony counting method. The colony-forming unit (CFU) on plates was counted, which indicates the intracellular MIC of nano-assemblies as well as % viability of bacteria after treatment with nano-assemblies.
To further confirm the intracellular bacterial viability, the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes) was used to visualize the viable and dead bacterial cells within macrophages. As described above, the macrophages ($10^4$ cells/mL) were seeded on 24-well plates, and the infection was established as described earlier in this section. Infected macrophages were incubated with a LIVE/DEAD BacLight bacterial viability staining solution for 30 min. After washing with 0.1 M HEPES buffer, cells were visualized under confocal microscopy.

3.2.26 Detection of phagosome acidification

THP-1 cells ($10^4$ cells/mL) were seeded on a 2 well chamber slide with PMA stimulation for 24 h incubation to differentiate into a macrophage. Following that, the macrophages were infected with \textit{Msmeg} and exposed to BQ, and (GM@BQ)L-Glu ± laser (50 $\mu$g/mL) for 6 h. These treated cells were then incubated with 1 $\mu$g/ml Acridine Orange (AO) for 15 min to detect acidic vesicular organelles (AVOs) under a confocal microscope. In AO-stained cells, the acidic compartments showed a degree of acidity which appeared as red fluorescence. The nucleus and cytoplasm emitted green fluorescence upon excitation with the same wavelength of light. As controls, macrophages and \textit{Msmeg} infected macrophages were incubated with 1 $\mu$g/ml Acridine Orange (AO) for 15 min and visualized under a confocal microscope.

3.2.27 Assessment of phagolysosome formation

To study whether nano-assemblies were internalized into the differentiated macrophages and simultaneously induced phagolysosome formation, we seeded \textit{Msmeg} infected macrophages in a 2-well chamber slide and incubated the cells with and (GM@BQ)L-Glu + laser (50 $\mu$g/mL) for 3 h at 37 °C. Then, the treated cells were
incubated with 50 nM ER-Tracker Red and LysoTracker Red D99 at 37 °C for another 3 h, then observed under a confocal microscope. ER-Tracker Red and LysoTracker Red D99 were used to track nano-assemblies inside cells. LysoTracker Red D99 selectively stains the acidic lysosomal compartment and detect phagolysosome formation.317

3.2.28 PT induced apoptosis in an infected host

To investigate the modulation of cell death by (GM@BQ)L-Glu-mediated PTT, the expression of Caspase-3/7 within each NIR-treated sample was examined. Activation of caspase-3 is an important event during apoptosis. Thus, caspase expression was analyzed by fluorescence microscopy using the Cell Event Caspase-3/7 Green Detection Reagent (ThermoFisher Scientific) to confirm cell death by apoptosis. For this experiment, THP-1 cells (10^4 cells/mL) were seeded on a 24 well plate with PMA stimulation for 24 h incubation to differentiate into a macrophage. Then, infected with Msmeq and treated with GM, BQ, and (GM@BQ)L-Glu (50 μg/mL) for 3 h at 37°C. Next, the cells were exposed to PTT treatment for [(GM@Dox) LI]-PF (NIR 808 nm laser, 500 mW, 15 min), and incubation was continued for another 3 h at 37 °C. Before use, the CellEvent Caspase-3/7 Green Detection Reagent was diluted with PBS to a 5 μM working solution. After the samples underwent PTT, the NP-containing media was removed and replaced with 100 μL of 5 μM CellEvent Caspase-3/7 Green Detection Reagent. Samples were allowed to incubate for an additional 30 min before analysis and then imaged using a fluorescence microscope. The fluorescent intensity was quantified at 525 nm, with excitation at 490 nm. Next, to check the viability of bacteria, the treated cells were lysed in distilled water for 3 h. The cell lysate was plated on agar, and the bacterial colonies were quantified. The number of colonies reflects the viable intracellular Msmeq present after treatment.
3.2.29 Cytotoxicity assay

THP-1 cells ($10^4$ cells/ mL) were seeded on a 24-well plate with PMA stimulation for 24 h incubation to differentiate into a macrophage, infected with $M$smeg and exposed to GM, BQ, and (GM@BQ)L-Glu ± laser with varying concentration (1-50 µg/mL) for 6 h. Finally, 10 µL of the MTT solution (1 mg/mL) was added to each well, and the cells were further incubated for 4 h. After incubation, the supernatant was discarded, and 150 µL of DMSO was added to each well. The absorbance intensity was determined at 590 nm by a microplate reader. Results were presented as the percentage (%) with respect to untreated control THP-1 cells. We carried out a similar assay to check the cytotoxic effect of NIR laser and nano-assemblies on healthy macrophages (without $M$smeg infection). THP-1 cells ($10^4$ cells/mL) were seeded on a 24-well plate with PMA stimulation for 24 h incubation to differentiate into a macrophage, then exposed to NIR laser and (GM@BQ)L-Glu (50 µg/mL) for 6 h. The rest of the step was similar as described above in the assay.

3.3 Results and Discussion

3.3.1 Determination of binding affinity between free ligands and receptors

We have developed an absorbance-based binding assay to determine the binding affinity of targeting ligands β-Glucan and FA with dectin-1 and folate receptors, present on macrophages, respectively. β-Glucan has been tagged with Congo red dye, which gives

![Figure 3.1](image). Absorbance-based binding assay for (a) β-Glucan and, (b) FA.
absorbance at 546 nm and FA gives absorbance at 310 nm.\textsuperscript{318, 319} In a typical assay, the standard calibration curve was prepared by varying the concentration of targeting ligands and recording the absorbance at 546 nm. Free ligand concentration was displayed on the x-axis, while ligand-receptor absorbance was plotted on the y-axis. (As shown in Appendix Figure B.1.). Similarly, the various concentrations of targeting ligands interacted with a fixed concentration of receptors present on macrophages and incubated for 3 h. After that, unbound ligands were washed off 3 times with HEPES buffer. Then the absorbance of the ligand-receptor complex was recorded at 546 nm. The concentration of ligand-receptor complex [LR] was calculated from standard calibration series as shown in Appendix Section B.1. The concentration of receptor [R] was also calculated from the standard calibration curve (Appendix Section B.2), the receptor binding saturation was achieved at 25 nM concentration of ligand, and ligand-receptor binding is 1:1, as reported previously.\textsuperscript{320, 321} Hence, the [R] was 25 nM $\sim 1.8 \times 10^7$ of dectin-1 receptor in one cell, and for FA, [R] was 40 mM $\sim 2.3 \times 10^{15}$ of folate receptor in one cell (Calculation is shown in Appendix Section B.2.). The binding affinity between ligand-receptor ($K_d$), calculated by plotting free ligand concentration [L] on the x-axis and [LR]/[R] on Prism software. The $K_d$ for $\beta$-Glucan- dectin-1 receptor and FA-folate receptor was found to be 3.6 nM and 11.1 mM, respectively, as shown in Figure 3.1.

3.3.2 Fabrication of HTNs

The stepwise synthesis of HTNs is illustrated in Scheme 3.1. The NIR light-sensitive agent, GNR, was synthesized via a seed method as described by El-Sayed \textit{et al.}\textsuperscript{48} with some minor modifications.\textsuperscript{74} Followed by mesoporous silica shell was grafted onto the surface of the GNR by a modified protocol from Matsuura \textit{et al.}\textsuperscript{74} CTAB was used as
a soft template, which creates the mesoporous silica shell structure during the polymerization of silane around the GNRs (to give GM). Simultaneously, the liposomes were synthesized by the extrusion method using the DPPC, cholesterol, and DSPE-PEG(2000) amine, along with Coumerine-6 dye. The Coumerine-6 is a green fluorescent molecule used to stain nano-assemblies. The phospholipid DPPC, which is used in an 85% molar ratio, makes this liposome thermo-sensitive due to its melting point at 43 °C. Cholesterol in the lipid layer enhances membrane fluidity. The large PEG group of DSPE-PEG(2000)amine is known to improve drug delivery efficiency, increase systematic circulation time, reduce immunogenicity, and offer stealth properties, which facilitates the binding of the targeting ligands onto the liposome. The liposome layer provides stability to the nano-assemblies, improving cellular uptake and offering surface to conjugates targeting ligands. Next, to achieve host target specificity and increase uptake efficiency into the macrophages, targeting ligands β-Glucan and folic acid conjugated separately to the surface of the liposome through EDAC chemistry and named as L-Glu
and L-FA, respectively (as shown in Appendix Scheme B.1 and Scheme B.2). β-Glucan is known for its binding to dectin-1 receptor which evade host immune mechanism by altering ROS and RNS production. On the other hand, folic acid has affinity towards folate receptors, which are overexpressed on a large number of macrophages. Finally, as-prepared GM was encapsulated within the ligand conjugated liposome by reacting them in an ice bath, where the amphiphilic phospholipids self-assemble around the GM to create a lipid layer and the final nano-assembly was abbreviated as (GM)L-Glu and (GM)L-FA. Also called as host targeting nano-assemblies (HTNs).

3.3.3 Morphology and size characterization of HTNs

The morphological characterization of HTNs was done by TEM. GNRs were synthesized via a seed method described by El-Sayed et al. with some minor modifications. As shown in Figure 3.2a, the average length and width of GNRs were 45 ± 4.4 nm and 11.8 ± 2.7 nm, respectively and the calculated average aspect ratio is 3.8:1. The GNRs displayed a uniform and well-disposed rod-like morphology. GNRs coated with a porous silica shell were synthesized by a modified protocol from Matsuura et al. CTAB was used as a soft template, which creates the mesoporous silica shell structure during the polymerization of silane around the GNRs (to give GM). TEM images (Figure 3.2b) show GNRs encapsulated in a mesoporous silica shell (GM). The silica shell has an average thickness of 16 ± 3 nm, and its porous structure can be clearly seen by TEM (Figure 3.2b). The GNR loaded mesoporous structure was wrapped by liposome to give (GM)L. The size of (GM)L was found to be 90 ± 3 nm (n = 20, n particle number) by TEM analysis and was calculated by Image J software. The liposomal layer was visible through TEM, as shown in Figure 3.2c. The shape of GM dictates the oblong shape of the liposomal layer. High-
angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) images (Figure 3.2e-h) show the presence of the elements of gold (Au), silica (Si), oxygen (O), and carbon (C) present in the layers of (GM)L. The merged image in Figure 3.2i clearly shows the individual layers overlaid on each other to show the final construct of (GM)L. The combined TEM (Figure 3.2c) and HAADF-STEM (Figure 3.2i) images clearly show liposome coating surrounding GM. The varying sizes of HTNs were analyzed by DLS. Two types of HTNs were prepared by using varying sizes of pore filters 800 and
1000 nm, and the DLS results were found to be $808 \pm 7$ and $1020 \pm 12$ nm, respectively.

### 3.3.4 Elemental analysis of HTNs

The chemical composition of GM and (GM)L was analyzed by Energy-dispersive X-ray spectroscopy (EDS). The EDS spectra of GM (Figure 3.3a) clearly displayed the peaks of gold (Au), oxygen (O), and silica (Si). Copper (Cu) peak was present in the EDS spectra (Figure 3.3a) because a Cu mesh TEM grid was used as a substrate. EDS spectrum of (GM)L (Figure 3.3b) shows the Au, Si, O, and C peaks. The presence of C peaks was used to indicate the existence of a liposomal layer.

### 3.3.5 Ligand density determination of HTNs

We measured the ligand density of both hosts targeting ligands on HTNs by TGA, as shown in Figure 3.4. To minimize the influence of volatiles, samples were dried under vacuum at room temperature for 3 days before TGA measurements. The weight loss difference and the percent weight of (GM)L-Glu at 700 °C were used to estimate the density of β-Glucan on (GM)L, according to the calculations (as shown in Appendix Section B.3.), given $53.7 \pm 0.5$ nmol/nm$^2$. Similarly, a density of $12.9 \pm 1.1$ nmol/nm$^2$ was obtained for FA on (GM)L by the weight loss difference between (GM)L and (GM)L-FA.
These results indicate that the ligand density of β-Glucan ligands on (GM)L was higher than FA. Higher molecular weight (MW) ligands have higher ligand loading on nanoparticles and have a higher binding affinity than low MW ligands.\(^{329, 330}\) Hence, considering β-Glucan (20K MW) as a host-targeting ligand is an attractive and beneficial candidate compared to lower MW of FA.

Next, we have determined carbohydrate (β-Glucan) ligand density with varying concentrations of β-Glucan on (GM)L. The Phenol-H\(_2\)SO\(_4\) assay, a colorimetric method widely used to measure carbohydrate concentrations in solution and on solid surfaces, was employed to measure the carbohydrate ligand density on the nano-assemblies.\(^{331, 332}\) Calibration curves were obtained by treating various concentrations of each carbohydrate with phenol/sulfuric acid, and the absorption at 490 nm was plotted against the carbohydrate concentration (Appendix Figure B.4.). The calibration curve was used to calculate the ligand density on (GM)L-Glu or HTNs. Here, we want to determine how the size of nano-assemblies and varying concentrations of ligands impacted ligand density. To
address that question, the ligand density experiments for (GM)L-Glu were carried out by dissolving freshly-prepared (GM)L-Glu with varying sizes 100 and 200 nm (varying ligand concentration 150-200 nM ~30–50 μg/mL) in 1 mL DMSO, and the solutions were treated with phenol/H₂SO₄ following the same protocol described methods. The un-functionalized (GM)L were treated similarly with phenol/H₂SO₄, and the absorbance at 490 nm was used as the background deducted from the total signals measured from the (GM)L-Glu.

Table 3.3.1. Ligand density as the function of the size of nano-assemblies and concentration of ligands by phenol sulphuric acid assay.

<table>
<thead>
<tr>
<th>Size of HTNs</th>
<th>Conc. of Ligands added (nM)</th>
<th>Conc. of ligands obtained (nM)</th>
<th>Ligand density (No. of glucan/ HTNs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>150</td>
<td>18.1 ± 4.1</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>175</td>
<td>44.9 ± 2.5</td>
<td>297</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>96.6 ± 3.1</td>
<td>645</td>
</tr>
<tr>
<td>200</td>
<td>150</td>
<td>2.8 ± 1.1</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>175</td>
<td>14.3 ± 2.1</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>29.6 ± 1.3</td>
<td>197</td>
</tr>
</tbody>
</table>

The density of β-Glucan ligands immobilized was then determined using the calibration curve. As shown in Table 3.3.1, smaller HTNs 100 nm size with higher concentration 96.6 ± 3.1 nM occupied 645 numbers of β-Glucan per (GM)L, while 200 nm size HTNs with higher concentration 29.6 ± 1.3 nM occupied only 197 β-Glucan per (GM)L (Calculations was in Appendix Section B.4). It has been previously studied that the smaller nanoparticles occupied more ligands due to higher surface curvature, which offers more available volume to accommodate bulky ligands.¹²⁰ Our results indicated that the smaller size of (GM)L-Glu
occupied more ligands than bigger size, and the ligand density on (GM)L is concentration-dependent.

3.3.6 Binding affinity by fluorescence competition binding assay

The fluorescence competition binding assay has been widely used to study the binding affinity of ligand conjugated nanoparticles. As shown in Figure 3.5a, we developed a fluorescence competition assay to determine the binding affinity of HTNs
conjugated targeting ligands with its receptors using a fluorescently labeled targeting ligand conjugated HTNs and a free competing ligand. In a typical assay, β-Glucan conjugated HTNs, (GM)L-Glu of varying concentrations, and a fixed concentration of a free competing ligand β-Glucan were incubated with THP-1 cells for 3 h at 37 °C. The unbound (GM)L-Glu and free ligand remained in the supernatant, corresponding to the amount of (GM)L-Glu that did not bind dectin-1 receptors. These unbound particles were washed off with HEPES buffer, and final fluorescence was measured with excitation at 457 nm and emission at 501 nm.

As shown in Figure 3.5b, the dose-response curve was obtained by plotting normalized fluorescent intensity against the log value of the varying (GM)L-Glu concentration using Prism 8 from GraphPad Software (San Diego, CA, USA). From this concentration-response curve, the IC$_{50}$ value was determined by running a competition binding experiment on Prism software, and the apparent dissociation constant K$_D$ was computed according to the Cheng Prusoff equation 3.4.

$$K_D = \frac{IC_{50}}{1 + \frac{[M]}{K_d}}$$  \hspace{1cm} (3.4)

Where [M] is the concentration of the free ligand, Kd is the dissociation constant of the free ligand β-Glucan-dectin-1 receptor (3.6 nM) and FA-folate receptor (11.3 mM), and K$_D$ is the apparent dissociation constant of the HTNs with its respective receptors, IC$_{50}$ values are calculated, and results are presented in Table 3.3.2.

We have carried out a similar assay to check binding affinity between FA conjugated HTNs, (GM)L-FA with folate receptors. As shown in Table 3.3.2, (GM)L-Glu showed higher affinity for the dectin-1 receptor (K$_D$, 0.11 ± 0.7 nM) as compared to
(GM)L-FA-folate binding affinity ($K_D$, 8.6 ±2.5 mM). We screened β-Glucan as well as FA as a host targeting ligands for binding affinity toward dectin-1 and folate receptors, respectively. The results revealed that β-Glucan had a stronger binding affinity and hence was selected for further studies.

<table>
<thead>
<tr>
<th>HTNs (100 nm)</th>
<th>IC$_{50}$</th>
<th>$K_D$</th>
<th>$K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(GM)L-Glu</td>
<td>6.38 nM</td>
<td>0.11 ± 0.7 nM</td>
<td>3.6 nM</td>
</tr>
<tr>
<td>(GM)L-FA</td>
<td>9.47 mM</td>
<td>8.6 ± 2.5 mM</td>
<td>11.3 mM</td>
</tr>
</tbody>
</table>

The binding affinity is sensitive to many factors, including the nano-assemblies' size and the density of ligands. In the present study, the ligand density and nanoparticle size were varied, and their impacts on the binding affinities of the resulting (GM)L-Glu were investigated. To check the effect of varying size and ligand density on binding affinity, the varying concentration of targeting ligand β-Glucan were added to the HTNs preparation and incubated with macrophages. The apparent $K_D$ value of the resulting Glu-HTNs was then determined using the fluorescence competition assay described above. The Table 3.3.3 show that the increased ligand concentration - increased ligand density - a high binding affinity. In terms of the impact of varying sizes on binding affinity, an increase in the size of HTNs and decreasing ligand density - a low binding affinity. Hence, we have selected 100 nm HTNs-Glu with higher ligand concentration (200 nM) which has 645 ligands, and showed $K_D$ of 0.11 ± 0.7 nM, which was shown 35 times stronger binding affinity as compared to free β-Glucan-dectin-1 receptor $K_d$ (3.6 nM).

The fluorescence-based competition binding assay was successfully used to determine the $K_D$ values and evaluate the binding affinity of HTNs. We have conducted
systematic studies to investigate the impacts of nano-assemblies size and ligand density on the binding affinity of (GM)L-Glu. Findings from this study conclude that (GM)L-Glu can be readily synthesized by varying size and ligand density and dramatically affects the binding affinity.

<table>
<thead>
<tr>
<th>Size (nm)</th>
<th>Concentration of ligands added (nM/mg of HTNs)</th>
<th>IC50 (nM)</th>
<th>Ligand density (No. of glucan/HTNs)</th>
<th>KD (nM)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>150</td>
<td>57.35</td>
<td>120</td>
<td>1.06 ± 0.5</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>175</td>
<td>31.34</td>
<td>297</td>
<td>0.58 ± 1.2</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>6.38</td>
<td>645</td>
<td>0.11 ± 0.7</td>
<td>3.6</td>
</tr>
<tr>
<td>200</td>
<td>150</td>
<td>512</td>
<td>19</td>
<td>9.54 ± 1.1</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>175</td>
<td>312</td>
<td>95</td>
<td>5.81 ± 1.4</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>100</td>
<td>197</td>
<td>1.86 ± 1.2</td>
<td>3.6</td>
</tr>
</tbody>
</table>

### 3.3.7 Surface charge of nano-assemblies

The altered surface zeta potential is shown in Figure 3.6a. The zeta potential of GNRs was 28 ± 0.7 mV, possibly due to the presence of positively charged CTAB on the surface. After silica coating, the surface charge was reduced to -23.5 ± 1.1 mV due to abundant Si-OH groups. Liposomal coating increased the zeta potential to +12.34 ± 0.5 mV, owing to the presence of amine groups from DSPE-PEG 2000 amine on the surface. The COOH-β-Glucan was conjugated to the abundant -NH₂ groups present on the surface of the liposome through EDC coupling chemistry. This is responsible for the enhancement of the zeta potential to +6.2 ± 0.8 mV.
3.3.8 Optical properties of nano-assemblies

The UV-vis spectra (Figure 3.6b) indicated two characteristic absorption peaks of GNRs: the transverse surface plasmon resonance (TSPR) peak at 514 nm and the longitudinal surface plasmon resonance (LSPR) peak at 790 nm. With the mesoporous silica coating, the LSPR peak underwent a red shift towards 805 nm due to the change in the local refractive index of the surrounding medium and changes in geometry. The laser was selected to match the new absorbance at 805 nm. There was no observable shift in the LSPR peak with the liposome wrapping and after ligand conjugation (Figure 3.6b).

3.3.9 Determination of triple helix structure of β-Glucan

Congo red assay has been widely used to confirm the triple helix structure of polysaccharides. The polysaccharides exist in an ordered three-dimensional structure, generally, triple-helical conformation, forming a complex with Congo red dye. The complex is stabilized by strong hydrogen bonds and hydrophobic interactions between the polysaccharide and the dye molecule. The complex formation of polysaccharides with Congo red is commonly evaluated by employing the shift in the visible absorption maxima.

Figure 3.6. (a) Zeta potential (b) UV-vis absorbance spectra of nano-assemblies.
(λ\text{max}) of the Congo red spectrum. The dye concentration was optimized to give a high and stable absorbance. The concentration of 50 μM gave the best result, and it was chosen for the analysis. The β-Glucan was allowed to complex with Congo red in a 50 μM concentration. Dextran was used as a random-coil control, and its absorbance was similar to the Congo red, which shows no complex formation. While the β-Glucan, COOH-β-Glucan, and (GM)L-Glu showed a bathochromic shift (10 nm), which indicates that this polysaccharide displayed a triple helical structure (Figure 3.7). Since COOH-β-Glucan and (GM)L-Glu also showed similar 10 nm bathochromic shift, which proved that even after chemical modification of β-Glucan and conjugation to (GM)L, the triple helix structure of β-Glucan exists.

### 3.3.10 FT-IR characterization of nano-assemblies

The FT-IR analysis were carried out after each fabrication step are presented in Figure 3.8. As shown in Figure 3.8a, GNRs show -CH₂ symmetric and asymmetric vibrations at 2847 cm⁻¹ and 2870 cm⁻¹, respectively. The presence of a peak at 1473 cm⁻¹
corresponds to the -CH₂ scissoring mode of vibration. In the fingerprint region, the bands at 960 and 817 cm⁻¹ are consistent with C-O stretching and in-plane C-H bending vibrations. The appearance of a peak at 1064 cm⁻¹ (Si-O-Si stretching) and the disappearance of symmetric (2847 cm⁻¹) and asymmetric (2870 cm⁻¹) vibrations, which were seen in GNR, proves the mesoporous silica coating is on the surface of GNRs. The liposomal coating was verified by symmetric and anti-symmetric -CH₂ stretching at 2850 cm⁻¹ and 2920 cm⁻¹, which are the characteristic peaks for the long carbon chain of the lipids used. Also, the -CH₂ scissoring vibration around 1467 cm⁻¹ and the PO₂ symmetric stretching vibration around 1039 cm⁻¹ are from phospholipids, confirming the successful liposome coating. In order to conjugate β-glucan on (GM)L, first, β-glucan is modified with succinic anhydride. Hence, FT-IR analysis of β-glucan and COOH-β-glucan carried out. As shown in Figure 3.8b, β-glucan showed the strong peak at 3320 cm⁻¹ attributed to O–H stretching, the peak at 2931 cm⁻¹ attributed to C–H stretching, and a peak at 1640 cm⁻¹ attributed to the bending of bound water molecules. When β-glucan is modified with

Figure 3.8. FT-IR analysis of (a) various nano-assemblies, (b) β-glucan and COOH-β-glucan.
succinic anhydride, a new peak appears at 1722 cm\(^{-1}\) (strong C=O stretching vibration). The FTIR results (Figure 3.8b) evidence that COOH-\(\beta\)-glucan is successfully synthesized. Furthermore, the FTIR spectra of (GM)L-Glu showed an intense broad peak at 1620 cm\(^{-1}\) (Figure 3.8a), which can be attributed to the new carbonyl (C=O) stretching of amide I absorption of the newly formed amide bond with the NH\(_2\) of liposomes and COOH-\(\beta\)-glucan. The rest of the characteristic peaks at 3252 cm\(^{-1}\) attributed to O–H stretching, the
peak at 2862 cm\(^{-1}\) attributed to C–H stretching, were from \(\beta\)-glucan, which proved that \(\beta\)-glucan is successfully conjugated on (GM)L.

3.3.11 Targeting efficiency/ Intracellular trafficking of HTNs

The intracellular distribution of fluorescently labeled HTNs was analyzed by confocal microscopy to confirm that HTNs were present in the cells and surrounding the \textit{Msmeg} rather than absorbed into the cell membrane. After green fluorescent dye (Coumerine-6) was encapsulated within HTNs, particle uptake (GM)L and (GM)L-Glu by macrophages were visualized. The nucleus of cells and \textit{Msmeg} were stained with blue Hoechst dye. After 6 h of incubation, the (GM)L-Glu were more efficiently taken up and surrounded by intracellular \textit{Msmeg} (shown in Figure 3.9a in box) than the (GM)L (Figure 3.9a). Figure 3.9a shows that the (GM)L-Glu were more rapidly recognized and taken up by \textit{Msmeg} infected macrophages than the (GM)L. Furthermore, as shown in Figure 3.9b (GM)L-Glu shows higher uptake, nearly 91%, while (GM)L showed only 20%. These results clearly depict that macrophage recognition, targeting capacity, and subsequent intracellular trafficking of tested nano-assemblies were positively correlated with the targeting efficiency of (GM)L-Glu. Furthermore, an endocytosis inhibition study was carried out to understand the intracellular trafficking mechanism of (GM)L-Glu. Macrophages were treated with a clathrin-mediated endocytosis inhibitor - chlorpromazine, a caveolin-mediated endocytosis inhibitor - nystatin, and a macropinocytosis inhibitor - wortmannin for 1 h before the addition of Coumarine-6 labeled (GM)L-Glu.\(^{334-336}\) The mechanism for the intracellular uptake of the (GM)L-Glu into the macrophage was monitored by confocal microscopy (Figure 3.10).
The fluorescence microscopy images reveal little or no visible green fluorescent Coumarine-6 labeled (GM)L-Glu in chlorpromazine treated macrophages (Figure 3.10), which indicates chlorpromazine treated macrophages inhibited uptake of (GM)L-Glu. However, uptake of (GM)L-Glu into macrophage was not hindered when treated with wortmannin and nystatin (Figure 3.10). These observations suggested that the trafficking of the (GM)L-Glu occurred through clathrin-mediated endocytosis.

3.3.12 HTNs triggered ROS and RNS induction within *Msmeg* infected macrophage

Pathogenic *Mtb* can scavenge (reactive oxygen and nitrogen species (ROS/RNS)) produced by host macrophages, thereby escaping host-initiated killing mechanisms.
Therefore, by initiating ROS and RNS production within infected host macrophages, we can restrict *Mtb* survival within macrophages. The polysaccharide β-glucan is very well known to activate macrophages to produce pro-inflammatory signals ROS/RNS.\(^{337,338}\) This host-targeting ligand β-glucan binds to the dectin-1 receptor on macrophage surfaces, subsequently activating various downstream signal transduction pathways that promote intracellular ROS/RNS production.\(^{337,338}\) Thus, we have studied if (GM)L-Glu can induce the production of ROS and RNS within infected macrophages (Figure 3.11). The oxidative burst induced by uptake of various nano-assemblies was evaluated by fluorescence imaging and quantitative fluorometric measurements following incubation with a cell-permeable fluorogenic dye 2,7'–dichlorofluorescein diacetate (DCFH-DA). As shown in Figure 3.11a, control- the *Msmeg* infected macrophage was not showing ROS production which proved that *Msmeg* has already evaded the killing mechanism of macrophage. β-glucan and COOH-β-glucan were showing green fluorescent, which indicates that β-glucan and chemically modified β-glucan (COOH-β-glucan) induce ROS within *Msmeg* infected macrophage. As shown in Figure 3.11b, (GM)L – Laser was not showing green fluorescent while (GM)L + Laser showed strong green fluorescent. This observation led to that that ROS can be produced in response to heat stress during PT treatment.\(^{100,302}\) (GM)L-Glu – Laser showed slight green fluorescent even in the absence of laser, which was due to targeting ability (COOH-β-glucan) of (GM)L-Glu. In contrast, (GM)L-Glu + Laser showed very strong green fluorescent, which proved the host targeting effect of (GM)L-Glu and PT treatment. The smaller box depicts the green fluorescent intracellular *Msmeg*, which indicated that (GM)L-Glu + Laser has produced more ROS, indicating macrophage activation and killed intracellular *Msmeg* (Figure 3.11b).
Figure 3.11. Free radical generation. (a) ROS determination by DCFH-DA mediated fluorescence observed within *Msmeg* infected macrophage as a control, *Msmeg* infected macrophage after exposure to β-glucan and COOH-β-glucan. (b) *Msmeg* infected macrophage after exposure to (GM)L ± Laser and (GM)L-Glu ± Laser. (c) Quantitative determination of ROS generation detected by fluorometric intracellular ROS assay. Data was presented as the mean ± SD (n=3). ns (non-significant), ** p < 0.01, *** p < 0.001, and **** p < 0.0001. A, **** p < 0.0001 indicated the data to be more significant when compared to *** p < 0.01 and ** p < 0.01 by One-way ANOVA. (d) RNS determination by nitrite assay.
The quantitative determination of ROS production data measured as fluorescence intensity is shown in Figure 3.11c. These results were consistent with the fluorescent images (Figure 3.11a and Figure 3.11b). It has been previously studied the correlation of reactive-nitrogen species (RNS ~ nitric oxide (NO)) production with mycobacterial infection and macrophage activation.\textsuperscript{339, 340} Activated macrophages have been shown to inhibit intracellular bacterial growth and kill at least 50\% of intracellular bacteria via nitric oxide synthase-2 (NOS2) dependent mechanisms.\textsuperscript{341} As shown in Figure 3.11d, (GM)L-Glu ± Laser exposure was seen to induce high amounts of NO (10 nmol/10\(^4\) cells) within \textit{Msmeg} infected macrophage, indicating macrophage activation by the exposure of (GM)L-Glu ± Laser as compared to (GM)L ± Laser (2 nmol/10\(^4\) cells). Here, we observed that the PT activity was not affecting the NO production. β-glucan and COOH-β-glucan had similar NO production activity (9.6 nmol/10\(^4\) cells), as (GM)L-Glu ± Laser which proved that the higher NO production was due to the presence of host targeting ligand β-glucan on (GM)L. The control, \textit{Msmeg} infected macrophage ± Laser, showed minimal NO production, proving that \textit{Msmeg} has already deactivated the macrophage. Thus, the significant induction of ROS and RNS upon exposure to (GM)L-Glu ± Laser is promising since it indicates the activation of infected macrophages by these nano-assemblies.

\subsection*{3.3.13 Synthesis and characterization of HTNs@BQ}

The stepwise synthesis of HTNs@BQ or (GM@BQ)L-Glu is explained in Scheme 3.2. The detailed synthesis of GNR, GM, liposome preparation, and ligand conjugation on liposome were explained in section 3.2. BQ, an anti-tuberculosis drug, was encapsulated into the porous structure of the GM to create GM@BQ. The thermo-sensitive liposome
was modified with COOH-β-glucan by EDAC chemistry to give L-Glu (as shown in Appendix Scheme B.1.). Finally, GM@BQ is encapsulated inside this prepared liposome L-Glu to create (GM@BQ)L-Glu, abbreviated as HTNs@BQ. The drug loading content (LC) and encapsulation efficiency (EE) are essential factors in nanoparticle-mediated delivery applications. The BQ loading and encapsulation efficiency (EE) was quantified using the standard calibration curve of BQ using RP-HPLC. EE% was calculated as 68.9 ± 0.8 % and 65.1.2 ± 3.5 % for GM@BQ and (GM@BQ)L-Glu, respectively. The LC was calculated to be 15.1 ± 0.5 % and 14.1 ± 1.1 % for GM@BQ and (GM@BQ)L-Glu, respectively.

The quantitative analysis of the HTNs@BQ after each synthesis step was done through TGA. The results are shown in Figure 3.12a. Percentage (%) weight loss between the GM@BQ and GM was calculated to determine the amount of BQ loaded onto GM. The BQ loading was found to be 14.5 ± 1.1% onto GM. Additionally, the percentage of
weight loss between (GM@BQ)L and GM@BQ showed that the lipid layer was $11 \pm 0.6\%$ of the weight of (GM@BQ)L. COOH-β-glucan loading on (GM@BQ)L was $38 \pm 1.5\%$.

$N_2$ adsorption-desorption isotherm and pore size distribution curves of GM and GM@BQ are given in Figure 3.12b. These nano-assemblies exhibit a typical type IV isotherm with a narrow pore size distribution, having a mean of $18.25 \pm 1.1$ Å and $11.13 \pm 1.4$ Å for GM and GM@BQ, respectively. This verifies the porous character of the mesoporous silica shell. Through BET, the total surface area and pore volume of GM were quantified to be $50.788 \pm 1.2$ m$^2$/g and $0.22 \pm 0.3$ cc/g, respectively. In contrast, GM@BQ, the total surface area, and pore volume were quantified as $41.014$ m$^2$/g and $0.16$ cc/g, respectively. The surface electrical charge, optical properties, and functional group analysis of (GM@BQ)L-Glu were shown in Appendix Figure B.6.

Figure 3.12. (a) TGA analysis of nano-assemblies, and (b) $N_2$ adsorption–desorption isotherm of GM and GM@BQ.
3.3.14 Colloidal stability study of nano-assemblies

The colloidal stability of the GM and (GM@BQ)L-Glu in the RPMI 1640 culture medium at 4, 25, and 37 °C was investigated for 5 days, as shown in Figure 3.13 and Figure 3.14. The LSPR peak intensity for GM@BQ was shown in Figure 3.13a, indicating that GM was not stable up to 5 days in any of the temperature conditions. In contrast, the LSPR peak intensity for (GM@BQ)L-Glu was pretty stable at 4 °C and 25 °C as compared to 37 °C, which proved that stability was due to having a liposomal coating (Figure 3.13b). Higher AI indicates a higher degree of aggregation, hence lower stability.\textsuperscript{291,292} As shown in Figure 3.14a, AI for GM was higher and increased with time in media in all the temperature conditions. While the AI values for (GM@BQ)L-Glu were low and constant.

Figure 3.13. Fluctuation of LSPR peak intensity in (a) GM and (b) (GM@BQ)L-Glu at 4, 25, and 37 °C in culture media.
with time in media at all temperature conditions (Figure 3.14b). Similarly, the constant zeta potential in media suggests that GM is not stable (Figure 3.14b). In contrast, (GM@BQ)L-Glu, has shown a constant zeta potential value +6.6 ± 0.9 mV up to 5 days in all temperature conditions (Figure 3.14b). These observations reveal that (GM@BQ)L-Glu is stable in cell culture media for up to 5 days. All these results together concluded that the (GM@BQ)L-Glu nano-assembly is stable and suitable for drug delivery applications.

3.3.15 NIR triggered drug release

The laser setup and photothermal properties of nano-assembly were explained in chapter 2, section 2.3.10. Since the GNR is used as a photothermal agent in (GM@BQ)L-Glu nano-assembly, the photothermal capacity was similar to previously developed nano-assemblies. The photothermal properties are concentration-dependent, as shown in Appendix Figure B.7. In-vitro drug release profile of BQ from GM@BQ ± Laser, (GM@BQ)L ± Laser, and (GM@BQ)L-Glu ± Laser was investigated using RP-HPLC for 12 h. As shown in Figure 3.15, BQ released at 6 h was found to be 70.2 ± 1.2% and 90.3 ± 1.5% for GM@BQ - Laser and GM@BQ + Laser, respectively. This observation
indicates the non-specific BQ release from the mesoporous structure of the silica shell. While \((\text{GM@BQ})\text{L} + \text{Laser}\) released 94.2 ± 1.3% of BQ, \((\text{GM@BQ})\text{L} - \text{Laser}\) showed only a 48.9 ± 1.2% BQ released in a similar time frame suggested that the liposome acts as a protective layer that reduces the non-specific release of the encapsulated BQ. \((\text{GM@BQ})\text{L}-\text{Glu} + \text{Laser}\) demonstrated a similarly high BQ release of 95.8 ± 1.0%. Only 50.7 ± 1.6% of BQ was released in the absence of laser (Figure 3.15). The results revealed that the liposome plays a role in reducing premature BQ release.

### 3.3.16 Intracellular antibacterial activity of nano-assemblies

The antibacterial activity of various nano-assemblies was evaluated by WST-8 colorimetric bacterial viability assay. The MIC was reported with respect to the encapsulated BQ in the nano-assemblies at their MIC concentrations, as shown in Table 3.3.4. The MIC of bare BQ was 40 ± 1.5 ng/mL, and the MIC of \((\text{GM@BQ})\text{L}-\text{Glu} + \text{Laser}\)
Table 3.3.4. MIC of the materials (MIC was reported with respect to encapsulated BQ in the nano-assemblies).*

<table>
<thead>
<tr>
<th>Materials</th>
<th>MIC (ng/mL) BQ encapsulated in nano-assemblies</th>
</tr>
</thead>
<tbody>
<tr>
<td>BQ</td>
<td>40 ± 1.5</td>
</tr>
<tr>
<td>GM@BQ</td>
<td>25 ± 1.8</td>
</tr>
<tr>
<td>GM@BQ + Laser</td>
<td>15 ± 1.5</td>
</tr>
<tr>
<td>(GM@BQ)L-Glu</td>
<td>30 ± 1.9</td>
</tr>
<tr>
<td>(GM@BQ)L-Glu + Laser</td>
<td>8 ± 1.8</td>
</tr>
</tbody>
</table>

was 8 ± 1.8 with respect to the encapsulated BDQ (Table 3.3.4). Results proved that encapsulated BQ in (GM@BQ)L-Glu + Laser is 5 -fold efficacious than the free drug BQ. The intracellular antimicrobial activity of nano-assemblies was tested in macrophages in-vitro by the micro broth dilution method. For that, macrophages were infected with *Msmeg*, followed by the treatment with (GM@BQ)L-Glu ± Laser and BQ of various concentrations. As shown in Table 3.3.5, Free BQ treatment showed 40 ± 1.3 ng/mL. *Msmeg* infected macrophages treated with (GM@BQ)L-Glu + Laser resulted in 4 ± 0.8 intracellular MIC, which was 10 times lower than free BQ. These results clearly indicated that (GM@BQ)L-Glu + Laser is 10 -fold more efficacious than the free drug BQ.

To explore whether (GM@BQ)L-Glu could eliminate the intracellular *Msmeg* upon NIR light irradiation, macrophages were infected by *Msmeg*, treated with (GM@BQ)L-Glu ± Laser and BQ at various concentrations. The survival of intracellular bacteria was measured by counting bacterial colony-forming units (CFU), as shown in Figure 3.16a. The colony count images, as shown in Figure 3.16a, displayed the minimum inhibitory concentration (MIC) of (GM@BQ)L-Glu + Laser was 4 ng/mL, and minimum biocidal concentration (MBC) was 2 ng/mL. In contrast, at those MIC and MBC concentrations,
(GM@BQ)L-Glu + Laser and free BQ showed growth of *Msmeg*. This clearly indicates that (GM@BQ)L-Glu + Laser reduced and killed the intracellular *Msmeg* compared to that in absence of NIR light and free BQ, indicating the efficient inhibition and killing of intracellular *Msmeg* by (GM@BQ)L-Glu upon NIR light irradiation.

Furthermore, nano-assemblies' quantitative intracellular antibacterial efficacy was studied using colony counts and expressed as a percentage of viable bacteria (Figure 3.16b). Intracellular *Msmeg* exposed to (GM@BQ)L-Glu + Laser at MIC concentration (4 ng/mL) exhibited 5 ± 2.1 % bacterial viability and (GM@BQ)L-Glu - Laser had 70 ± 4.1 % bacterial viability. This observation clearly indicated that laser irradiation is vital to the antibacterial effect of the nano-assembly. Free BQ had 82 ± 4.6 % bacterial viability, which clearly explained that (GM@BQ)L-Glu + Laser nano-assembly was efficacious as compared to free BQ.

To further confirm the intracellular antibacterial activity of (GM@BQ)L-Glu, LIVE/DEAD BacLight bacterial viability assays were carried out and visualized under a confocal microscope to distinguish between dead (labeled in red) and live bacteria (labeled in green) within macrophages after treatment (Figure 3.17). Free BQ and (GM@BQ)L-Glu

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Table 3.3.5. Intracellular MIC of the materials (MIC was reported with respect to encapsulated BQ in the nano-assemblies).

<table>
<thead>
<tr>
<th>Materials</th>
<th>Intracellular MIC (ng/mL) BQ encapsulated in nano-assemblies</th>
</tr>
</thead>
<tbody>
<tr>
<td>BQ</td>
<td>40 ± 1.3</td>
</tr>
<tr>
<td>GM@BQ</td>
<td>23 ± 1.1*</td>
</tr>
<tr>
<td>GM@BQ + Laser</td>
<td>10 ± 1.3*</td>
</tr>
<tr>
<td>(GM@BQ)L-Glu</td>
<td>28 ± 1.4*</td>
</tr>
<tr>
<td>(GM@BQ)L-Glu + Laser</td>
<td>4 ± 0.8*</td>
</tr>
</tbody>
</table>
+ Laser was incubated with Msmeg infected macrophage followed by stained with SYTO9/PI, and results were observed through confocal microscopy. Msmeg infected macrophage was not treated with any nano-assembly and hence showed green fluorescent bacteria inside macrophage with no red fluorescent, indicating the viability of bacteria inside the macrophage. Msmeg infected macrophages, when treated with BQ, displayed intense green and less red fluorescence, indicating the presence of more live intracellular bacteria than dead bacteria. In contrast, Msmeg infected macrophage, when treated with (GM@BQ)L-Glu + Laser, had a higher amount of red fluorescence along with high bacterial aggregation as indicated by the white arrows (rod-shaped bacteria with red fluorescence inside the macrophage) clearly seen (Figure 3.17). This result reveals that (GM@BQ)L-Glu + Laser has killed intracellular Msmeg.
3.3.17 HTNs@BQ triggered phagosome acidification and phagolysosome formation in *Msmeg* infected macrophage

*Mtb* manipulates host immune response in two ways; (1) inhibition of phagosome maturation/acidification (2) phagolysosome inhibition, thereby avoiding its killing. Under normal conditions, the V-ATPase pump present on the cell membrane of the phagosome pumps protons into the phagosome.\(^\text{146}\) This increases the acidity of the phagosome and is called the maturation of phagosome or acidification of phagosome.\(^\text{146}\) In the case of *Mtb*

![Confocal microscopic images](image)

Figure 3.17. Confocal microscopic images of the viable (green) and dead (red) intracellular *Msmeg* within macrophage after the treatment with the BQ, and (GM@BQ)L-Glu + Laser. (scale bars represent 5 μm)
infection, *Mtb* releases the protein tyrosine phosphatase (PTPA) protein. PTPA protein binds to a subunit of the V-ATPase pump, which inhibits the activity of the V-ATPase. Hence, inhibits phagosome acidification and inhibits phagosome maturation. In common bacterial infection, the fusion of phagosome with lysosome leads to the formation of the phagolysosome. Upon initiation of this process—increases ROS/RNS production, bactericidal enzymes, peptidase, reduces pH. All these processes together kill intracellular bacteria. However, in *Mtb* infection, phagolysosome formation is inhibited by *Mtb*. In this process, the phagosome and lysosome are prevented from fusing. Also, intracellular *Mtb* can inhibit ROS/RNS generation and prevent a decrease in pH. Therefore, *Mtb* can survive inside a phagosome by inhibiting phagolysosome formation. Recently, Tailleux and co-workers reported that antibiotic bedaquiline (BQ) activates host immune response by promoting phagosome acidification and phagolysosome formation. Therefore, we hypothesize that our BQ loaded HTNs also induce phagosome acidification and phagolysosome formation upon the release of BQ, as shown in Scheme 3.3.

After treating with (GM@BQ)L-Glu + Laser, the activation of macrophages can be verified by confocal microscopy. First, we have observed the phagosome maturation/acidification by treating *Msmeg* infected macrophages with free BQ and (GM@BQ)L-Glu ± Laser treatment followed by staining with acridine orange (AO) dye. Macrophage alone and *Msmeg* infected macrophages were treated directly with AO dye. In AO-stained cells, the acidic compartments were seen to be bright red fluorescence, the intensity of the fluorescence being proportional to the degree of acidity. The nucleus and cytoplasm emitted green fluorescence upon excitation with the same wavelength of light.
As shown in Figure 3.18a, macrophages treated with AO stain displayed bright red fluorescence, indicating that macrophages without Msmeq infection have acidic compartments, thus capable of acidifying phagosome and killing bacteria. In contrast, the Msmeq infected macrophage showed only green fluorescent and no red fluorescence, which indicated that the macrophage had been arrested by Msmeq and stopped phagosome...
maturation/acidification. *Msme* infected macrophage, when treated with BQ and (GM@BQ)L-Glu - Laser showed less red fluorescence indicative of less acidity. However, after treatment with (GM@BQ)L-Glu + Laser, the macrophages displayed very strong and bright red fluorescence, suggesting that (GM@BQ)L-Glu + Laser has enhanced phagosome acidification within *Msme* infected macrophages. The pH changes from neutral (visualized as green) to acidic (red color), indicating the recruitment of vacuolar V-ATPases on the phagosome membrane and the progression of phagosome maturation.\textsuperscript{342,343} The quantitative estimation of red fluorescence intensity as shown in Figure 3.18b is consistent with confocal images (Figure 3.18a).

Phagolysosome formation induced by coumarin-6-labeled (GM@BQ)L-Glu + Laser was investigated using confocal microscopy. For that, *Msme* infected macrophages were exposed to (GM@BQ)L-Glu + Laser. As shown in Figure 3.19, the cell nucleus and

![Figure 3.19. Phagolysosome formation in *Msme* infected macrophage. Confocal microscopy imaging of (a) Colocalization of (GM@BQ)L-Glu (green), ER-Tracker (red), Hoechst-stained nucleus, and *Msme* (blue) in the same vesicles in macrophages after treatment with (GM@BQ)L-Glu + Laser. (b) Colocalization of (GM@BQ)L-Glu (green), Lysosome tracker D99 (red), Hoechst-stained nucleus, and *Msme* (blue) in the same vesicles in macrophages after treatment with (GM@BQ)L-Glu + Laser. Scale bar 50 μm.](image-url)
Scheme 3.3. Working hypothesis of how (GM@BQ)L-Glu induce (a) phagosome maturation/acidification and, (b) phagolysosome formation.

*Msmeg* were stained with Hoechst (blue), ER-Tracker Red, and LysoTracker Red D99 dyes, which are highly selective for acidic organelles, were used as pH-dependent markers of endosomal and lysosomal compartments, respectively. The coumarin-6-labeled (GM@BQ)L-Glu were seen in green fluorescence. After 6 h of incubation with coumarin-6-labeled (GM@BQ)L-Glu + Laser, significant colocalization of green fluorescent nano-assemblies into endosome and lysosome compartment as seen (see merged 1 Figure 3.19). The merged image 2 in Figure 3.19 showed colocalization of nano-assemblies in both endosomal and lysosomal compartments surrounding the intracellular *Msmeg* in macrophage. Most bacteria reside in the arrested phagolysosomal compartment during *Mtb* infection, and it is necessary to deliver the nano-assemblies in this environment. Our data suggest that nano-assembly (GM@BQ)L-Glu was trafficked through β-Glucan-dectin-1(ligand-receptor) mediated endocytosis. Furthermore, the liposomal coating of the
nano-assembly (GM@BQ)L-Glu promotes facile uptake and subsequent trafficking deep into intracellular compartments (Scheme 3.3). The *in-vitro* drug release profile of the (GM@BQ)L-Glu validates that the thermo-sensitive liposomal shell melts, and BQ is released upon NIR laser irradiation. The release of BQ induces phagosome maturation and phagolysosomal formation (Scheme 3.3).

3.3.18 PT induces cell death within *Msmeg* infected macrophage

Two different forms of cell death are commonly observed in *Mtb* infected macrophages (i) Necrosis: a death modality defined by cell lysis. Necrosis is a mechanism used by bacteria to exit the macrophage, evade host defenses, and spread. (ii) Apoptosis: a form of cell death that maintains an intact plasma membrane. Therefore, apoptosis of infected macrophages is a promising host defense mechanism to clear intracellular *Mtb*. By sequestering *Mtb* within apoptotic cells, the spread of bacteria to healthy cells can be prevented. It has been previously studied that NIR laser-induced PTT causes cell death via apoptosis in macrophage and cancer cells. Activation of caspase-3 is an important event during apoptosis. Thus, caspase expression was analyzed by fluorescence microscopy using CellEvent Caspase-3/7 Green Detection Reagent in order to confirm cell death within *Msmeg* infected macrophage. Caspase-3/7 expression was analyzed in the *Msmeg* infected macrophage after (GM@BQ)L-Glu mediated PTT in order to confirm cell apoptosis (Figure 3.20). The fluorescence images as shown in Figure 3.20a displayed little green fluorescence, when *Msmeg* infected macrophage was treated with BQ and (GM@BQ)L-Glu – Laser. In contrast, the strong green fluorescence was observed when *Msmeg* infected macrophage treated with (GM@BQ)L-Glu + Laser which clearly
suggested PT induced apoptosis as compared to (GM@BQ)L-Glu – Laser treatment. As shown in Figure 3.20b, the quantified data from the fluorescence images presents a 3-fold increase in green fluorescence intensity for (GM@BQ)L-Glu + Laser compared to
(GM@BQ)L-Glu – Laser. In comparison to free BQ, (GM@BQ)L-Glu + Laser represented a 7-fold increase in green fluorescence intensity. Since caspase-3/7 indicates apoptotic cell death activation, the data verify that the peak of apoptotic cell death occurs after exposure to (GM@BQ)L-Glu + Laser.

To confirm the viability of bacteria after inducing apoptosis, treated macrophages were lysed, and lysates were plated on agar plates. The colony count images of treatment with (GM@BQ)L-Glu ± Laser, and BQ at MIC (4 ng/mL) and MBC (2 ng/mL) concentration of (GM@BQ)L-Glu + Laser were shown in Figure 3.21. The photographs in Figure 3.21a displayed that NIR laser as control has an abundance of Msmeg growth,
indicating that NIR laser alone was not killing intracellular *Msmeg*. Treatment with (GM@BQ)L-Glu + Laser at MIC (4 ng/mL) concentration had inhibited intracellular *Msmeg* and at MBC (2 ng/mL) concentration completely eradicated intracellular *Msmeg*. Figure 3.21b showed the percentage viability of *Msmeg* within macrophage at MIC (4 ng/mL) and MBC (2 ng/mL) concentration of (GM@BQ)L-Glu ± Laser and BQ. Those were calculated from the counting colony-forming units. *Msmeg* infected macrophages, after treatment with (GM@BQ)L-Glu + Laser at MBC concentration, had only 1% viable of intracellular *Msmeg*. When treated with (GM@BQ)L-Glu - Laser viable bacteria was at 45%, and with free drug BQ only, the viable bacteria was at 70%. These results demonstrate the targeting ability and high antibacterial efficacy of the laser-irradiated (GM@BQ)L-Glu, which kills 99% intracellular *Msmeg*.

3.3.19 Cytotoxicity

The cytotoxic effect of nano-assemblies and laser on healthy macrophage and *Msmeg* infected macrophage were carried out by MTT assay. *Msmeg* infected macrophages were incubated with various concentrations (1-50 μg/mL) of GM, BQ, and (GM@BQ)L-Glu ± Laser. As shown in Figure 3.22a, in the absence of NIR laser GM and (GM@BQ)L-Glu did not show any cytotoxicity. A similar result was found after treatment with BQ. However, the cell viability of *Msmeg* infected macrophage after treatment with GM and (GM@BQ)L-Glu + Laser was nano-assemblies’s concentration-dependent (Figure 3.22b). (GM@BQ)L-Glu + Laser with higher concentration (50 μg/mL) showed only 1% cell viability of *Msmeg* infected macrophage, suggesting that intracellular *Msmeg* were killed after treatment.
This result supports the fact that the apoptosis of infected macrophages is an innate defense to prevent bacteria from spreading to healthy cells and eventually clearing intracellular bacteria.\textsuperscript{350-352}

We also observed the cyto-phototoxicity of nano-assemblies on healthy macrophages. For this experiment, healthy macrophage without \textit{Msmeg} infection was exposed to \pm Laser and (GM@BQ)L-Glu \pm Laser. As shown in Figure 3.22c, NIR laser-
treated macrophages and (GM@BQ)L-Glu ± Laser treated macrophages did not show any cyto-phototoxicity. Healthy macrophages are professional phagocytes capable of inducing phagosome acidification and phagolysosome formation. Hence (GM@BQ)L-Glu ± Laser nano-assemblies were taken up by lysosome and degraded. This is why we did not observe any cyto-phototoxic effect on the healthy macrophage.

3.4 Conclusion

In summary, we screened host-targeted nano-assemblies (HTNs) (a) to favorably modulate the response of macrophages through specific ligand-receptor binding so that it promotes higher cellular uptakes of HTNs and subsequently enhance ROS/RNS, (b) to induce killing of intracellular Msmeg via NIR-triggered drug BQ release that can promote host defense mechanism and (c) to eradicate intracellular Msmeg by inducing cell death of Msmeg infected macrophages. For host (macrophage) targeting, host-specific ligands, such as β-glucan (Glu), and folic acid (FA), were investigated. Free ligands and receptor binding affinity was evaluated. Based on our results, we found β-Glucan conjugated HTNs have a stronger binding affinity towards dectin-1 receptors than FA-folate interactions. Hence, β-Glucan was selected as a host-targeting ligand for further study. Next, we systematically studied the impact of varying sizes and ligand density on binding affinity. The nano-assemblies were composed of a gold nanorod (GNR) core (act as a photosensitizer) and mesoporous silica (MS) shell containing TB antibiotic (BQ). Then after assembly would be wrapped within a thermo-sensitive liposome (L) shell that has tagged with host targeting ligands. Upon NIR laser exposure, the designed HTNs released BQ and activated macrophages by enhancing phagosome maturation and inducing phagolysosome formation. This is how (GM@BQ)L-Glu + laser altered host (macrophage) immune
mechanism. BQ encapsulated in (GM@BQ)L-Glu was found to be 10-fold more efficacious intracellularly compared to free drug equivalent. The final nano-assembly was capable of targeting and killing 99% intracellular \textit{Msmeg} residing within macrophages. Our work will collectively impact the field by investigating the essential roles of cutting-edge nanotechnology in host-targeted therapies for treating intracellular bacterial infection. This may potentially reduce the drug dosage required, shorten the duration of treatment, and reduce the emergence of drug resistance.
CHAPTER 4. TARGETED DELIVERY OF LIPOSOMAL HYBRID GOLD NANO-ASSEMBLY FOR ENHANCED PHOTOTHERMAL THERAPY AGAINST LUNG CARCINOMAS

4.1 Introduction

Despite the tremendous efforts devoted to identifying an effective approach to fight against cancer, precise cancer treatment is still a challenging task. Current therapeutic mainstays available for cancer treatment, such as chemotherapy, surgery, and radiotherapy, are known for their significant after-effects. These conventional therapies are not site (tumor) specific and cause detrimental effects on surrounding cells, leading to further complications such as toxicity, limited bio-distribution, and poor healing. Thus, it is crucial to develop an effective solution that can tackle the fundamental challenges of these conventional treatments and provide targeted tumor-specific outputs without any substantial damage to the viability of the surrounding tissues.

Nanomaterials that respond to externally applied physical stimuli such as light, ultrasound, and magnetic fields have shown great potential for controlled and targeted delivery of therapeutic agents by harnessing the unique optical, magnetic, and physicochemical properties of metallic inorganic nanoparticles. In recent years, photothermal therapy (PTT) has been widely explored. In PTT, nanomaterials act as a
photothermal agent due to their optical properties in the near-infrared (NIR) region. Photothermal heating occurs due to the absorption of NIR light by an ensemble of electrons on the surface of specific conductive nanomaterials and the subsequent dissipation of that energy as heat. A heat source is applied to the site of interest during PTT, and thermal ablation is used to kill tumor cells. However, as a standalone treatment option, the use of PTT is limited due to the lack of effect on metastatic abrasions outside of the area of irradiation, which may lead to disease recurrence. To overcome these limitations and increase PTT's efficacy, researchers have combined the use of NP-mediated photothermal therapy with secondary therapeutic strategies. In nanoparticle-mediated PTT, photoactive NPs are delivered to the tumors. When these NPs are exogenously irradiated with laser light, synchronized oscillation of the conduction-band electrons occurs, which generates heat. Increased temperature in the tumor causes cellular damage, such as protein denaturation, DNA repair deformities, changes in the permeability of the cell membrane, and subsequent tumor regression without damaging the surrounding healthy tissue.

Plasmonically active metal nanoparticles, such as gold nanoparticles (GNPs), black phosphorus, carbon tubes, and polydopamine nanoparticles, are particularly effective photothermal agents because of their large absorbance cross sections and tunable optical properties, and highly efficient conversion of light into heat. Colloidal gold has been shown to have localized surface plasmon resonance (LSPR), which means that gold nanoparticles can absorb light at specific wavelengths, resulting in photoacoustic and photothermal properties, making them useful for hyperthermic cancer treatments and medical imaging. By modifying the shape and size of GNPs can change their LSPR photochemical activities and their photothermal and photoacoustic properties. Gold
nanostructures have many advantages that make them suitable for photothermal cancer treatment, including being administered locally while minimizing non-specific distribution, low cytotoxicity, the ability to be activated via near-infrared (NIR) laser light, and the ability to penetrate deep into biological tissues.\textsuperscript{363} They can also be modulated to create multifaceted cancer PTT and drug delivery systems, and hence gold nanostructures have been a promising photothermal candidate for nanocarrier-mediated PTT applications in cancer therapy.\textsuperscript{364} GNPs can absorb near-infrared (NIR) light through the surface plasmon oscillation of free electrons.\textsuperscript{365} Additionally, by altering the synthesis conditions, the aspect ratio of the nanoparticles can be controlled, which leads to precise control over the absorption spectrum of GNPs. This controlled, fine-tuned regulation permits the nanoparticles to absorb light of a particular wavelength and to exhibit excellent specificity as a photothermal agents.\textsuperscript{361-366} Among available gold (Au) nanoplatforms, Au nanoshells,\textsuperscript{367} Au nanospheres,\textsuperscript{368} Au nanorods,\textsuperscript{369} and Au nano-cages\textsuperscript{370} are used most extensively in PTT. Studies have also exhibited the improved photothermal response of gold nanocarriers with compatible outer shell coatings.\textsuperscript{371-374} Chen et al. reported that the GNPs conjugated to the anti-cancer drug methotrexate (MTX) have demonstrated high levels of tumor retention and enhanced therapeutic efficacy in a lung carcinoma mouse model, compared with an equal dose of free MTX. Another study by Zhang et al. has loaded doxorubicin on mesoporous silica-coated gold nanorods for light-mediated drug delivery carriers for lung cancer treatments. The synergistic effects of chemotherapy and hyperthermia-based treatments have been shown to increase cancer cell death. The multifunctional polymeric nanoparticles formulation was developed by Cheng et al. for combined chemotherapy and PT-based lung cancer treatment.\textsuperscript{375} They have developed a
complex nano-assembly in which paclitaxel-loaded polymeric nanoparticles were conjugated with amine-terminal iron oxide nanoparticles ($\text{Fe}_3\text{O}_4$) and quantum dots (QDs) to obtain QD/$\text{Fe}_3\text{O}_4$/Taxol-loaded polymeric nanoparticles with optical and MR imaging functionalities. Subsequently, poly(styrene sulfonate) (PSS)-coated gold nanorods were introduced to attach to the QD/$\text{Fe}_3\text{O}_4$/Taxol-loaded polymeric nanoparticle surface. These nano-assemblies simultaneously achieve the photothermal ablation of tumor tissue and destroy spherical polymeric nanoparticles, efficiently releasing encapsulated Taxol. However, all of these reported treatments still lack a specific targeting ability towards tumor cells. Therefore, the incorporation of small targeting molecules provides unprecedented opportunities to design targeted nanocarriers to enhance efficiency and reduce adverse effects. In contrast to normal cells, cancer cells overexpress multiple surface receptors. To exploit this concept, we used a dual ligand-targeting approach to develop a target-specific drug delivery nano-assembly.

In previous work, we have utilized a similar nano-assembly for the targeted antibiotic delivery. Utilizing this system, the antibacterial activity was found 20 folds more efficacious than the free drug equivalent. Gold nanorods (GNRs) were coated with mesoporous silica shells (GM) that enhanced drug loading capability. Anticancer drug doxorubicin (Dox) was loaded into a mesoporous silica shell (GM@Dox). Doxorubicin is an anthracycline antibiotic known as a novel antitumor drug to treat various solid malignant tumors. However, free doxorubicin has its own side effects when used in cancer therapy. To reduce the side effects and improve the nano-assemblies' efficacy and stability, GM@Dox was encapsulated further in a liposome. In liposome preparation, a thermosensitive phospholipid (DPPC) was used in a higher molar ratio, making this liposome
thermo-sensitive since the melting point of DPPC is \( \sim 43 \) °C. Encapsulating nano-assemblies inside a liposome also offers a controlled drug release and prevents any premature drug release before the nano-assembly approaches the target site. A near-infrared dye, IR 780, was used as a photosensitizer to enhance the photothermal activity with increased reactive oxygen species (ROS)-generation under laser irradiation. Hence, the IR 780 dye was loaded with the liposome, and then GM@Dox was encapsulated inside the liposome. The nano-assemblies were abbreviated as [(GM@Dox) LI]. Following that, cationic peptide GE-11 and folic acid were conjugated to the surface of the nano-assemblies to specifically target the epidermal growth factor receptor (EGFR) and folate receptor, respectively, which are overexpressed on cancer cells. This dual ligand-targeting approach enhances the cancer-targeting specificity, minimizes non-specific attacks on
surrounding tissues, and improves the therapeutic efficacy of anticancer drugs by enhancing cellular uptake.\textsuperscript{210} The final nano-assemblies are abbreviated as [(GM@Dox) LI]-PF, where GE-11 peptide is indicated as P and folic acid as F. The developed nano-assemblies were tested \textit{in-vitro} and showed a 20-fold increase in effectiveness compared to free Dox. [(GM@Dox) LI]-PF represents a simple, safe, and effective nanocarrier, which can be used for a combination of targeted drug delivery and PTT treatment.

As shown in Scheme 4.1., we anticipate that our engineered nano-assemblies [(GM@Dox) LI]-PF would effectively bind to cancer cells through receptors-directed clathrin-mediated endocytosis pathway by targeting ligands GE-11 and folic acid. Then upon NIR laser irradiation, the heat generated by GNR and IR 780 dye would melt the thermo-sensitive liposome, to release encapsulated Dox. The chemo-photothermal effect of the nano-assemblies would activate apoptotic cell death events, such as increasing ROS production, reducing GSH and cellular ATP. Combinedly this would increase mitochondrial dysfunction, to cause apoptosis through caspase activation.

4.2 Experimental

4.2.1 Materials and Instruments

Chemicals used for gold nanorod and mesoporous silica shell synthesis were similar as described in Section 2.2.1. Coumerine-6, 4’6’-diamidino-2-phenylindole (DAPI, 98%), folic acid, β-Glucan, succinic anhydride, Phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, USA). Lipids such as 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG 2000 amine), used for liposome preparation were similar as explained in section 2.2.1.
Doxorubicin, chlorpromazine nystatin, and wortmannin were purchased from Sigma-Aldrich (St. Louis, USA). GE11 peptide (Tyr-His-Trp-Tyr-Gly-Tyr-Thr-Pro-Gln-Asn-Val-Ile) was obtained from Phoenix Pharmaceuticals, Inc (California, USA).

A549 lung cancer cells were purchased from American Type Culture Collection (ATCC, CCL-185) (Virginia, USA). The CellEvent™ Caspase 3/7 Green Detection Reagent, Live/dead® viability kit, MitoProbe™ JC-1 assay kit, Tubulin Tracker™ Green Detection kit, and Scientific and Invitrogen™ ATP Determination kit was purchased from ThermoFisher Scientific (New Jersey, USA). Fluorometric intercellular ROS kit and MES buffer were purchased from Sigma-Aldrich (St. Louis, USA). The GSH-Glo™ Glutathione assay was purchased from Promega Corporation (Wisconsin, USA). (3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) cell proliferation kit, 4’,6-diamidino-2-phenylindole (DAPI), and Calcein-AM were purchased from ThermoFisher Scientific (Massachusetts, USA). Ham’s F- 12K nutrient mixture with L-glutamine (F-12K, 1X), fetal bovine serum (FBS, 10%), trypsin EDTA (2.21 mM), and penicillin-streptomycin (Pen-Strep, 1X) were obtained from Corning (New York, USA). The instruments were used for characterization of nano-assemblies were similar as described in Section 2.2.1.

4.2.2 Synthesis of gold nanorods (GNRs)

GNRs were synthesized as described in chapter 2 (See Section 2.2.2).

4.2.3 Synthesis of mesoporous silica shell coated gold nanorods (GMs)

GMs were synthesized as described in chapter 2 (See Section 2.2.3).

4.2.4 RITC-labelled GMs synthesis

RITC-labelled GMs were synthesized as explained in chapter 2 (See Section 2.2.4).
4.2.5 Surface area and pore volume determination of nano-assemblies

The surface area of GMs and GM@Dox were determined using the Brunauer–Emmett–Teller (BET) method. The cumulative pore volume was calculated from the adsorption branch of the isotherm using the Barrette–Joyner–Halenda (BJH) model.

4.2.6 Dox encapsulation in GMs

GMs (40 mg) were mixed with Dox (10 mg) in 20 mL of water while stirring overnight at 37 °C. The ratio of GMs and Dox used in the entire study was 4:1 w/w (GMs 40 mg: Dox 10 mg). The Dox-loaded GM samples (GM@Dox) were collected by centrifugation at 12,000 rpm for 10 min and vacuum dried to obtain a dry powder. The encapsulation efficiency and drug loading of Dox in GM@Dox were determined by using a standard calibration curve. To construct a standard calibration curve for Dox, Dox (1 mg) was resuspended in 1 mL of phosphate-buffered saline (PBS) and then diluted to concentrations of 1, 5, 10, 25, 50, 75, and 100 μg/mL. The calibration curve was prepared by recording the absorbance value of Dox at 480 nm using UV-Vis spectroscopy, as shown in Appendix Figure C.1.

\[
\text{Drug encapsulation efficiency} = \frac{\text{Initial amount of drug added} - \text{Amount of drug in supernatant}}{\text{Initial amount of drug added}} \times 100\% \quad (4.1)
\]

\[
\text{Drug loading content} = \frac{\text{Initial amount of drug added} - \text{Amount of drug in supernatant}}{\text{Nanoparticles weight}} \times 100\% \quad (4.2)
\]
4.2.7 Loading of IR 780 dye inside the liposome

IR 780 dye was loaded in the liposome preparation step. Since IR 780 is hydrophobic, it was dissolved with the lipids used to prepare the liposome. Liposomes were prepared by a previously published lipid film-based method. Briefly, DPPC (85% molar ratio), cholesterol (10% molar ratio), DSPE-PEG(2000) amine (5% molar ratio), and IR 780 dye (0.8 mg) was dissolved in 2 mL of chloroform and evaporated in a rotary evaporator, yielding a thin lipid film. This lipid film was rehydrated in 2 mL of PBS (0.5 x, pH 7.4) and extruded 15 times through a polycarbonate membrane (pore size 800 nm) using a mini extruder. Here, the prepared liposome was a thermo-sensitive liposome, as the amount of DPPC used in liposome preparation was the highest in molar ratio compared to cholesterol and DSPE-PEG(2000) amine. As previously studied in our recent paper, these liposomes become permeable at temperatures ~ 50 °C. To check the photothermal effect of IR 780, two different ratios of lipid: IR 780 (100:1 and 100:5 weight ratio) were prepared using a similar lipid film-based method. The resultant product, liposome-IR 780 (LI), was stored at 4 °C until further use.

4.2.8 Encapsulation of GM@Dox inside LI

To prepare [(GM@Dox) LI], 1 mg of GM@Dox was resuspended in LI (2 mL, 10 mg/mL) in PBS and mixed for 20 min in an ice bath. [(GM@Dox) LI] particles were separated from empty liposomes by centrifugation at 12,000 rpm for 5 min with repeated (3x) washing in PBS. The resultant [(GM@Dox) LI] was lyophilized and dried at −20 °C until further use.

The IR-780 loading and encapsulation efficiency was calculated using a standard calibration curve of IR 780 at 780 nm using microplate reader as shown in Appendix Figure...
C.2. The Dox loading and encapsulation efficiency were determined by a standard calibration curve of Dox, as shown in Appendix Figure C.1.

4.2.9 Conjugation of GE-11 and FA on [(GM@Dox) LI]

GE-11 and folic acid (FA) were conjugated to the liposomal shell by amide conjugation. EDC was used to activate the carboxylic groups of GE-11 peptide and FA. Once activated, these groups would react with the amine group of DSPR-PEG (2000) amine in the TSL shell. GE-11 (435 µL) and FA (30 µL) were dissolved in 0.1 M MES containing 0.5 M NaCl at pH 6.0 (reaction buffer) and then incubated with a 10-fold molar excess of EDC (pH = 4.0) and 25-fold molar excess of sulfo-NHS for 30 min. Here the concentration of GE-11 to FA was kept 100:1 molar ratio. Amine group-containing liposomes [(GM@Dox) LI] were added to the solution having carboxylic acid-activated GE-11 and FA and then reacted for 24 h at 37 °C. The molar ratio of GE-11:liposome was kept at 10:1. After 10 h, the reaction mixture was purified by centrifugation at 10,000 rpm for 15 min at 25 °C to remove excess unreacted EDC. The purified [(GM@Dox) LI] conjugated with peptide (GE-11) and FA was abbreviated as [(GM@Dox) LI]-PF. This was lyophilized and stored at −20 °C until further use. The Dox loading and encapsulation efficiency were determined by a standard calibration curve of Dox as shown in Appendix Figure C.1. Similarly, IR 780 loading and encapsulation efficiency were calculated by the standard calibration curve of IR 780 as shown in Appendix Figure C.2.

4.2.10 Quantification of peptide GE-11 conjugation on nano-assemblies

The amount of peptide GE-11 bound on the surface of nano-assemblies was quantified by the Micro BCA method as per the manufacturers’ instructions. 150µl of Micro BCA working solution was added to 150µl PBS (pH 7.0), [(GM@Dox) LI]-PF (1
mg/ml), and [(GM@Dox) LI] (1 mg/ml, served as blank). After 60 min of incubation, the absorbance was measured at 562 nm using a microplate reader. The results were compared to a standard calibration curve of GE-11 solution in PBS (pH=7.0), ranging from 1.0-10 µg/ml as shown in Appendix Figure C.4.

4.2.11 Quantification of folic acid on nano-assemblies

The amount of folic acid conjugated onto the nano-assemblies was quantified by constructing a calibration curve of folic acid at 310 nm using a microplate reader. The absorbance of 300µL of [(GM@Dox) LI]-PF (1 mg/ml) and [(GM@Dox) LI] (1 mg/mL, served as blank) were measured using a microplate reader. The results were compared to a standard calibration curve of folic acid solution in PBS (pH=7.0) ranging from 10µg/mL to 50µg/mL, as shown in Appendix Figure C.3.

4.2.12 TGA analysis of nano-assemblies

TGA was carried out under argon (99.999%), where dried GM, GM@Dox, [(GM@Dox) LI], and [(GM@Dox) LI]-PF (ca. 1 mg) were heated at a rate of 5 °C/min to 100 °C and then kept isothermal at 100 °C for 15 min followed by ramping 5 °C/min to 700 °C. The Dox loaded in GM was calculated by analyzing the weight loss difference between GM and GM@Dox. Similarly, the LI content was analyzed by measuring the weight loss difference between GM@Dox and [(GM@Dox) LI]. Finally, the amount of GE-11 and folic acid was calculated by measuring the weight loss difference between [(GM@Dox) LI] and [(GM@Dox) LI]-PF.

4.2.13 Stability analysis of nano-assemblies

The stability studies were carried out by following the protocol in our previously reported study. Briefly, GM and [(GM@Dox) LI]-PF solution were stored at 37 ° C in
cell culture media for 5 days. The nano-assemblies were monitored daily, and their stability was characterized by UV absorbance to track the longitudinal surface plasmon resonance (LSPR) intensity, aggregation index (AI), and zeta potential to monitor the change in surface charge.

4.2.14 NIR laser irradiation studies

The laser setup is explained in detail in chapter 2 (See Section 2.2.11) and our previously reported study. First, the enhanced photothermal effect due to GNR and IR 780 was evaluated by comparing PT effects of various nano-assemblies such as GM@Dox, LI, and [(GM@Dox) LI]-PF. All three nano-assemblies were exposed to an 808 nm NIR laser (350 mW) for 15 min, and the changes of temperature were recorded using the thermocouple monitor.

Furthermore, various weight concentrations of [(GM@Dox) LI]-PF ranging from 10, 50, 70, and 100 µg/mL (calculated with respect to the weight of GM) were irradiated with the 808 nm NIR laser (350 mW) for 15 min. Finally, two different concentrations of liposome: IR 780 (100:1 and 100:5 weight ratio) were also irradiated with the 808nm NIR laser (350 mW) for 15 min to investigate the role of IR 780 in photothermal activity.

4.2.15 NIR responsive in-vitro drug release of nano-assemblies

1 mg of [(GM@Dox) LI]-PF (having 50 µg of Dox/mg of nano-assemblies) was suspended in 3 mL of PBS buffer at pH 7.5 and 5.5 (release media). The nano-assemblies were incubated at 37 °C for 120 min with or without laser irradiation for 15 min. (808 nm, 350 mW). At the pre-determined time point, the samples were taken out and centrifuged at 15,000 rpm for 15 min. The supernatant was collected and replaced with the same volume of fresh, prewarmed (37 °C) release media. The pellet was resuspended in the new release
media and incubated further. The collected supernatant was quantified by using a standard calibration curve for Dox.

A similar experiment was carried out for GM@Dox ± Laser, [(GM@Dox) LI] ± Laser, and [(GM@Dox) LI]-PF ± Laser to study the drug release profile of different nano-assemblies. Various nano-assemblies (having 50 µg of Dox/mg of nano-assemblies) were suspended in 3 mL of PBS buffer at pH 5.5 then incubated at 37 °C for 6 h, with or without 808 nm NIR laser irradiation (350 mW) for 15 min. At the indicated time point, samples were taken out and then centrifuged, and the supernatant was collected and replaced with prewarmed release media. The pellet was resuspended in the new release media and further incubated. The Dox release was quantified from a standard calibration curve of Dox.

4.2.16 Intracellular trafficking of nano-assemblies in A549 cells

Calcein AM and DAPI were utilized to label the cytoplasm and nucleus of A549 cells, respectively. A549 cells were seeded in 2-well chamber slides and cultured overnight with a density of 1 × 10³ cells/well. Then, the cells were treated with [(GM@Dox) LI]-PF (having 50 µg of Dox/mg of nano-assemblies) for 3 h. The cells were cultured for another 3 h following 808nm NIR laser (350 mW) irradiation for 15 min. Afterward, the cells were washed twice with PBS and subsequently incubated with Calcein AM and DAPI for 30 min at 37 °C. After washing twice with PBS, the cells were observed by a fluorescence microscope. The experiments were performed twice, with and without Laser. Similarly, to study the targeting ability of folic acid and GE-11 peptide, the intracellular uptake between RITC-GM@Dox and RITC-[(GM@Dox) LI]-PF was carried out for 6 h, washed with PBS, then observed by a fluorescence microscope.
4.2.17 Cellular uptake mechanism of nano-assemblies

The cellular uptake mechanism of GM@Dox and [(GM@Dox) LI]-PF in A549 cells was determined under different endocytosis-inhibited conditions. The cells were seeded into 24-well plates at a density of $1 \times 10^3$ cells/well for 24 h and then pre-treated with different endocytosis inhibitors for 30 min. The final concentration of specific endocytosis inhibitors was listed as follows: chlorpromazine at 20 µg/mL, nystatin at 20 µg/mL, and wortmannin at 1 µg/mL. The cells were further incubated with 50 µg/mL RITC-GM@Dox and RITC-[(GM@Dox) LI]-PF for 3 h at 37 °C, washed with PBS and then lysed with 0.5% Triton X-100 in 0.2 M NaOH solution. A fluorescence microplate reader was used to measure the red fluorescence intensity of RITC-GM@Dox and RITC-[(GM@Dox) LI]-PF with excitation and emission wavelengths set at 544 and 576 nm, respectively. The cellular uptake efficacy was expressed as the test wells' fluorescence percentage over the control wells.

4.2.18 NIR-triggered cellular drug release of nano-assemblies

A549 cells were seeded in 2-well chambered cover glasses with a density of $1 \times 10^3$ cells/well. After being incubated overnight at 37 °C, the medium was replaced by a fresh medium containing [(GM@Dox) LI]-PF (having 50 µg of Dox/mg of nano-assemblies) and incubated for 3 h. After the 3 h incubation, the cells were exposed to an 808 nm NIR laser (350 mW) for 15 min. The cells were washed with PBS (2x) and imaged at 0 and 15 min using an inverted fluorescence microscope. The red fluorescent intensity from Dox was used to observe the NIR-triggered Dox release. The mean fluorescent intensity of the released intracellular Dox was quantified using Fiji-win64 software.
4.2.19 Cell cytotoxicity and phototoxicity studies

A549 cells were seeded in a 24-well plate (1×10³ cells/well) and incubated for 24 h. The cells were treated with free Dox, GM@Dox, [(GM@Dox) LI] and [(GM@Dox) LI]-PF (with varying encapsulated Dox concentrations inside the nano-assemblies: 1, 5, 10, 25, and 50 μg/mL) and incubated for 3 h with 5% CO₂ and 37 °C. A549 cells were treated with GM carriers with varying concentrations of 1, 5, 10, 25, and 50 μg/mL. After 3 h of incubation, the cells were irradiated with 808nm laser (350 mW) for 15 min and further incubated for 3 h. A 100 μL aliquot of MTT (5 μg/mL) was added to each well, and the plate was further incubated for 3 h. The culture medium in each well was replaced with 100 μL of DMSO, and the plate was gently agitated for 10 min. Similarly, cells were treated without NIR laser after incubating with nano-assemblies. The percentage viability was calculated by comparing untreated cells to treated cells, and the absorbance was measured at 590 nm using a plate reader.

4.2.20 Live-dead assay

A549 cells were cultured in a 24-well plate (1×10³ cells/well). When the cells reached 80% confluency, the cells were incubated with free Dox, GM@Dox, [(GM@Dox) LI], and [(GM@Dox) LI]-PF (having 50 μg of Dox/mg of nano-assemblies) for 3 h. Similarly, the cells were treated with 50 μg /mL of GM carriers. After 3 h of incubation, the cells were irradiated with 808nm laser (350 mW) for 15 min and further incubated for 3 h. Then, cells were washed twice with cold PBS and incubated for 30 min with the live-dead reagent (2 μM Calcein AM and 4 μM ethidium bromide) according to the manufacturer’s instructions. The samples were washed twice with PBS and imaged by fluorescence microscopy.
4.2.21 Intracellular ROS determination

The generation of reactive oxygen species (ROS) in cells was measured using a ROS-sensitive fluorescent dye, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), by the previously reported method.\textsuperscript{378} This dye can be oxidized to 2′,7′-dichlorofluorescein (DCF) by ROS, which exhibits increased green fluorescence intensity. Briefly, A549 cells (1×10\textsuperscript{3} cells/well) were treated with free Dox, GM@Dox, [(GM@Dox) LI], and [(GM@Dox) LI]-PF (having 50 μg of Dox/mg of nano-assemblies) for 3 h. The cells were treated with GM carriers at 50 μg/mL. The cells were then treated with or without 808 nm NIR laser (350 mW) for 15 min and were allowed to continue incubating for another 3 h. After treatment, the cells were washed twice with 1× PBS. Then, the treated cells were incubated with 10 mM of DCFH-DA for 10 min, and the generated intracellular ROS was examined under a fluorescence microscope. The fluorescence intensity was quantified at 525 nm, with excitation at 490 nm.

4.2.22 Intracellular reduced glutathione (GSH) measurement

Intracellular GSH levels were determined using a GSH-Glo glutathione assay kit (Promega, Madison, WI, USA), following the manufacturer’s instructions. Briefly, cells (1×10\textsuperscript{3} cells/well) were seeded in 24-well plates and treated with free Dox, GM@Dox, [(GM@Dox) LI], and [(GM@Dox) LI]-PF (having 50 μg of Dox/mg of nano-assemblies) at 37 °C for 3 h. Similarly, the cells were treated with 50 μg/mL with GM carriers. The cells were then treated with or without 808nm NIR laser (350 mW) for 15 min and were allowed to continue incubating for another 3 h. The cells were washed with PBS, and GSH-Glo reagent was added to each well for 30 min at room temperature, and the luminescent signal was measured with a microplate reader.
4.2.23 Assessment of mitochondrial membrane potential

A mitochondrial potential-sensitive probe, JC-1, was used in this study to evaluate mitochondria in the cells after treatment with the laser-irradiated nano-assembly. For this study, A549 cells (1×10³ /well) were seeded in 24-well plates. After 24 h, the old medium was replaced with a fresh medium containing free Dox, GM@Dox, and [(GM@Dox) LI]-PF (having 50 µg of Dox/mg of nano-assemblies) and incubated for 3 h. Then the cells were treated with a GM carrier with 50 µg/mL. The cells were then treated with or without 808nm NIR laser (350 mW) for 15 min and were incubated for another 3 h. After treatment, the cells were washed twice with 1×PBS and incubated with 100 µL of JC-1 dye (2 mg/ml) for 20 min at 37 °C. Finally, images were taken in the green and red fluorescence channels by fluorescence imaging. The images were obtained at 488 nm excitation and 530 nm emission for green (JC-1 monomers) and 543 nm excitation and 590 nm emission for red fluorescence (JC-1 aggregates).

4.2.24 Measurement of cellular ATP

A549 cells were seeded at a density of 1 × 10³ cells/well and then incubated for 24 h before experiments. Cells were then treated with free Dox, GM@Dox, [(GM@Dox) LI], and [(GM@Dox) LI]-PF (having 50 µg of Dox/mg of nano-assemblies) and incubated for 3 h at 37 °C. Similarly, the cells were treated with a GM carrier with 50 µg/mL. Subsequently, the cells were treated with or without 808nm NIR laser (350 mW) for 15 min and were incubated for another 3 h. Then, the cells were washed with PBS and harvested with trypsin for 1 min at 37 °C. Cold PBS was added to terminate the reaction, and the cells were collected by centrifugation. The ATP level was assessed using the adenosine 5’-triphosphate (ATP) bioluminescent assay kit (Sigma-Aldrich).
4.2.25 Caspase 3 & 7 activity

To investigate the modulation of cell death by [(GM@Dox) LI]-PF-mediated PTT, the expression of Caspase-3/7 within each NIR-treated sample was examined. Activation of caspase-3 is an important event during apoptosis. Thus, caspase expression was analyzed by fluorescence microscopy using the Cell Event Caspase-3/7 Green Detection Reagent (ThermoFisher Scientific) to confirm cell death by apoptosis. This reagent is an amino acid peptide (DEVD) conjugated to a nucleic acid-binding dye. Activated Caspase-3/7 cleaves at the DEVD peptide sequence, which allows the conjugated dye to bind to DNA and fluoresce green. For this experiment, A549 cells were seeded at a density of $1 \times 10^3$ cells/well and then incubated for 24 h. The cells were then treated with free Dox and [(GM@Dox) LI]-PF ± Laser (having 50 $\mu$g of Dox/mg of nano-assemblies) and incubated for 3 h at 37 °C. A GM carrier (50 $\mu$g/mL) was used to treat the cells. Next, the cells were exposed to PTT treatment for [(GM@Dox) LI]-PF (NIR 808 nm laser, 350 mW, 15 min), and incubation was continued for another 3 h at 37 °C. Before use, the CellEvent Caspase-3/7 Green Detection Reagent was diluted with PBS to a 5 $\mu$M working solution. After the samples underwent PTT, the NP-containing media was removed and replaced with 100 $\mu$L of 5 $\mu$M CellEvent Caspase-3/7 Green Detection Reagent. Samples were allowed to incubate for an additional 30 min before analysis and then imaged using a fluorescence microscope. The fluorescent intensity was quantified at 525 nm, with excitation at 490 nm.

4.2.26 Morphological assessment of apoptotic cells

A549 cells were grown on sterile chamber glass slides overnight, and the cells were then treated with [(GM@Dox) LI]-PF + Laser (having 50 $\mu$g of Dox/mg of nano-assemblies), incubated for 3 h at 37 °C. Then, the cells were exposed to PTT treatment for
[(GM@Dox) LI]-PF (NIR 808 nm laser, 350 mW, 15 min), and incubation was continued for another 3 h at 37 °C. At the end of the incubation, the cells were fixed with 4% paraformaldehyde and then permeabilized with Triton X-100 (0.1% in PBS). The cells were finally stained using DAPI in PBS (2.5 µg/mL) and allowed to stand for 20 min in the dark. Finally, morphological changes were viewed using fluorescence microscopy.

4.2.27 Tubulin assay

Tubulin polymerization was determined using a biochemical detector following the manufacturer’s protocol. Briefly, A549 cells (10^3 cells/well) were incubated with Dox and [(GM@Dox) LI]-PF ± Laser (having 50 µg of Dox/mg of nano-assemblies) for 3 h at 37 °C. Similarly, the cells were treated with a GM carrier with 50 µg/mL. After that, [(GM@Dox) LI]-PF-treated cells were exposed to an 808nm NIR laser for 15 min and further incubated for 3 h. The culture medium was removed, and the cells were washed twice with PBS and stained with diluted tubulin tracker green reagent for 30 min at 37 °C. Cell nuclei were stained with DAPI for 15 min. Blue fluorescence and green fluorescence were observed under an inverted fluorescence microscope.

4.3 Results and Discussion

4.3.1 Synthesis of [(GM@Dox) LI]-PF

The design and synthetic strategy of [(GM@Dox) LI]-PF is illustrated in Scheme 4.2. The photothermal agent, GNR, was synthesized via a seed method as described by El-Sayed et. al., with some minor modifications. To achieve an effective Dox loading and controlled drug release, a porous silica shell was grafted onto the surface of the GNR by a modified protocol from Matsuura et al. CTAB was used as a soft template, which creates the mesoporous silica shell structure during the polymerization of silane around the GNRs
(to give GM). Afterwards, doxorubicin (Dox), a known broad-spectrum antitumor drug, is loaded into the GNR-coated mesoporous silica shell, which is referred as GM@Dox. Dox mainly inhibits topoisomerase II upon intercalation with DNA and induce cell death via the production of reactive oxygen species (ROS), inducing oxidative DNA lesions and lipid peroxidation.  

Furthermore, IR 780 dye was loaded in the liposome to enhance the photothermal performance, which was referred to as Liposome-IR 780 (LI). IR 780 is a hydrophobic dye widely used as a photosensitizing agent in PTT, and it has been extensively explored for NIR fluorescence imaging and approved by the FDA for clinical applications. This synergistic combination of GNR and IR-780 dye in our nano-assembly would facilitate a higher photothermal impact using low power laser intensity (350mW). The liposomes were synthesized by the extrusion method using the DPPC, cholesterol, and DSPE-PEG(2000) amine, along with IR 780. The as-prepared GM@Dox was encapsulated within the liposome by reacting them in an ice bath, where the amphiphilic phospholipids self-
assemble around the GM@Dox to create a lipid layer [(GM@Dox) LI]. The phospholipid DPPC, which is used in an 85% molar ratio, makes this liposome thermo-sensitive due to its melting point at 43 °C. Cholesterol in the lipid layer enhances membrane fluidity and Dox retention. The large PEG group of DSPE-PEG(2000)amine is known to improve drug delivery efficiency, increase systematic circulation time, reduce immunogenicity, and
offer stealth properties, which facilitates the binding of the targeting ligands onto the liposome. The liposome layer acts as a gatekeeper that restricts the premature release of Dox. The liposomal coating also helps to stabilize the GM@Dox and improves the cellular uptake of nano-assemblies.

Next, to achieve target specificity and increase uptake efficiency into the cancer cells, the cationic peptide, GE-11 (P), and folic acid (F) were conjugated to the surface of the liposome through EDAC chemistry, and the final nano-assembly was abbreviated as [(GM@Dox) LI]-PF. GE-11(YHWYGYTPQNV1) is known for its binding affinity for the EGFR, which directs the nano-assemblies towards the cancerous cells. GE11 is a dodecapeptide that binds specifically to EGFR, which is overexpressed in many tumors of epithelial origin, including breast, lung, and ovarian cancers. On the other hand, folic acid has an affinity towards folate receptors, recognized biomarkers for tumor cells, due to their overexpression on many tumors of epithelial origin, including breast, lung, and ovarian cancers. Therefore, these folate receptors are an attractive candidate for drug targets. The use of folic acid as a cancer cell-targeting ligand has become more advantageous due to its non-immunogenicity, stability, tissue permeability, and ease of conjugation into NPs. We have successfully attached both targeting ligands to the liposomal layer of the [(GM@Dox) LI]-PF nano-assemblies and showed the enhanced cancer-targeting effects of these nano-assemblies compared to the drug-loaded particles (GM@Dox).
4.3.2 Morphology studies of nano-assemblies

The morphological analysis of nano-assemblies was carried out using TEM and HAADF-STEM. As shown in Figure 4.1a, the average length and width of GNRs were found to be 42 ± 3.8 nm and 11.2 ± 1.1 nm, respectively, and the calculated average aspect ratio was 3.8:1. The GNRs displayed a uniform and well-dispersed rod-like morphology, visible through Transmission Electron Microscopy (TEM) (Figure 4.1a). Dox was encapsulated into the porous structure of the GM to create GM@Dox. TEM images (Figure 4.1b) show GNRs encapsulated in a mesoporous silica shell loaded with Dox (GM@Dox). The silica shell has an average thickness of 15 ± 3 nm, and its porous structure can be clearly seen by TEM. The photothermal effect of GNR has been enhanced using an additional photosensitizer: IR 780 (I) and the dye loaded inside the liposome. Then, GM@Dox was encapsulated inside the liposome, and the entire assembly was called [(GM@Dox) LI]. The liposomal layer is visible through TEM, as shown with a red double-headed arrow (Figure 4.1c). The shape of GM@Dox dictates the oblong shape of the liposomal layer. High-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) images (Figure 4.1e-i) display the presence of gold (Au), silicon (Si), oxygen (O), and carbon (C) elements in the layers of [(GM@Dox) LI]. The merged image in Figure 4.1i depicts the individual layers overlaid on each other to confirm the structure of the final construct of [(GM@Dox) LI]. HAADF images reveal the presence of carbons from the long hydrocarbon chains of the lipids used to form the liposome. This proves the presence of a liposomal layer surrounding GM@Dox, as indicated by a red double-headed arrow in the merged image of Figure 4.1i. The combined TEM (Figure 4.1c) and HAADF-STEM (Figure 4.1i) images display the liposomal coating around the GM@Dox.
4.3.3 Elemental analysis of nano-assemblies

Energy-dispersive X-ray spectroscopy (EDS) is an analytical method for the chemical characterization of materials. The chemical composition of GM@Dox and [(GM@Dox) LI] was analyzed by energy-dispersive X-ray spectroscopy (EDS). The EDS spectrum of GM@Dox (Figure 4.2a) clearly displayed the peaks of gold (Au), oxygen (O), and silica (Si). A copper (Cu) peak was present in the EDS spectra due to using a Cu mesh TEM grid as a substrate. The EDS spectrum of [(GM@Dox) LI] (Figure 4.2b) shows the peaks for elements Au, Si, O, and C. The presence of C peaks was used to indicate the existence of a liposomal layer. A liposome is composed of lipids with a long hydrocarbon chain, for example, DPPC, which has a 40-carbon alkyl chain. The HAADF-STEM image in Figure 4.2b shows the clear presence of C in the outermost liposomal layer.

4.3.4 Surface charge

The zeta potential was measured after the addition of each layer to monitor the change in surface charge. The altered surface zeta potential is shown in Figure 4.3a. The zeta potential of GNRs was 28 ± 0.59 mV, possibly due to the presence of positively
charged CTAB on the surface. After silica coating, the surface charge was reduced to -23.5 ± 0.8 mV due to abundant Si-OH groups. The free cationic Dox has a surface charge of 18 ± 1.8 mV in water; therefore, after loading Dox to GM, the zeta potential increased to -10.27 ± 0.76 mV. Liposomal coating increased the zeta potential to +7.25 ± 0.6 mV, owing to the presence of amine groups from DSPE-PEG 2000 amine on the surface. The cationic peptide GE-11 and folic acid were conjugated to the abundant -NH₂ groups present on the surface of the liposome through EDC coupling chemistry. This is responsible for the final increment of the zeta potential to +15.6 ± 0.88 mV.

4.3.5 Optical properties of nano-assemblies

The UV-vis spectra (Figure 4.3b) indicated two characteristic absorption peaks of GNRs: the transverse surface plasmon resonance (TSPR) peak at 514 nm and the longitudinal surface plasmon resonance (LSPR) peak at 790 nm. With the mesoporous silica coating, the LSPR peak underwent a red shift towards 800 nm due to the change in the local refractive index of the surrounding medium and changes in geometry. The laser
was selected to match the new absorbance at 800 nm. After Dox loading, there was no change in the observed LSPR peak. The absorption peaks of [(GM@Dox) LI] at 740 nm belong to the characteristic absorption of IR 780 dye, and the peak at 800 nm belongs to the GM@Dox. For [(GM@Dox) LI]-PF, the observed absorption peaks were similar to [(GM@Dox) LI] (Figure 4.3b). The strong absorption of [(GM@Dox) LI]-PF in NIR regions indicated PTT potential upon laser irradiation. Many studies have previously reported that both GNR and IR 780 can act as outstanding PTT agents.\textsuperscript{386,387}

### 4.3.6 Functional group characterization of nano-assemblies

The FT-IR spectra labeled with the characteristic peaks of the different components of each fabrication step are presented in Figure 4.4. As shown in Figure 4.4a, GNRs show -CH\(_2\) symmetric and asymmetric vibrations at 2847 cm\(^{-1}\) and 2870 cm\(^{-1}\), respectively. The presence of a peak at 1473 cm\(^{-1}\) corresponds to the -CH\(_2\) scissoring mode of vibration. In the fingerprint region, the bands at 960 and 817 cm\(^{-1}\) are consistent with C-O stretching and in-plane C-H bending vibrations. The appearance of a peak at 1064 cm\(^{-1}\) (Si-O-Si stretching) and the disappearance of symmetric (2847 cm\(^{-1}\)) and asymmetric (2870 cm\(^{-1}\)) vibrations, which were seen in GNR, proves the mesoporous silica coating on the surface of GNRs. The spectrum of free Dox showed characteristic peaks at 3311 cm\(^{-1}\) (OH and NH, stretch), 2898 cm\(^{-1}\) (CH stretch, aromatic), 1730 cm\(^{-1}\) (C=O stretch, ketone), 1615 cm\(^{-1}\) (C=O stretch), 1,282 cm\(^{-1}\) (C-O-C, stretch), 1115 cm\(^{-1}\) (C-O stretch, tertiary alcohol), 1070 cm\(^{-1}\) (C-O stretch, secondary alcohol), and 988 cm\(^{-1}\) (C-O stretch, primary alcohol).\textsuperscript{388} Similar trends in characteristic peaks were seen after loading Dox into GM, which confirms the successful loading of Dox.
Next, as shown in Figure 4.4b, the IR 780 loading inside the liposome and the liposome wrapping on GM@Dox were analyzed by comparing FTIR spectra of GM@Dox, [(GM@Dox) L], [(GM@Dox) LI], and IR 780. The symmetric and anti-symmetric -CH$_2$ stretching at 2850 cm$^{-1}$ and 2920 cm$^{-1}$ were the characteristic lines for the long carbon chain of the lipids used. Also, the C=O stretching vibration around 1737 cm$^{-1}$ and the PO$_2$
symmetric stretching vibration around 1111 cm\(^{-1}\) are from phospholipids, confirming the successful liposome coating. IR 780 loading inside TSL was proved by comparing FTIR spectra of IR 780 and [(GM@Dox) LI]. As shown in Figure 4.4b, the trend of characteristic peaks of [(GM@Dox) LI] was similar to free IR 780, confirming the loading of IR 780 dye inside the liposome.

Furthermore, the FTIR spectra of [(GM@Dox) LI]-PF and free GE-11 were recorded to verify that the GE11 peptide had been conjugated to [(GM@Dox) LI]. The intense broad band at 3289 cm\(^{-1}\) in Figure 4.4c represents N-H stretching vibrations, indicating the presence of amino groups. We assign two sharp absorption bands at 1647 and 1515 cm\(^{-1}\) to the amide I and amide II groups of the GE-11 peptide, respectively.\(^{389}\) By contrast, the absorption bands for the amide I and amide II groups of the [(GM@Dox) LI]-PF nano-assemblies appeared at 1637 and 1553 cm\(^{-1}\), respectively (Figure 4.4c). The shifts of these two amide absorption bands to the higher wavenumber suggest that conformational changes occurred to the GE-11 peptide upon binding to [(GM@Dox) LI]. The presence of C=O groups in free GE-11 peptide at 1444 cm\(^{-1}\) corresponds to the symmetrical stretching vibrations of carboxylate groups, while in [(GM@Dox) LI]-PF nano-assemblies, this shift to a higher wavenumber 1517 cm\(^{-1}\) suggests that conformational change occurred to the GE-11 peptide upon binding to [(GM@Dox) LI].

The conjugation of folic acid onto [(GM@Dox) LI] was investigated by comparing free folic acid and [(GM@Dox) LI]-PF, as shown in Figure 4.4d. The characteristic IR absorption peaks at 1690 and 1601 cm\(^{-1}\) are observed in the spectrum of free folic acid, due to C=O amide stretching of the \(\alpha\)-carboxyl group and absorption of aromatic C=C group respectively.\(^{390,391}\) While the absorption bands of the [(GM@Dox) LI]-PF nano-assemblies
appeared at 1732 and 1636 cm\(^{-1}\), respectively. Again, the wavenumber shifting to a higher range indicated that the folic acid's conformational changes upon conjugation with [(GM@Dox) LI]. From all these FTIR spectra, we have successfully confirmed the fabrication of the nano-assemblies after each step of the synthesis.

4.3.7 Porosity analysis of mesoporous silica shell

\(\text{N}_2\) adsorption-desorption isotherm and pore size distribution curves of GM and GM@Dox are given in Figure 4.5a and as shown in Appendix Table C.1. These nano-assemblies exhibits a typical type IV isotherm with a narrow pore size distribution, having a mean of 19.13 ± 1.1 Å and 12.19 ± 0.8 Å for GM and GM@Dox, respectively. This verifies the porous character of the MS shell. Through BET, the total surface area and pore volume of GM were quantified to be 53.852 ± 1.5 m\(^2\)g\(^{-1}\) and 0.23 ± 0.1 cc/g, respectively. However, for GM@Dox, the total surface area and pore volume were quantified as 42.060 m\(^2\)g\(^{-1}\) and 0.17 cc/g, respectively.
4.3.8 TGA of various nano-assemblies

The quantitative analysis of the nano-assemblies after each synthesis step was done through TGA. The results are shown in Figure 4.5b and Table 1. Percentage (%) weight loss between the GM@Dox and GM was calculated to determine the amount of Dox loaded onto GM. The Dox loading was found to be $32 \pm 1.1\%$ onto GM. Additionally, the percentage weight loss between [(GM@Dox) LI] and GM@Dox showed that the lipid layer and IR-780 together is $10 \pm 1.6\%$ of the weight of [(GM@Dox) LI]. The loading content of IR 780 onto [(GM@Dox) LI] was found to be $45.2 \pm 1.1\%$ using a plate reader, based on calculations from the standard calibration curve of IR 780. The combined results of TGA analysis and UV-vis spectroscopy indicated that 4.5% of IR 780 (TGA- total lipids + IR 780 was $10 \pm 1.6\%$ ~UV-vis 45% of IR-780 from plate reader) and 5.5% of lipids were present on [(GM@Dox) LI]. Finally, peptide GE-11 and folic acid loading on [(GM@Dox) LI] were quantified by TGA analysis between the [(GM@Dox) LI]-PF and [(GM@Dox) LI]. The total ligand loading was found to be $12 \pm 1.5\%$ on [(GM@Dox) LI]-PF.

4.3.9 Quantification of targeting ligands on nano-assemblies

The total GE-11 peptide conjugated on [(GM@Dox) LI]-PF was $41.5 \pm 1.0\%$ by a Micro BCA assay. The amount of GE-11 was calculated using the standard calibration curve of GE-11, using the Micro BCA assay as shown in Figure C.4. Folic acid conjugation on [(GM@Dox) LI]-PF was found to be $58 \pm 0.8\%$ by measuring at 310 nm with a plate reader. The amount of folic acid was calculated from the standard calibration curve of folic acid, as shown in Figure C.3. These data correlated with the TGA data, which gave the total amount of ligands on [(GM@Dox) LI] $12 \pm 1.5\%$. This means that 5% of
GE-11 (TGA- total ligand 12 ± 1.5 % ~ UV-vis 42% GE-11 from Micro BSA assay) and 7% of FA (TGA-total ligand 12 ± 1.5 % of ~ 58% folic acid from plate reader) were present on [(GM@Dox) LI]-PF.

4.3.10 Encapsulation efficiency and drug loading content

The encapsulation efficiency (EE) and loading content of Dox and IR 780 were calculated using the standard calibration curve of Dox and IR 780 using a microplate reader. (Table 4.3.1). The higher Dox loading in GM@Dox may be due to the porous structure of the silica shell and the hydrophilic nature of Dox. The EE of Dox in [(GM@Dox) LI] and [(GM@Dox) LI]-PF had negligible differences. The LC of Dox in GM@Dox, [(GM@Dox) LI], and [(GM@Dox) LI]-PF were found to be 34.6 ± 0.5%, 32.8 ± 0.9%, and 30.1 ± 0.9%, respectively. The high EE and LC of Dox in all the nano-assemblies was primarily due to the electrostatic interactions between the cationic anticancer drug Dox and the negatively charged Si-OH porous walls of the mesoporous silica shell. The high EE and LC of IR 780 in [(GM@Dox) LI] and [(GM@Dox) LI]-PF may be due to the strong hydrophobic interaction between IR 780 and the lipophilic phospholipids in the liposome composition.

Table 4.3.1. Encapsulation efficiency and loading content of Dox and IR 780 in nano-assemblies

<table>
<thead>
<tr>
<th>Nano-assemblies</th>
<th>EE (%)</th>
<th>LC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM@Dox</td>
<td>98.9 ± 0.8 (Dox)</td>
<td>34.6 ± 0.5 (Dox)</td>
</tr>
<tr>
<td>[(GM@Dox) LI]</td>
<td>95.3 ± 0.7 (Dox)</td>
<td>32.8 ± 0.9 (Dox)</td>
</tr>
<tr>
<td></td>
<td>99.5 ± 0.5 (IR 780)</td>
<td>45.2 ± 1.1 (IR 780)</td>
</tr>
<tr>
<td>[(GM@Dox) LI]-PF</td>
<td>93.9 ± 0.9 (Dox)</td>
<td>30.11 ± 0.9 (Dox)</td>
</tr>
<tr>
<td></td>
<td>97.9 ± 0.8 (IR 780)</td>
<td>43.62 ± 0.5 (IR 780)</td>
</tr>
</tbody>
</table>
4.3.11 Colloidal stability

The colloidal stability of the GM@Dox and [(GM@Dox) LI]-PF in the culture medium was investigated, as shown in Appendix Figure C.5. The LSPR peak intensity, aggregation index, and zeta potential of [(GM@Dox) LI]-PF were stable for 5 days in the culture medium. This reflects the stability afforded by the liposomal layer in [(GM@Dox) LI]-PF. GM@Dox, however, was comparatively unstable. The reducing LSPR peak intensity, high aggregation index, and changing zeta potential of GM@Dox were evidence of its instability. The final [(GM@Dox) LI]-PF nano-assembly is stable and suitable for cancer applications.

4.3.12 Targeting efficiency of nano-assemblies

Moreover, to check the targeting efficiency of [(GM@Dox)LI]-PF, we have treated A549 cells with GM@Dox and [(GM@Dox)LI]-PF and incubated them for 6 h. As shown in Figure 4.6, cells treated with [(GM@Dox)LI]-PF show strong, intense red fluorescence
(red fluorescence from Dox), while GM@Dox-treated cells have very little to no red fluorescence, which confirms that the targeting capacity of [(GM@Dox)LI]-PF helped more nano-assemblies to get inside the cells. These data suggest that [(GM@Dox)LI]-PF could deliver the drug with enhanced efficiency due to the active targeting of GE-11 toward EGFR and folic acid to folate receptors (FR) on cancer cell membranes. The EGFR and FR have recognized biomarkers for cancer cells due to their overexpression on multiple tumors, including liver cancer. \(^{290, 392, 393}\) Cancer cell-directed enhanced GE-11 and folic acid synergistically facilitated drug delivery effects to increase therapeutic efficiency and limit multidrug resistance.

### 4.3.13 Enhanced photothermal performance

The enhanced photothermal performance of GNR and the encapsulated IR 780 under laser irradiation was investigated. The temperature variations of GM@Dox, LI, and [(GM@Dox)LI]-PF exposed to laser irradiation at 808 nm and 350 mW for 15 min were tested and are displayed in Figure 4.7a. The temperature increases after laser irradiation of the [(GM@Dox)LI]-PF was 60 ± 5.1 °C, which was higher than that of both GM@Dox (45.2 ± 1.5°C) and LI (40 ± 1.1°C). The photothermal activity of the nano-assembly was enhanced by 15 ± 4.5 °C even with the use of lower laser intensity, 350 mW. This was done by co-encapsulating GNR and IR 780 inside a liposome layer. Furthermore, the [(GM@Dox)LI]-PF exhibited concentration-dependent photothermal properties, demonstrating a linear increase in temperature with the increasing concentration Figure 4.7b. Even at very low concentrations (10 μg/mL), the temperature increased to 45 ± 1.3°C. As shown in Figure 4.7c, two different weight ratios of liposome: IR 780 (LI) were investigated to check the PT activity. With higher IR 780 weight ratios, higher PT activity
was observed. The greatly enhanced photothermal properties of the \([(GM@Dox) LI]\)-PF was attributed to the LSPR of the GNR, which increased the light absorption efficiency of IR 780.
4.3.14 PTT-induced drug release studies

We examined the photothermally triggered Dox release from the nano-assemblies in PBS at different pH values. As shown in Figure 4.8a, after 2 h of on-off NIR laser irradiation, the accumulated release of Dox in [(GM@Dox) LI]-PF reached 35.2 ± 1.0% and 12.1 ± 1.1% in PBS at pH 5.5 and 7.5, respectively. In contrast, in pH 7.5 PBS buffer solution, which mimics normal physiological conditions, the Dox release was (2.7 ± 1.0%) in the absence of laser irradiation over a similar time frame (2 h). The higher release of Dox at a lower pH was attributed to the protonation of silicates at a lower pH, resulting in the dissociation of the electrostatic interaction between the negatively charged porous silica shell and Dox consequently boosting the release of the drug\textsuperscript{394}. In addition, when exposed to NIR laser irradiation, the drug release can be controlled remotely through the melting of
thermo-sensitive liposomes and the weakened interaction between the mesoporous silica matrix and the drug.

PTT-induced drug release studies were carried out on various nano-assemblies in the presence (+) or absence (-) of NIR laser. As shown in Figure 4.8b, cumulative Dox released within 6 h was found to be 30.1 ± 1.2% and 46.3 ± 1.3% for GM@Dox - Laser and GM@Dox + Laser, respectively. This indicates that the subsequent temperature increases with laser irradiation increased the non-specific release of Dox. While \[(GM@Dox) LI\] + Laser released 90.7 ± 1.0% of Dox, \[(GM@Dox) LI\] - Laser showed only a 12.1 ± 0.8% Dox release in a similar time frame. This observation suggested that the liposome acts as a protective layer that reduces the non-specific release of the encapsulated Dox. Additionally, as shown in Appendix Figure C.6., comparing Dox released by \[(GM@Dox) L\] + Laser (75.3 ± 1.2%) and \[(GM@Dox) L\] + Laser (90.7 ± 1.0%) suggests that the enhanced photothermal performance of the nano-assemblies is indicative of encapsulated IR 780 dye, which increased Dox release rate by 1.2-fold. \[(GM@Dox) LI\]-PF + Laser demonstrated a similarly high Dox release of 95.3 ± 3.6%. Only 15.1 ± 1.5% of Dox was released in the absence of Laser (Figure 4.8b). It is apparent from the above results that the liposome plays a role in reducing premature Dox release. With laser irradiation, the temperature of the \[(GM@Dox) LI\]-PF nano-assembly was increased to 60 ± 5.1 °C. This was accompanied by the increase in the Dox release. At this elevated temperature, the phospholipid membrane becomes permeable due to gel-to-liquid phase transition. Due to the combined hyperthermia generated by GNR and IR-780 under the laser, the thermo-sensitive liposomal layer melts, allowing Dox release.
4.3.15 Cellular internalization of nano-assemblies

The cellular uptake and distribution of the nano-assemblies in A549 cells were observed with an inverted fluorescence microscope. After 6 h of incubation in A549 cells,
the subcellular localization of free Dox and [(GM@Dox) LI]-PF ± Laser irradiation were imaged (Figure 4.9a). In the case of free Dox, the low fluorescence intensity indicated that only a minimal amount of Dox was distributed in the cytoplasm. However, in the case of [(GM@Dox) LI]-PF – Laser, moderate fluorescence indicated that slightly more Dox entered the cells, owing to the targeting ability of the dual peptides, GE-11 and folic acid. The lower amount of Dox fluorescence response around the nucleus also suggested that the [(GM@Dox) LI]-PF prevented Dox from leaking out. Upon NIR laser irradiation, stronger fluorescence was found with [(GM@Dox) LI]-PF + laser group, which can be ascribed to the quick release of Dox. Dox distribution in the cytoplasm was seen mainly surrounding the nucleus. This indicated that the GE11- and folic acid-modified liposome encapsulation can enhance the cellular uptake efficiency of the nano-assemblies, and the NIR laser irradiation could control the release of the drug. Additionally, we have also studied the cellular uptake mechanism by which nano-assemblies are transported into the A549 cells, which were described in detail in Appendix Section C.1 and Figure C.7. Briefly, nano-assembly-mediated drug delivery occurred preferably through clathrin-mediated endocytosis, and nano-assembly internalization was enhanced due to the liposome and targeting ligands.

4.3.16 NIR-triggered drug release in cells

The intracellular drug release under continuous laser irradiation was monitored by fluorescence microscopy. Before laser irradiation, little or no Dox was seen distributed in the cells (Figure 4.9b). After NIR laser radiation for 15 min, Dox was seen to be released in the cells. Dox was seen dispersed homogeneously in the cytoplasm and surrounding the nucleus (Figure 4.9b). The mean fluorescence intensity of released Dox from [(GM@Dox)
LI]-PF + or - laser treatment was quantified, and the results were shown to correspond to the fluorescence images (Figure 4.9c). Therefore, NIR laser irradiation plays an essential role in controlling the release of Dox in A549 cells. The intracellular on-demand drug release is critical to increasing therapeutic efficiency, reducing side effects, and reducing drug resistance.

### 4.3.17 Cellular cyto-phototoxicity studies

The therapeutic potential of various nano-assemblies in presence or absence of laser was evaluated. A549 cells were incubated with free Dox, GM, GM@Dox, [(GM@Dox) LI], and [(GM@Dox) LI]-PF for 3 h and then irradiated with 808 nm NIR laser for 15 minutes. The obtained cell viability data are shown in Figure 4.10a. GM showed no obvious cytotoxicity without the laser, but in the presence of laser irradiation, GM displayed slight cytotoxicity at a 50 μg/mL concentration. The results showed that without laser exposure, only GM@Dox (percentage viability 80 ± 3 % at 50 μg/mL) showed a cytotoxic effect on A549 cells. Lower cell viability in the presence of GM@Dox was possibly due to continuous non-specific Dox release from mesoporous silica.

Compared to GM@Dox, both [(GM@Dox) LI] (90 ± 2.5 %) and [(GM@Dox) LI]-PF (89 ± 3.1 %) had higher cell viabilities. However, in the presence of laser irradiation, [(GM@Dox) LI] and [(GM@Dox) LI]-PF had much higher toxicity toward A549 cells. [(GM@Dox) LI] + Laser had 25 ± 3.1 % cell viability, and [(GM@Dox) LI]-PF + Laser had 8 ± 3 % cell viability. GM@Dox + Laser only had 61 ± 2.7 % cell viability, and Dox alone had 89 ± 2.8 % cell viability. Therefore [(GM@Dox) LI]-PF + Laser was 20-fold more effective when compared to Dox alone. This is due to the combined chemophotothermal effect and higher internalization afforded through GE-11 and folic acid.
A live/dead assay was also conducted to visualize the chemo-phototherapeutic effect of the nano-assemblies. The cells were stained with Calcein-AM to indicate intracellular esterase activity in live cells and stained with ethidium homodimer-1 (EthD-1) to indicate the loss of plasma membrane integrity in dead cells. Living cells are indicated with green fluorescence, and red fluorescence indicates dead or late apoptotic cells. Stained
cells were observed using an inverted fluorescent microscope. As shown in Figure 4.10b, the viability of cells treated with [(GM@Dox) LI] + Laser and [(GM@Dox) LI]-PF + Laser was lower than that of cells treated with GM@Dox + Laser under the same conditions. The fluorescence responses of the live/dead cells are shown in Figure 4.10b, which are consistent with the results of the MTT assay (Figure 4.10a). In the absence of NIR laser excitation, neither [(GM@Dox) LI] nor [(GM@Dox) LI]-PF exhibited apparent cytotoxicity. The results reflect the combined chemo-photothermal effect of both [(GM@Dox) LI] and [(GM@Dox) LI]-PF.

4.3.18 Intracellular ROS determination

Cellular ROS generation mainly occurs in the mitochondria, and mitochondrial damage is closely correlated with high ROS levels. Additionally, Dox has an affinity to bind specifically to the abundant phospholipid cardiolipin located in the inner mitochondrial membrane, which leads to mitochondrial accumulation of the drug. Hence, disrupting the electron transport chain (ETC) causes ROS production. To evaluate the photothermal effect of the nano-assemblies in vitro, ROS (typically $^{1}\text{O}_2$) was monitored in living cells using carboxy-H$_2$DCFDA as a fluorescent indicator. This nonfluorescent molecule is readily converted to a green, fluorescent carboxy-DCF. This occurs when the acetate groups are removed by intracellular esterases and oxidation by singlet oxygens ($^{1}\text{O}_2$). This enables living cells to exhibit bright green fluorescence, indicating ROS production in cells. A549 cells were incubated with free Dox, GM, GM@Dox, [(GM@Dox) LI], and [(GM@Dox) LI]-PF, followed by the addition of carboxy-H$_2$DCFDA. After being continuously irradiated for 15 min, the samples were imaged through a fluorescence microscope (Figure 4.11a), and fluorescence intensity was
quantitatively measured by a microplate reader (Figure 4.11b). The cells treated with GM solution exhibited negligible fluorescence in the absence of laser irradiation. A slight green fluorescence was displayed when cells were treated with GM@Dox + Laser, probably due to premature Dox release. Higher green fluorescence intensity was seen in cells treated with [(GM@Dox) LI]-PF + Laser. This can be attributed to the enhanced photothermal effect due to the additional photosensitizer, IR 780. IR 780 has been used previously in PTT and as a generator of ROS. ROS, such as $^1$O$_2$, plays a vital role in PTT, damaging the
cell structure or affecting cell function. Similarly, with [(GM@Dox) LI]-PF + Laser, the fluorescence intensity of the treated cells was strong compared to cells treated with GM@Dox + laser and [(GM@Dox) LI] + laser. We hypothesized that upon NIR exposure, GNR and IR 780 would induce a hyperthermic condition that will melt the thermo-sensitive liposome, thus delivering more Dox molecules into the mitochondria, thereby inducing higher levels of ROS.

4.3.19 Intracellular GSH determination

It is well-known that ROS can cause a drop in GSH levels either by oxidation or by reaction with the thiol group of GSH. GSH is a non-enzymatic antioxidant that functions as the first line of the cellular defense mechanism against oxidative injury in all cell compartments. Previous research has shown that ROS generation following GSH depletion induces mitochondrial damage and up-regulation of pro-apoptosis mediators. Here, we wanted to evaluate further whether the laser-treated nano-assemblies could down-regulate the intracellular GSH level. A fluorescent thiol probe was used to indicate the intracellular GSH level with or without laser-treated nano-assemblies. As shown in Figure 4.11c, without laser treatment, high GSH levels in the tumor cells were observed, while with laser treatment, gradual GSH depletion was observed in GM@Dox (53.0 ± 4.2 %), [(GM@Dox) LI] (31.8 ± 3.5 %), and [(GM@Dox) LI]-PF (16.2 ± 3.3 %). The intracellular GSH level for free Dox was found to be 93.8 ± 3.8 %, which is much higher compared to [(GM@Dox) LI]-PF + Laser (16.2 ± 3.3 %).

4.3.20 Cellular ATP determination

After treatment with various nano-assemblies, the cellular ATP level was analyzed to demonstrate the apoptotic pathway mediated via mitochondria. As shown in Figure
4.11d, the energy level in A549 cells remained higher, which was seen in the increased ATP level in cells treated with nano-assemblies in the absence of laser irradiation compared to laser. However, GM@Dox + laser, [(GM@Dox) LI] + Laser, and [(GM@Dox) LI]-PF + Laser caused a constant decrease in the intracellular ATP, which was related to the decreased proliferation rates. After treatment with Dox only, the ATP level was 86.8 ± 3.1 %, while with [(GM@Dox) LI]-PF + Laser was 7.1 ± 3 %. The up-regulation of ROS, downregulation of GSH, and decrease in the cellular ATP level are initiators to prompt mitochondrial depolarization.408, 409

4.3.21 Mitochondrial function assessment

Next, mitochondrial function was checked qualitatively by fluorescence imaging using JC-1 as a probe. Mitochondria are central organelles in which the intrinsic mitochondrial pathway of apoptosis can be directly triggered by injury.410, 411 Many mitochondria-related critical events may result in cell apoptosis, such as mitochondrial
membrane potential loss, electron transport chain destruction, caspase-activating protein release, and disruption of the micro skeleton structure of tubulin.  

\[(\text{GM@Dox}) \text{ LI}\]-PF + Laser-induced mitochondrial dysfunction was first investigated by evaluating the mitochondrial membrane potential using JC-1 staining (Figure 4.12). The JC-1 probe is capable of examining mitochondrial damage. The green JC-1 monomer can enter the cytoplasm and aggregate in normal mitochondria, forming numerous red J-aggregates. In contrast, the fluorescence transformation from red to green indicates a loss of membrane potential in the mitochondria, suggesting significant mitochondrial damage. Very little green fluorescence due to damaged mitochondria was seen after treatment with GM, Dox, GM@Dox, and \[(\text{GM@Dox}) \text{ LI}\]-PF, in the absence of Laser. Red fluorescence indicative of healthy mitochondria was noticeable even after 6 h incubation of GM, Dox, GM@Dox, and \[(\text{GM@Dox}) \text{ LI}\]-PF, in the absence of Laser. An increase of green fluorescence could be observed after treatment with laser-irradiated GM@Dox and\[(\text{GM@Dox}) \text{ LI}\]-PF. The green fluorescence intensity of A549 cells treated with \[(\text{GM@Dox}) \text{ LI}\]-PF + Laser was much stronger than GM@Dox + Laser, indicating significant mitochondrial dysfunction. Dox itself could also cause mild mitochondrial dysfunction based on JC-1 staining, but extreme mitochondrial dysfunction could be triggered by \[(\text{GM@Dox}) \text{ LI}\]-PF + Laser, which was due to combinational therapy of chemo-photothermal therapy and peptide targeting capability against A549 cells.

4.3.22 Apoptosis detection

Apoptosis of cancerous cells was first assessed by Caspase 3 &7 assay. The activity of various caspases was used as an apoptosis-specific target for the direct visualization of apoptosis since their activity changes correlate with the apoptosis stages. Caspase-3 and
caspase-7 are cysteine-aspartic acid proteases that can carry out apoptosis directly, following sequential activation of caspase-8 or caspase-9 activation.\textsuperscript{415} Hence, the caspase-3/7-specific cleavable peptide substrate, Asp-Gly-Val-Asp (DEVD), has been commonly used as a caspase-cleavable apoptosis imaging probe to track caspase activity in both \textit{in-vitro} and \textit{in-vivo} conditions of tumor cells.\textsuperscript{416,417}

As shown in Figure 4.13a, no green fluorescence was observed with the GM-treated cells, while A549 cells treated with Dox alone showed slight green fluorescence. Upon NIR laser irradiation, a higher level of strong green fluorescence was observed for [(GM@Dox) LI]-PF + Laser compared to [(GM@Dox) LI]-PF – Laser. [(GM@Dox) LI]-PF + Laser treatment caused morphological changes in cells. Shrinking and rounding of cells were observed, as is typical of apoptosis and the activation of caspase3/7 (see merged image Figure 4.13a). These results suggest that [(GM@Dox) LI]-PF + Laser induces apoptosis through caspase-3/7 activation. The quantified data from the fluorescence images show a 20-fold increase in green fluorescence intensity for [(GM@Dox) LI]-PF + laser compared to GM alone (Figure 4.13b). Since caspase-3/7 activation indicates apoptotic cell death activation, the data verify that the peak of apoptotic cell death occurs after laser exposure to the nano-assemblies, which suggests that the combined approach of chemotherapy nano-assembly-mediated PTT causes the apoptosis of A549 cells.

To further confirm apoptosis detection, DAPI staining was utilized to check the morphology of the A549 cell nucleus with or without treatment with [(GM@Dox) LI]-PF + Laser (Figure 4.13c). A549 cells treated with [(GM@Dox) LI]-PF + Laser showed apparent apoptotic features compared to the cells not given treatment. A549 cells treated with [(GM@Dox) LI]-PF + Laser showed an increase in the number of cells with small,
Figure 4.13. Apoptosis mechanism of A549 cells evaluated by Caspase-3/7 assay. (a) Cells are shown in a bright field, and green fluorescent images correspond to the expression of Caspase-3/7. Morphological changes such as shrunken cells and the aggregation of cell nuclei in A549 cells were observed in laser-treated [(GM@Dox) LI]-PF compared to cells treated with carrier GM. (b) Quantitative analysis of green fluorescence density, indicative of Caspase-3/7 expression for cells treated with nanomaterials, was detected by a fluorometric assay. The data are presented as the mean ± SD (n=3). N.S = non-significant. A ** represents p < 0.01 and *** rep p < 0.001, indicating that data are statistically significant by one-way ANOVA compared with free Dox. (c) DAPI fluorescence image of apoptotic A549 cells treated with [(GM@Dox) LI]-PF + laser, white arrows indicate nuclear disintegration (ND), red arrows indicate cell membrane blebbing (BL) and yellow arrows indicate nucleus condensation (NC). Scale bar = 10 mm.
condensed nuclei (nuclei condensation, NC), nuclei disintegration (ND), and membrane blebbing (BL), which indicates cell structure loss (Figure 4.13c). These morphological changes indicated that \([(\text{GM@Dox}) \text{ LI}]\)-PF + laser-treated A549 cells have undergone apoptosis. Untreated control cells remained intact, and evenly shaped nucleus compared to \([(\text{GM@Dox}) \text{ LI}]\)-PF + Laser treated A549 cells.

4.3.23 Tubulin assay

A tubulin assay was carried out, as microtubules are natural biopolymers that are produced by the polymerization of α- and β-tubulins, one of the components of the cytoskeleton and they contribute to cellular transport, cell motility, and mitosis.\(^{418}\) Previously, it has been reported that PTT inhibits cancer cell migration and invasion by disturbing the cytoskeleton.\(^{419-421}\) To confirm the effect of PTT induced by \([(\text{GM@Dox}) \text{ LI}]\)-PF in the presence and absence of laser irradiation on microtubule integrity, the \([(\text{GM@Dox}) \text{ LI}]\)-PF nano-assemblies were incubated with A549 cells and was analyzed by fluorescence microscopy.

As shown in Figure 4.14, the microtubules of the GM-treated A549 cells (control) were outstretched and slender, exhibiting a well-organized cytoplasmic network. Compared to the control, almost no noticeable morphological change in the microtubules was observed when incubated with free Dox and \([(\text{GM@Dox}) \text{ LI}]\)-PF – Laser. However, enhanced damage and disruption were seen in the microtubule skeleton structure when cells were incubated with \([(\text{GM@Dox}) \text{ LI}]\)-PF + Laser. The peripheral microtubules were shrunk, condensed, and rounded up into clusters. Damaged microtubules cause failed replication and eventually lead to cell apoptosis. Based on the above results, we concluded that the \([(\text{GM@Dox}) \text{ LI}]\)-PF + Laser (a) amplifies mitochondrial oxidative stress (mtROS)
(b) reduces GSH and ATP levels (c) disturbs the mitochondrial membrane potential (d) upregulates caspase-3/7, and (e) disrupts the microtubule network which are all indications of programmed cell death.

4.4 Conclusion

In summary, we have fabricated dual-targeted nano-assemblies that offer combinational therapy: chemotherapy and PTT against NSCLC. The photothermal activity of the nano-assembly was enhanced by 15 ± 4.5 °C even with the use of lower laser intensity, 350 mW. This was done by co-encapsulating GNR and IR 780 inside a liposome layer. Dox was loaded into the mesoporous silica coating surrounding the GNR. IR 780
dye was encapsulated inside the liposome, and the entire nano-assembly was conjugated with the tumor-targeting ligands GE-11 and folic acid. The nano-assemblies are efficiently internalized inside the cancer cells through clathrin-mediated endocytosis via targeting ligands. The burst of the TSL layer was driven by PT activity triggered by NIR laser irradiation. Subsequently, Dox was released from the mesoporous pores due to a weakly acidic condition-induced protonation in the tumor micro-environment. The encapsulated Dox was 20-fold times more efficacious compared to free Dox. We have also investigated the cancer cell death mechanism after treating the cells with the nano-assemblies. The results indicate that the cancer cells undergo apoptosis via an intrinsic mitochondrial pathway that can be directly triggered by the chemo-photothermal treatment given by nano-assemblies. Compared with monotherapy, the application of multiple treatments could reduce the required dose of the drug to achieve the goal of overcoming drug resistance in cancer. Due to the synergistic effect of chemo-photothermal therapy, this proposed multifunctional targeted drug delivery system shows great promise in improving the therapeutic effect, providing a powerful platform for optimal therapeutic effect at low doses.
CHAPTER 5. RAPID DIAGNOSTICS OF MYCOBACTERIA USING THE LECTIN CONJUGATED MULTI-CORE SHELL MAGNETIC NANOPARTICLES

5.1 Introduction

Tuberculosis (TB) is an infectious disease generally caused by *Mycobacterium tuberculosis* (*Mtb*) that exists as a latent and active condition. Latent TB occurs when mycobacteria infect the body but remain silently in an inactive state, representing the silent epidemic.\(^4\) According to the WHO Global Tuberculosis Report for 2020, approximately almost 10 million new cases and 1.6 million deaths are caused by TB, making it one of the most virulent diseases.\(^5\) Effective diagnosis of TB allows for early treatment and potential prevention of disease spreading.\(^6\) Those diagnosed with latent TB infections may elect to take medications to reduce the risk of TB becoming active. Diagnosis of active TB provides opportunities to prevent the spread of tuberculosis, such as covering the mouth when coughing, wearing a mask, and reducing contact with others while undergoing treatment, which generally involves the administration of multi-drug regime antibiotics.\(^7\) Common detection methods include the tuberculin skin test, amplification tests, nucleic acid, culture methods, and conventional microscopy.\(^8\) However, these methods are often time-consuming, labor-intensive, and require expensive laboratory equipment. Hence, rapid detection for *Mtb* is needed, which can be relatively inexpensive to manufacture, provide accurate and reliable results, operate without requiring clinical personnel and instruments
A variety of nanomaterials have been developed for the rapid capture and detection of TB. Examples are silica nanoparticles, quantum dots, gold nanoparticles, and magnetic nanoparticles.\textsuperscript{426} Qin \textit{et al.} reported a study in which mesoporous silica nanoparticles coupled with fluorescent dye for the detection of \textit{Mtb} and the detection window has been 2 h.\textsuperscript{427} A study was reported by Liandris and his co-workers, who utilized quantum dots of CdSeO$_3$ coupled with streptavidin and species-specific probes, which detect surface antigen of mycobacterium species in 2 h.\textsuperscript{428} The gold nanosphere (AuNPs) was firstly reported in TB diagnosis by Baptista \textit{et al.} and they have coupled DNA (oligonucleotide derived from the gene sequence of the \textit{Mtb} RNA polymerase subunit) onto AuNPs for the colorimetric detection of \textit{Mtb} with 15 minutes detection time.\textsuperscript{429} Magnetism-based detection techniques in the past have employed magnetic nanoparticles to magnetically separate analyte from the sample using an external magnet.

We have developed a simple and rapid diagnostic test for detecting mycobacteria using magnetic nanoparticles.\textsuperscript{430} \textit{Mycobacterium smegmatis} (\textit{Msmeg}) is a mimic of \textit{Mycobacterium tuberculosis} (\textit{Mtb}) that is often utilized in the study of tuberculosis due to its faster doubling time and lower biosafety level facility requirements. In addition, \textit{Msmeg} shares many homologous genes and similar cell wall composition as \textit{Mtb}. Therefore, the diagnosis method could also diagnose a TB infection rapidly. The nanoparticles (NPs) have a core-shell structure. The core consists of superparamagnetic nanoparticles (MNPs) and a silica shell. Superparamagnetism is a phenomenon that occurs primarily in nanoscale, single-domain MNPs. When exposed to an external magnetic field, this MNPs develops a strong internal magnetization as a result of electron exchange coupling within the domain and thus becomes superparamagnetic.\textsuperscript{431} Superparamagnetic properties of MNPs were used
as an external cue to capture bacteria and silica coating offers stability and surface for further modification. These multi-core shell nanoparticles (multiple MNPs core with single silica shell – multi-core shell nanoparticles) are addressed as silica magnetic nanoparticles (SMNPs). The ligand NHS-PEG-Silane coupled to a surface of silica shell (SMNP-PEG-NHS). The reaction is between the hydroxyl group of silicates on the silica surface and ethoxy silane of NHS-PEG-Silane. Following conjugation of NHS-PEG-Silane lectins such as *Concanavalin A* (Con A) and *Aleuria aurantia* (AAL), *Wisteria floribunda* (WFL) (AAL), and *Bovine serum albumin* (BSA) conjugated separately on SMNP-PEG-NHS. NPs were abbreviated as SMNP-Con A, SMNP-AAL, SMNP-WFL, and SMNP-BSA.

The mycobacteria have a unique cell wall that distinguishes them from other bacteria, as shown in Scheme 5.1. The cell wall of mycobacteria consists of five layers such as outermost layers, mycomembrane, arabinogalactan, peptidoglycan, followed by the plasma membrane (See Scheme 5.1). Mannose and arabinose are among the most abundant carbohydrates present on the outermost layer, and an important virulence factor of *Mtb*, which is found in lipoarabinomannan (LAM). The outermost layer also contains arabinomannan, protein, and lipids. The outer layer is followed by the mycomembrane, which consists of mycoloyal residue (mycolic acids), distributed as a thick layer, while the internal layers of mycobacteria consist mostly of peptidoglycan covalently linked to the arabinogalactan layer. We chose plant lectins Con A which has affinity toward mannose, AAL has binding preference to arabinose, and WFL has binding affinities to GalNAc. The reported binding affinity of Con A towards mannose carbohydrate epitope was 2.89 μM. AAL, and WFL binds to arabinose and *N*-acetylgalactosamine (GalNAc) with binding
affinity 16 μM and 92.3 μM, respectively. 435, 436 WFL and GalNac interaction used as a positive control as GalNac residue was absent in cell wall of mycobacteria. 437 Similarly, BSA protein act as a negative control. Lectins typically have multiple carbohydrate-binding sites, and as a result, lectin binding to mycobacterial cell wall frequently results in strong binding due to crosslinking with cell surface carbohydrate epitopes. 113 Multivalent lectins with proper presentation and a lectin with two or more carbohydrate-binding sites are required for multivalent interactions. 113 Multivalent interaction between nanoparticles conjugated lectins and carbohydrate epitopes present on the cell wall of mycobacteria is
possible by taking advantage of the size and larger surface area of nanomaterials that can be used as a scaffold. Hence, lectin such as Con A and AAL conjugated SMNP would specifically bind to mannose/arabinose carbohydrates epitopes present on the LAM of bacterial cell wall. This binding would then cause the bacteria to fall out of the solution and present as a magnetic precipitate within 1 minute. This precipitate could therefore be used to determine the presence of mycobacteria. We conducted a systemic study to prove our developed rapid diagnostic test for TB can detect bacteria within 1 minute, with the lowest detection limit being 10 CFU/mL.

5.2 Experimental

5.2.1 Materials and Instruments

Tetraethyl orthosilicate (TEOS, 98%), IGEPAL® CO-520, oleylamine (70%), oleic acid (90%), iron (III) acetylacetonate (97%), benzyl ether (98%), ammonium hydroxide (28-30%), cyclohexane (99.5%), concanavalin A lectin (ConA) from Canavalia ensiformis, and N-(2-Hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES solution) (1M, pH 7.0-7.6) were purchased from Sigma-Aldrich (Missouri). Absolute ethanol (200-proof), hexane (4.2%), and Hoechst dye were purchased from ThermoFisher Scientific (Massachusetts). 1,2-Hexadecanediol was purchased from TCI America (Oregon). NHS-PEG-silane (1000 Da) was purchased from Nanocs (Massachusetts). Aleuria aurantia Lectin (AAL), Wisteria floribunda Lectin (WFL), and Bovine serum albumin (BSA) were purchased from Vector Laboratories (California). Mycobacterium smegmatis (ATCC® 700084™) and Streptococcus mutans (ATCC® 25175™) were purchased from American Type Culture Collection (Virginia). Difco™ Middlebrook 7H9 broth, Difco™ Middlebrook 7H10 agar, BBL™ Middlebrook ADC enrichment, BBL™
Middlebrook OADC enrichment, BBL™ Brain Heart Infusion (BHI), and Difco™ BHI agar were purchased from Becton Dickenson (New Jersey). Double distilled water was used throughout these experiments unless otherwise specified.

Fluorescence microscopic images were obtained using an Olympus CKX53 inverted fluorescence microscope (Tokyo, Japan) equipped with a Lumenera Infinity 3 camera (Ottawa, Canada). Dynamic light scattering (DLS) measurements were taken using a Malvern Zetasizer (Malvern Panalytical Inc., Massachusetts). Transmission electron microscopy (TEM) images of the nanoparticles were obtained on an FEI Tecnai G2 Osiris S/TEM (Oregon). Attenuated total reflectance Fourier-transform infrared (ATR FTIR) spectroscopic analyses were done using Nicolet iS50 ATR FTIR (ThermoFisher Scientific). Thermogravimetric analyses (TGA) were performed by a Discovery Q550 TA Instrument (Delaware). Optical density measurements were taken from the Spectramax M2 plate reader (Molecular Devices, California). The Msmeg samples were incubated in a Corning LSE shaking incubator (New York). The bacteria samples and the related glassware were autoclaved in a BioClave autoclave (Ward’s Science, New York) All aseptic bacterial techniques were conducted under a 1300 Series A2 biosafety cabinet (ThermoFisher Scientific).

5.2.2 Fabrication of magnetic nanoparticles (MNPs)

The synthesis of MNPs followed the previously reported procedure of Sun et al. Iron (III) acetylacetonate (0.706 g, 2.0 mmol), 1,2-hexadecanediol (2.584 g, 10.0 mmol), oleic acid (2.239 mL, 6.0 mmol), and oleylamine (2.820 mL, 6 mmol) in dibenzyl ether (20 mL) were stirred under a blanket of nitrogen. The mixture was then heated to 200 °C for 2 h, followed by heating at 280 °C for 1 h, and finally stirred overnight
at 400 rpm. The resulting MNP solution was purified by adding ethanol (200-proof, 40 mL), and the mixture was centrifuged at 7000 rpm for 10 min. The black precipitate was re-dispersed in hexane (30 mL) containing oleic acid (0.05 mL) and oleylamine (0.05 mL), and the mixture was centrifuged at 6000 rpm for 10 min. The precipitate was discarded, the supernatant was collected, and ethanol was added. After centrifugation, the precipitate was re-dispersed in hexane.

5.2.3 Synthesis of multi-core shell silica-coated magnetic nanoparticles (SMNPs)

A modified Stöber process was employed to introduce a silica coating to the MNPs. IGEPAL (0.225 g) was dissolved in cyclohexane (11 mL) and sonicated for 10 min. MNPs (0.5 mg) was then dispersed in the IGEPAL and cyclohexane mixture and stirred at room temperature for 10 min. TEOS (100 μL) and NH₄OH (25%, 100 μL) were added dropwise to the solution, and the mixture was stirred overnight. The product was purified by centrifugation at 15000 rpm for 10 min and re-dispersion in ethanol for 3 times to give SMNPs. To prepare the fluorescent magnetic nanoparticles, RITC-APTMS precursor was first prepared. Briefly, rhodamine isothiocyanate (RITC) (8.0 mg, 0.015 mM in ethanol) was stirred overnight with (3-aminopropyl)trimethoxysilane (APTMS) (5.2 μL, 0.03mM) in anhydrous ethanol (15.0 mL) at 42 °C to obtain the RITC-APTMS precursor. As-prepared RITC-APTMS precursor was then grafted to the magnetic nanoparticles through the reaction between APTES and hydroxyl groups on the SMNPs. The rest of the procedures were similar as described above for SMNP preparation. Similarly, FITC-SMNP was prepared.
5.2.4 Functionalization of SMNPs with NHS-PEG-Silane

To determine the mass of NHS-PEG-silane required to completely encapsulate the SMNPs, the surface area of the nanoparticles was calculated from the hydrodynamic radius and concentration of SMNPs and multiplied by the footprint of NHS-PEG-silane. This value was then multiplied by the volume of SMNPs to give the amount of NHS-PEG-silane required to functionalize the SMNPs. SMNPs were mixed with the calculated amount of NHS-PEG-silane, and the mixture was stirred overnight. The solution was purified by centrifugation at 15,000 rpm for 10 min and redispersion in ethanol. The purified NPs abbreviated as SMNP-PEG-NHS.

5.2.5 Conjugation of proteins with SMNP-PEG-NHS

The carbonyl groups present on the surface of functionalized SMNPs were used for conjugation with the amine groups of the various proteins. The proteins Con A, AAL WFL, BSA, and mycobacterial polyclonal antibody (pAb) were mixed with functionalized SMNPs and stirred overnight. The amount of each protein needed to conjugate 1mg/mL of functionalized SMNPs was calculated based on the hydrodynamic size and concentration of functionalized SMNPs. This value was multiplied by the volume of functionalized SMNPs to give the total protein required for conjugation of the entire batch of functionalized SMNPs. Finally, the solution mixtures were purified by centrifugation at 15,000 rpm for 10 min and redispersion in water. The purified final NPs abbreviated as SMNP-PEG-NHS- Con A or SMNP-Con A, SMNP-PEG-NHS- AAL or SMNP-AAL, SMNP-PEG-NHS- WFL or SMNP-WFL, and SMNP-PEG-NHS- BSA or SMNP-BSA, respectively.
5.2.6 **Morphology and size analysis by dynamic light scattering and electron microscopy**

The size and zeta potential of the nanoparticles (MNPs, SMNPs, SMNP-PEG-NHS, and protein conjugated SMNPs) were determined by DLS. TEM images of the nanoparticles were obtained at an accelerating voltage of 200 kV. To prepare samples for TEM, 200 mesh carbon thin film-coated Cu grids were drop-casted with a dilute solution of the nanoparticle and vacuum dried overnight.

5.2.7 **Fourier-transform infrared spectroscopy (FT-IR)**

Nanoparticle samples were prepared for ATR FT-IR by centrifugation and drying to remove the solvent. The successful synthesis of SMNPs, SMNP-PEG-NHS, and proteins conjugated SMNPs was then verified using ATR FT-IR.

5.2.8 **Thermogravimetric analysis**

TGA of the nanoparticles MNPs, SMNPs, SMNP-PEG-NHS, and SMNP-Con A was performed under argon (99.999%). Approximately 1 mg dried nanoparticles were heated at a rate of 5°C/min to 100°C and then held isothermal for 15 min, followed by heating at a rate of 5°C/min to 700°C. The MNPs loaded in silica shell was calculated by analyzing the weight loss difference between MNPs and SMNPs. Similarly, the ligand (NHS-PEG-Silane) content was analyzed by measuring the weight loss difference between SMNPs and SMNP-PEG-NHS. Finally, the amount of Con A was calculated by measuring the weight loss difference between SMNP-PEG-NHS, and SMNP-PEG-NHS-Con A.

5.2.9 **Bacterial culture**

*Mycobacterium smegmatis* (*Msmeg*) was grown in sterilized Middlebrook 7H9 medium supplemented with 10% Middlebrook ADC enrichment and 0.05% glycerol at
37°C while shaking at 150 rpm. Once an optical density (OD$_{650}$) of 2.0 was reached, the bacterial concentrations were determined by serial dilution and plating on Middlebrook 7H10 agar plates with Middlebrook OADC enrichment. *Streptococcus mutans* (*S. mutans*) was grown in BHI and plated on BHI agar following a similar protocol to *Msmeg*.

5.2.10 Hoechst staining

Once OD$_{650}$ of 2.0 was reached, *Msmeg* bacteria cells (2 mL, ~10$^9$ bacteria cells) were harvested, centrifuged at 5,000 rpm, and re-dispersed in 1 mL of HEPES buffer with Ca$^{+2}$ (0.1mM) and Mn$^{+2}$ (0.1mM). Then Hoechst dye (12.5 µL, 20µM) was added and incubated at 37°C for 1 h. To remove the excess dye, Hoechst-dyed bacteria were centrifuged at 5,000 rpm for 10 min and the supernatant was discarded. The pellet was re-dispersed in fresh HEPES buffer (5 mL) containing Ca$^{+2}$ (100 µL,0.1 mM) and Mn$^{+2}$ (100 µL, 0.1 mM).

5.2.11 Magnetic precipitation assay

SMNPs conjugated with proteins (Con A, AAL, WFL, BSA or polyclonal antibody) (90µL, 15 mg/mL) were mixed with Hoechst-dyed *Msmeg* (10 µL, ~10$^9$ CFU/mL) in a microcentrifuge tube and rubbed in between palms for a minute. The formation of a brown precipitate within less than a minute was observed. The magnetic precipitation was confirmed using a neodymium iron boron magnet (max. pull 69 lb). The same process was followed with the *S. mutans* bacteria. The precipitate from the magnetic precipitation assay was separated and smeared on a glass slide and observed using a fluorescence microscope. An aliquot of 400 µL fluorescent FITC-SMNPs-Con A was mixed with 400 µL of Hoechst-dyed *Msmeg* (~10$^9$ bacteria cells/mL) in HEPES with Ca$^{+2}$ (0.1mM) and Mn$^{+2}$ (0.1mM) and incubated at 37°C for 1 h while shaking at 180 rpm. Wet
and dried samples of the mixture of fluorescence-labeled bacteria and nanoparticles were observed under fluorescence microscopy to see the interactions between bacteria and nanoparticles.

5.2.12 Colony counting to determine the CFU/mL

After OD_{650} of 2.0 was obtained, a 100 μL aliquot of Msmeg suspension was taken and serially diluted 10^8-fold in OADC-enriched Middlebrook 7H9 broth. From the diluted solution, 50 μL was spread on OADC-enriched Middlebrook 7H10 agar plates. Colonies were counted after 48 h of incubation at 37°C. After multiplying the number of colonies by dilution factor it was reported as log colony-forming units (CFUs)/mL. A similar protocol was followed to count the colonies of S. mutans grown in BHI and plated on BHI agar.

5.2.13 Bacterial capture efficiency of SMNP-Con A

We have tested the bacterial capture efficiency of nano-assemblies at various concentration series. Hoechst-dyed Msmeg (10 μL, ~10^3 CFU/mL) bacterial suspension was incubated with different amounts (5, 10, 15, 25, 50, 75, and 100 mg/mL) of SMNP-Con A in a microcentrifuge tube and rubbed in between palms for a minute. The formation of a brown precipitate within less than a minute was observed. The magnetic precipitation was confirmed using a neodymium iron boron magnet. After magnetic separation by the nanoparticles, the supernatant was discarded. 100 μL of HEPES buffer was added to the microcentrifuge tube to dissolve the magnetic pellets stuck on the wall of the microcentrifuge tube, then sampled and analyzed for bacterial concentration via a plate counting method. The bacterial-capture efficiency of the NPs was tested by counting the number of CFU on the agar plates.
5.2.14 Ability of SMNP-Con A to detect lowest concentration of bacteria

SMNP-Con A (90µL, 15 mg/mL) was incubated with 1 mL of bacterial suspension at very low concentrations (10 and 10² CFU/mL). After magnetic separation by the nanoparticles, the supernatant was discarded. 100 µL of HEPES buffer was added to the microcentrifuge tube to dissolve the magnetic pellets stuck on the wall of the microcentrifuge tube, then sampled and analyzed for bacterial concentration via a plate counting method. The bacterial-capture efficiency of the NPs was tested by counting the number of CFU on the agar plates. To check bacteria-NPs interaction at the lowest concentration of bacteria 10 CFU/mL), precipitate from magnetic precipitation assay was separated and smeared on a glass slide and observed using a fluorescence microscope. Briefly, 400 µL fluorescent RITC-SMNPs-Con A was mixed with 400 µL of Hoechst-dyed Msmeq (~10⁹ bacteria cells/mL) in HEPES with Ca²⁺ (0.1mM) and Mn²⁺ (0.1mM) and incubated at 37°C for 1 h while shaking at 180 rpm. Wet and dried samples of the mixture of fluorescence-labeled bacteria and nanoparticles were observed under fluorescence microscopy to see the interactions between bacteria and nanoparticles.

Scheme 5.2. Stepwise synthesis of protein (Con A) conjugated SMNP.
5.3 Results and Discussion

5.3.1 Synthesis and characterization of nano-assemblies

The stepwise synthesis of lectin conjugated SMNPs was illustrated in Scheme 5.2. Firstly, MNPs were synthesized by the thermal decomposition method as reported by Sun et al.\textsuperscript{438} High-temperature reaction of iron(III) acetylacetonate (Fe(acac)\textsubscript{3}), with 1,2-hexadecanediol in the presence of oleic acid and oleylamine leads to monodisperse magnetite (Fe\textsubscript{3}O\textsubscript{4}) nanoparticles (MNPs). Fe(acac)\textsubscript{3} act as an ideal precursor due to high yields and low cost. 1,2- hexadecanediol, reacted well with Fe(acac)\textsubscript{3} to yield MNPs. Oleic acid and oleylamine are necessary for the formation of particles. The MNPs synthesis by thermal decomposition method controls the NPs size to get monodisperse MNPs with 5 to 10 nm in diameter. The uniform shape and size distribution of MNPs was analyzed by transmission electron microscopy (TEM) and showed an iron oxide core of about 10.2 ± 0.7 nm in diameter on average (Figure 5.1a and Table 5.3.1). The crystal lattice structure
of the MNPs could clearly be seen (Figure 5.1b), with the distance between planes being 3.5 Å. The size of the nanoparticles was further analyzed by DLS which was found to be 11.7 ± 2.3 nm (Table 5.3.1). The multi-core shell structure of the SMNPs was confirmed by high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) and energy-dispersive X-ray spectroscopy (EDS) (Appendix Figure D.1.). SEM analysis of SMNPs was also shown in Appendix Figure D.2.

Table 5.3.1. Characterization of nanomaterials by TEM and DLS

<table>
<thead>
<tr>
<th>Nanomaterials</th>
<th>TEM (nm)</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNP</td>
<td>10.2 ± 0.7</td>
<td>11.7 ± 2.3</td>
<td>NA</td>
</tr>
<tr>
<td>SMNP</td>
<td>35.4 ± 3.2</td>
<td>166.5 ± 7.2</td>
<td>-35.1 ± 0.7</td>
</tr>
<tr>
<td>SMNP-PEG-NHS</td>
<td>-</td>
<td>238.6 ± 13</td>
<td>-28.9 ± 1.4</td>
</tr>
<tr>
<td>SMNP-PEG-NHS-Con A</td>
<td>-</td>
<td>462.5 ± 12</td>
<td>-17.5 ± 0.7</td>
</tr>
</tbody>
</table>

Next magnetic nanoparticles were coated with silica by the reverse micelle method. Magnetic nanoparticles were previously coated with hydrophobic ligands and then dispersed in an organic media. The dispersion is then contacted with aqueous alkaline ammonium hydroxide solution containing the surfactant, IGEPAL with constant stirring, determining the microemulsion formation. In the final stage, a silica precursor, generally TEOS is added and allowed to react during a determinant period of time. The growth of the silica coating on the magnetic surface is regulated by experimental parameters such as TEOS concentration, amount of NH₄OH, IGEPAL concentration and MNPs concentration. The volume of TEOS precursor is an important factor that affected the thickness of the silica shell of superparamagnetic MNPs. When TEOS is added to the mixture, it converts to hydrolyzed TEOS adsorbed onto the superparamagnetic MNPs surface, followed by condensation reaction to form the silica shell. As shown in Appendix Table D.1., as the amount of TEOS increases (100 to 200 mL) the size of the
silica shell increases and hence increases in size. The NH$_4$OH content also affects the size of the silica shell. As shown in Appendix Table D.1 and Table D.2., the increase in the content of both TEOS and NH$_4$OH can increase the shell thickness of SMNPs, but a good match between these two components is essential to avoid the formation of core-free silica particles. It has been previously reported, IGEPAL self assembles into micelles in cyclohexane due to its hydrophilic groups.$^{441,443}$ The oleic acid that adsorbed on the surface of superparamagnetic MNPs was exchanged to IGEPAL in the cyclohexane. At volume concentration that is higher than the surfactant CMC (0.22 to 0.45 g), superparamagnetic SMNPs was well obtained with diameters of 182.8 ± 6 nm with 0.22 g of surfactant (Appendix Table D.3.). However, IGEPAL micelles could fuse at high concentrations, resulting in aggregated SMNPs. MNPs concentration also affects the formation of SMNPs. As shown in Appendix Figure D.3., as the amount of MNPs increases, the size of SMNP increases as more than one MNPs are covered with one silica shell and form multi-core silica nanoparticles. We have conducted a systematic study to understand the impact of each reagent on silica coating surrounding MNPs and from all of the above observations, we have selected, 0.02 mg of MNPs, 0.22 g of IGEPAL, 100 µL of NH$_4$OH, and TEOS were used for further study. As shown in the TEM image, SMNPs have a diameter of 35.4 ± 3.2 nm and indicated that multiple MNPs cores were encapsulated in the silica shells (Figure 5.1c). The multi-core shell structure of the SMNPs was confirmed by high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) and energy-dispersive X-ray spectroscopy (EDS) (Appendix Figure D.1). Furthermore, the size of the SMNP was analyzed by DLS (Table 5.3.1). The hydrodynamic diameter of SMNPs was determined to be 166.5 ± 7.2 nm.
NHS-PEG-Silane is a surface reactive PEG derivative that is used to modify, silica shell surface via the reaction between hydroxyl group and ethoxy silane.\textsuperscript{444} In addition to that, pegylation can greatly suppress the non-specific binding of charged molecules to the modified surfaces, provide stability and surface to further conjugation with biomolecules such as proteins, aptamers, and antibodies.\textsuperscript{445, 446} Finally, SMNP-NHS-PEG Silane was used for crosslinking between primary amines (NH\textsubscript{2}) in lectin. The N-hydroxysuccinimide ester (NHS) groups at SMNP-NHS-PEG Silane end react specifically and efficiently with N-terminal amino groups of lectins and form stable amide bonds.\textsuperscript{447} After functionalization with NHS-PEG-silane to form PEG-SMNP, the hydrodynamic diameter increased to 238.6 ± 13 nm. The conjugation of the protein Con A further increased the hydrodynamic diameter of the final nano-assembly to 462.5 ± 12 nm.

The zeta potential of the nanoparticles was also measured after the addition of each layer to monitor changes in surface charge. The zeta potential of MNPs could not be obtained due to their hydrophobicity, but the zeta potential of SMNPs was determined to be -35.1 ± 0.7 mV due to the presence of Si-OH groups. The surface electrical charge of nanomaterials also represents the stability of colloidal system.\textsuperscript{119} As shown in Appendix Table D.1, Table D.2, and Table D.3, the impact of varying concentrations of TEOS, IGEPAL, and NH\textsubscript{4}OH on silica shell formation as well as the stability of SMNP. After functionalization with NHS-PEG-silane, the nanoparticles showed a more positive zeta potential of -28.9 ± 1.4 mV, due to the addition of amine groups on the surface of PEG-SMNPs. Finally, the conjugation of proteins to the amine groups through NHS ester coupling chemistry further increased the zeta potential to -17.5 ± 0.7 mV.
To further confirm the successful synthesis of the nanoparticles, FTIR analysis was carried out after the addition of each layer. Characteristic peaks for the nanoparticles were identified after each step in the synthesis (Figure 5.2a). SMNPs showed characteristic peaks at 1045 cm\(^{-1}\) and 880 cm\(^{-1}\) due to the asymmetric stretching vibration of Si-O-Si bending and the asymmetric bending vibration of Si-OH bending respectively. After functionalization with NHS-PEG-Silane, the spectrum of SMNP-PEG-NHS continued to show the characteristic peaks for SMNPs and displayed additional peaks that are characteristic of PEG-NHS. Characteristic peaks of SMNP-PEG-NHS due to the functionalization with NHS-PEG-silane were observed at 1179 cm\(^{-1}\) and 1640 cm\(^{-1}\) corresponding to C-N and C=O stretching vibrations, respectively. The success of protein conjugation was confirmed with FTIR. Characteristic peaks of Con A were due to the presence of amides, which showed peaks between 1391 cm\(^{-1}\) and 1555 cm\(^{-1}\) due to N-H bending vibrations and between 1563 cm\(^{-1}\) and 1633 cm\(^{-1}\) due to C=O stretching vibrations. Similar spectra were observed for control proteins, which are displayed in Appendix Figure D.4. These peaks confirmed the presence of the protein on the surface of the nanoparticles.

Analysis of the ligand content of the nanoparticles was completed after each step of synthesis using TGA, as shown in Figure 5.2b. Percentage weight loss was calculated at each step to determine the amount of ligand conjugated to the nanoparticle. The percentage of weight loss between MNPs and SMNPs was found to be 2.55 ± 0.6%. Additionally, the percentage of weight loss between SMNPs and SMNP-PEG-NHS showed NHS-PEG-Silane ligand was determined to be 22.81 ± 1.5%. Similarly, the percentage weight loss
between the protein-conjugated nano-assemblies and SMNP-PEG-NHS was found to be 9.83 ± 0.8%.

5.3.2 Rapid bacterial detection by magnetic precipitation assay

The rapid detection of *Msmeg* bacteria was carried out by our developed magnetic precipitation assay as shown in Scheme 5.3. Briefly, SMNPs conjugated with proteins (Con A, AAL, WFL, BSA, or polyclonal antibody) with concentration (90μL, 15 mg/mL) were mixed with an aliquot of *Msmeg* spiked sputum (10 μL, ~10⁹ CFU/mL) and incubated at room temperature for 1 min. Subsequently, a magnet was used to capture the “magnetized” bacteria (magnetic nanoparticles bounded to the cell surface of *Msmeg*) onto the wall of the tube. As shown in Figure 5.3a and Figure 5.3b, the brown precipitates were observed in less than 1 minute after rubbing the eppendorf between the palm when SMNP-Con A or SMNP-AAL was used. The lectins, Con A and AAL recognize the mannose and arabinose
carbohydrate epitopes, found in the cell wall of the Mycobacteria.

Multivalent SMNP-Con A/AAL bind to the mycobacteria bacterial cell wall and crosslink the bacteria within minutes to form a visible precipitate without the need for any instrumentation. The ability of SMNP-Con A or SMNP-AAL to form a precipitate with mycobacteria was compared with SMNP conjugated with mycobacterial polyclonal antibody (SMNP-pAb). The brown precipitate observed with SMNP-pAb was similar to the precipitate observed with SMNP-ConA/AAL. Therefore the ability of Con A and AAL to detect mycobacteria is similar to polyclonal antibody (pAb). However, when Msmeg
was incubated with SMNP-WFL and SMNP-BSA, no precipitate was seen. (Figure 5.3d and Figure 5.3e). WFL was used as a positive control which has binding affinity towards GalNac carbohydrate residue which are absent on mycobacterial cell wall. BSA is used as a negative control. We have also conducted control experiment by incubating *Streptococcus mutans* bacteria with SMNP-Con A, as *S. mutans* species are abundantly found in oral mucosa. As shown in Figure 5.3f, no precipitate was observed when sputum was spiked with *S. mutans*. To observe the bacterial precipitate under fluorescence microscope, *Msmeg* was stained with Hoechst dye (blue) and SMNPs-ConA/AAL/pAb was fluorescently tagged to green fluorescent FITC dye. As shown in Figure 5.4, SMNP-
Con A/AAL/pAb, crosslinked to the Msmeg to form large fluorescent agglomerates.

5.3.3 Detection of low concentration of Msmeg bacteria

To further study the bacteria and SMNP-Con A interactions at various concentration series of SMNP-Con A, we incubated Msmeg at a constant concentration of
CFU/mL with various concentrations of SMNP-Con A ranging from 5 to 100 mg/mL. The SMNP-bound Msmeg were then magnetically captured, separated, and plated on agar plates. The magnetic capture efficiencies of bacteria by NPs are plotted as shown in Figure 5.5. The capture efficiency was calculated as shown in equation 5.1. At 5 mg/mL
of SMNP-ConA the capture efficiency was $11 \pm 1 \%$. The capture efficiency increased in a concentration-dependent manner as shown in Figure 5.5a. However, at SMNP-Con A of 5 and 10 mg/mL the magnetic precipitate was slight and was not discernible by eye. However after a threshold value of 15 mg/mL concentration of SMNP-Con A, the magnetic precipitates were sufficiently large enough for visual detection. At 15 mg/mL concentration the capture efficiency of bacteria was found to be $25 \pm 1 \%$. Additionally, as seen from the agar plates assays a dose-dependent increase of bacterial colonies with SMNP-Con A was
demonstrated in Figure 5.5b. The control was *Msmeg* \((10^3 \text{ CFU/mL})\) without incubation with SMNP-Con A. To obtain the lowest detection limit of *Msmeg* that can be detected by SMNP-Con A, we incubated SMNP-Con A (90\(\mu\)L, 15 mg/mL) with 1 mL of bacterial suspension at very low concentrations 100 and 10 CFU/mL of *Msmeg*. Then the captured *Msmeg* were plated on agar. As shown in Figure 5.6a, SMNP-Con A treated with 100 and 10 CFU/mL of *Msmeg* had capture efficiency 45 ± 3% and 11 ± 2%, respectively. Using SMNP-Con A at 15 mg/mL we could capture *Msmeg* even at 10 CFU/mL.

To further confirm the capturing ability of Con A, we incubated *Msmeg* (10 CFU/mL) with the control SMNP and SMNP-Con A (15 mg/mL). The *Msmeg* was stained with Hoechst dye (blue). SMNP-Con A and SMNP were tagged with red fluorescent RITC. As shown in Figure 5.7, SMNP-Con A, crosslinked the *Msmeg* and form large

Figure 5.7. Fluorescent image of *Msmeg* bacteria binding to RITC- SMNP-Con A, at lowest concentration of bacteria (10 CFU/mL), where NPs stained with RITC and *Msmeg* bacteria stained with Hoechst dye. Scale bar is 10 mm.
agglomerates that were visualized using a fluorescence microscope. However, when *Msmeg* was incubated with SMNP no agglomerates were seen.

### 5.4 Conclusion

In summary, we have developed a simple and rapid diagnostic, magnetic precipitation assay for TB. Con A conjugated SMNP bind to mannose carbohydrates which are prevalent in the bacterial cell wall of *Msmeg*. This binding would then cause the bacteria to crosslink followed by forming large agglomerates that appeared as a magnetic precipitate within 1 minute. This precipitate could therefore be used to determine the presence of mycobacteria sputum. SMNP-Con A can effectively detect as low as 10 CFU/mL of mycobacteria *Msmeg* even in presence of other bacterial species in the sputum matrix.
CHAPTER 6. CONCLUSION

The aim of this thesis is to engineer drug delivery nanomaterial-based platforms to target and eradicate intracellular bacterial infections. Nanotechnology has the capability to offer novel therapeutic approaches that can be used in conjunction with small-molecule therapies to reduce drug resistance in infectious diseases. In the thesis, we looked into two disease types; bacteria-based infectious diseases such as tuberculosis (TB) and lung carcinomas.Treating intracellular bacterial infections like TB is extremely difficult because mycobacteria develop strategies to evade the immune system and thus remain silent in the host.

Our multifaceted platform offers (1) small molecule drug therapy combined with photothermal therapy (PTT), (2) targeted drug delivery to the site of infection (3) drug release through external near-infrared laser light stimulation. This platform consists of gold nanorods (GNRs) as a core that acts as a photothermal agent. GNRs coated with mesoporous silica shell that offers drug loading surface. This is followed by thermo-sensitive liposome (TSL) encapsulation of drug-loaded nano-assembly, which prevents premature drug release and provides stability. Finally, mycobacteria targeting peptide NZX conjugated on liposome. Upon irradiation with NIR laser light, GNRs convert light into heat, causing melting of TSL, which induces the release of antibiotics. Mycobacteria
targeting peptide NZX guides the nano-assemblies to approach the bacterial cell surface. The drug encapsulated in nano-assemblies was 20-fold more efficacious than free drug equivalent.

Using another engineered nano-assembly: host-targeted nano-assemblies (HTNs), we were able to target intracellular mycobacteria (\textit{Msmeg}) residing within the macrophage. Intracellular mycobacteria are intelligent enough to alter the function of immune cells, making treatment of intracellular bacterial infection very challenging. Our engineered HTNs can increase the response of macrophages against intracellular mycobacteria by inducing the production of ROS/RNS and reactivating macrophages’ own host defense mechanisms. These events finally lead to the apoptosis of \textit{Msmeg} infected macrophages. HTN’s were capable of killing 99\% of intracellular \textit{Msmeg} residing within macrophages.

Carrying forth therapeutics, we have also engineered similar nano-assembly to deliver chemotherapeutics to lung carcinomas. A dual-targeted nano-assemblies combined therapeutic features: chemotherapy and PTT were designed to target non-small cell lung carcinoma (NSCLC). IR 780 photosensitizer was used additionally with GNR to enhance PTT using lower-powered laser irradiation. IR 780 dye was encapsulated inside a TSL, and the entire nano-assembly was conjugated with the dual tumor-targeting ligands GE-11 and folic acid. The encapsulated drug doxorubicin (Dox) was 20-fold times more efficacious than free Dox.

We have also developed a simple and rapid diagnostic magnetic precipitation assay to detect mycobacteria in sputum. \textit{Mycobacteria tuberculosis} (\textit{Mtb}) is the major causative pathogen in tuberculosis (TB). The rapid detection of \textit{Mtb} is crucial for TB treatment. We have utilized lectin-carbohydrate interaction to recognize specific carbohydrate epitopes
found abundantly in the cell wall of mycobacteria to design a detection technique. The lectin conjugated magnetic nanoparticles (SMNPs) were synthesized and conjugated plant lectins Con A and AAL, which bind to mannose and arabinose. Lectin conjugated SMNPs would bind to mycobacteria in sputum and crosslink to form large agglomerates. These agglomerates are magnetic and would be precipitated within minutes. Therefore, it could be used to determine mycobacteria presence in sputum, which can be taken as an indicator for TB detection. Even in the presence of other bacterial species in sputum, the lectin conjugated SMNP can detect mycobacteria Msmeg at a detection level as low as 10 CFU/mL.

As discussed above, our engineered nano-assemblies have improved drug efficacy by offering on-site delivery and combination therapy. Our rapid diagnostic platform also offers quick verification of pathogens in sputum. Nanotechnology has a lot to offer in theranostics, and we will look to continue to improve existing processes with nanotechnology.
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Figure A.1. Calibration curve of bedaquiline (BDQ) by HPLC.

\[ y = 84773.06x + 872.43 \]
\[ R^2 = 0.99954 \]
Figure A.2. Photographs of stability studies for GNR@MSNP and GNR@MSNP@TSL at 37 °C in mammalian cell culture media.
A.1 Laser triggered TSL melting

To support the laser triggered photothermal (PT) activity-based melting of TSL, a fluorescent dye (green, fluorescent calcein) release assay was designed to demonstrate the PT triggered melting of TSL. TSL-calcein was prepared by encapsulating calcein in TSL by thin-film method followed by extrusion. Similarly, GNR@MSNP@BDQ@TSL-calcein was prepared. The release of calcein was measured in proportion to the fluorescence intensity of the solution. TSL-calcein and GNR@MSNP@BDQ@TSL-calcein were exposed to NIR laser for 15 min, and the fluorescence intensity was quantified at 5 min intervals, and the temperature change was recorded. The percentage of calcein released from GNR@MSNP@BDQ@TSL-calcein with laser trigger was calculated using the following equation 1. Before laser irradiation, blank fluorescence ($I_{pre}$) was measured at an excitation wavelength of 490 nm, emission wavelength 515 nm. Fluorescence intensity ($I_{post}$) was measured after laser irradiation (808 nm, 15 min). The fluorescence intensity ($I_{100}$), due to a 100% release of calcein from TSL-calcein, was recorded by solubilizing TSL in detergent Triton-X100.

$$\% \text{ Calcein release} = \frac{I_{post} - I_{pre}}{I_{100} - I_{pre}} \times 100\%$$  \hspace{1cm} (1)

The melting of TSL was evaluated by calcein dye leakage assay, as shown in Appendix Figure A.3. The fluorescent intensity from GNR@MSNP@BDQ@TSL-calcein increased continuously after laser irradiation compared with TSL-calcein (Figure A.3.a). The PT effect of GNRs caused a rise in temperature up to $60 \pm 3 \, ^\circ C$ for 15 min., which helps permeate the TSL and increase the release of calcein
(Figure A.3.a and Figure A.3.b). Figure A.3.a shows the increase in calcein release upon an increase in laser irradiation time, and Figure A.3.b displays calcein intensity with the temperature change. When the temperature reaches and surpasses the melting point (48 ± 3 °C) of TSL, the maximum release of calcein occurs (Figure A.3.c). It is noteworthy that fluorescence intensity increased rapidly from a temperature range of 40 – 55 °C, where the range includes the melting point of TSL. The observed melting point of TSL was quantified to be 48 ± 3 °C. The percentage release of calcein was quantified, as shown in Figure A.3.c. Calcein release from TSL-calcein was low (2.5 ± 1.1%) when compared to GNR@MSNP@BDQ@TSL-calcein (70.11 ± 1.3%) after 15 min of laser irradiation. This release profile (Figure A.3.c) shows that 35% of calcein from 70% total release was between the temperature range of 40 – 55 °C. Since the measured melting point of TSL was 48 ± 3 °C, the higher calcein release can be positively correlated to the melting of the TSL. In addition, the continuous temperature increase in the system can be seen only in the presence of GNRs in GNR@MSNP@BDQ@TSL-calcein (Figure A.3.b), which confirms the PT effect of GNRs. From these observations, it is apparent that calcein release increased in the range of the melting point of TSL, which confirms the correlation of PT activity of GNRs with the subsequent melting of TSL.
Figure A.3. Change in calcein release from TSL-calcein and GNR@MSNP@BDQ@TSL-calcein. (a) Change in fluorescent intensity with laser irradiation times for TSL-calcein and GNR@MSNP@BDQ@TSL-calcein. (b) Change in fluorescence intensity with temperature in TSL-calcein and GNR@MSNP@BDQ@TSL-calcein. (c) Percentage release of calcein from TSL-calcein and GNR@MSNP@BDQ@TSL-calcein after irradiating with laser for 15min.
A.2 Morphological changes of *Msmeg* through TEM

The morphological changes of *Msmeg* after treatment with various nano-assemblies were observed through TEM. *Msmeg* (10^8 CFU/mL) was treated with GNR@MSNP@BDQ and GNR@MSNP@BDQ@TSL@NZX and exposed to NIR laser for 15 min, followed by incubation (37 °C, 4 h). Bacteria were pelleted and washed by centrifugation at 5000 rpm for 10 min and fixed with 2.5% glutaraldehyde for 4 h at 4 °C. After washing 3x with HEPES, the bacteria were dehydrated in a graded series of ethanol (30%–100% v/v) for 30 min and imaged through TEM operating at 200 kV. Figure A.4.a-a shows that laser irradiated GNR@MSNP@BDQ has minimal damage to the cell membrane of bacteria. In contrast, when laser irradiated GNR@MSNP@BDQ@TSL@NZX is treated with *Msmeg*, increased surface aggregation of GNR@MSNP@BDQ@TSL@NZX is seen on *Msmeg*. The surface aggregation compromises the outer cell membrane integrity and eventually complete degradation of *Msmeg* (Figure A.4.a-b, red arrows indicate degraded cells). The loss of outer membrane integrity is due to the physical aberrations caused by nano-assemblies pressing hard against the surface. NZX mediated adhesion is the probable cause for the high interaction between *Msmeg* and the GNR@MSNP@BDQ@TSL@NZX. These TEM studies show possible NZX peptide-mediated adhesion of GNR@MSNP@BDQ@TSL@NZX and high antibacterial activity upon NIR laser exposure (Figure A.4.a-b). The schematic illustration in Figure A.4.b shows NZX mediated adhesion of GNR@MSNP@BDQ@TSL@NZX on *Msmeg* surface (pink block), PT activity,
and disintegration of TSL upon NIR irradiation (red block) and BDQ release and lysis of *Msmeg* (grey block).

Figure A.4. Morphological study of bacteria after treatment with various nano-assemblies upon laser exposure. (a) Morphological changes observed under TEM, The scale bars are 1 μm. (b) Schematic illustrated the mechanism of action of final nano-assembly GNR@MSNP@BDQ@TSL@NZX upon interacting with *Msmeg*.
A.3 Cell cytotoxicity assay conducted on host A549 cells.

The live-dead assay was conducted on lung cells treated with GNR@MSNP, GNR@MSNP@BDQ, and GNR@MSNP@BDQ@TSL@NZX after laser irradiation. A549 cells were seeded onto 24 well plates at $1 \times 10^3$ cells/well (1 mL per well). After reaching 90% confluence, cells were treated with (0.5 mg/mL) of GNR@MSNP@BDQ and final nano-assembly GNR@MSNP@BDQ@TSL@NZX for 18 h at 37 °C. Treated cells were exposed to NIR laser 808 nm, 500 mW for 15 min. A549 cells treated with nano-assemblies were stained with SYTO 9 and propidium iodide (PI) for 30 min at 37°C. Finally, the live and dead cells were examined under an inverted fluorescence microscope.

The live-dead assay consists of two fluorescent dyes (propidium iodide and SYTO 9) that differentially label live (green) and dead (red) cells. Due to the compromised plasma membrane of the dead cell, propidium iodide (PI) intake increases, which subsequently binds to the nucleic acids, resulting in red fluorescence. SYTO 9 dye labels intact plasma membrane; hence viable cells appear in green fluorescence. As shown in Figure A.5, lung cells incubated with GNR@MSNP@BDQ and GNR@MSNP@BDQ@TSL@NZX even after laser irradiation appears to be live (green fluorescent). A live-dead assay makes it apparent that laser irradiation has minimal cytotoxic effects on the lung cells.
Figure A.5. Fluorescence microscopy images of the live/dead assay. Live/dead assay performed on A549 cells treated with different nano-assemblies for 18 h with laser exposure. Scale bar: 50 μm.
APPENDIX B

B.1 β-Glucan concentration determination

The calibration curve for β-Glucan was carried out, the various concentration of free β-Glucan (5-50 nM ~ 1-10 ug/mL) interacted with Congo red 5 µg/mL (Congo red used to tag β-Glucan) for 30 minutes at room temperature with constant stirring. The absorbance comes from Congo red itself at 536 nm which is subtracted from the test samples (β-Glucan tagged with Congo red). Then absorbance was recorded at 546 nm as the Congo red-tagged β-Glucan had abs at 546 nm (verified it from Congo red assay), Free ligand (β-Glucan) concentration vs absorbance was plotted as shown in Figure B.1.a. Similarly, a calibration curve for folic acid (FA) was carried out, the various concentration of free FA 5-50 mM ~ 2-20 mg/mL was prepared. The calibration curve was prepared by recording the absorbance value at 310 nm using UV-Vis spectroscopy, as shown in Figure B.1.b.

![Figure B.1. Calibration curve for (a) β-Glucan and (b) FA.](image)
B.2 Determination of dectin-1 receptor concentration

Jiang et al. have studied the binding affinity of β-Glucan toward dectin-1 receptor and explored their binding sites. They found that the binding of β-Glucan ligand and dectin-1 receptors is ratiometrically 1:1 and a calculated $K_D$ of 0.003 μM. The binding is therefore monovalent. The concentration of receptor [R] was also calculated from the standard calibration curve, as shown in Figure B.2. The standard calibration curve was prepared by varying the concentration of targeting ligands and recording the absorbance at 546 nm. Free ligand concentration was displayed on the x-axis, while ligand-receptor absorbance was plotted on the y-axis. As observed in Figure B.2.a, binding saturation was achieved at 25 nM concentration of free, β-Glucan. Since binding is monovalent, the receptors' concertation is approximated to be 25 nM. The amount of β-Glucan moles was calculated as follows and was found to be $3 \times 10^{-11}$ moles. The molecular weight of β-Glucan $2 \times 10^5$ gmol$^{-1}$. The amount of receptors was calculated as following and was calculated to be $1.8 \times 10^{11}$ molecules of dectin-1 receptors.

$$25 \text{nM} = 6 \mu\text{g} \beta-\text{Glucan}$$

$$= 6 \mu\text{g}/2 \times 10^{11} \text{gmol}^{-1}$$

$$= 3 \times 10^{11} \text{moles}$$

$$= 3 \times 10^{11} \text{moles} \times 6.022 \times 10^{23}$$

$$= 1.8 \times 10^{11} \text{molecules of } \beta-\text{Glucan; hence dectin-1 receptors}$$

Each well was seeded with $10^4$ cells. Therefore in 1 cell, $1.8 \times 10^7$ molecules of dectin-1 receptors can be approximated. Similarly, we calculated the number of folate receptors on the macrophage, and it was found to be $2.3 \times 10^{15}$ molecules of folate receptors.
Figure B.2. Saturation curve for (a) β-Glucan and (b) FA.

Figure B.3. Calibration curve for BQ by RP-HPLC.
Scheme B.2. Folic acid conjugation on liposome by EDAC chemistry.
B.3 Calculation of ligand density by TGA.

Ligand density ($\rho_L$) is defined as the amount (mol) of ligand per unit area (nm$^2$) of nano-assemblies.

$$\rho_L = \frac{n_p}{A}$$  \hspace{1cm} (1)

Where $n_p$ is the amount of ligand on one (GM)L (gold nanorod coated mesoporous silica shell encapsulated inside liposome) and A is the surface area of one (GM)L. Since (GM)L were ellipsoid in shapes due to the oblong shape of gold nanorods. The mean length (a), width(b), and depth(c) of (GM)L ellipsoid shaped nano-assembly was found to be 46, 31, and 30 nm, respectively, and were calculated from analyzing TEM images using ImageJ software. Hence, surface area (A) of ellipsoid shape (GM)L was found to be 15803 nm$^2$ calculated using equation 2.

$$\text{Surface area (A)} = 4\pi\left(\frac{(ab)^{1.6}(ac)^{1.6}(bc)^{1.6}}{3}\right)^{1.6}$$  \hspace{1cm} (2)

The volume of ellipsoid shape (GM)L was $1.79 \times 10^5$ nm$^3$ and was calculated using equation 3.

$$\text{Volume (V)} = \frac{4}{3} \pi abc$$  \hspace{1cm} (3)

The number of (GM)L nano-assemblies (N) in 1 mg of (GM)L was calculated using equation 4.

$$N = \frac{1}{\rho_p \times V}$$  \hspace{1cm} (4)
As reported in literature, the density of gold nanoparticles is 19.7 g/cm³. Hence, the density of (GM)L (ρP) is 19.7 g/cm³ and N was calculated by using volume (V) from equation 3.

Using TGA, the ligand (β-Glucan) concentration C_L (mol per mg) of (GM)L-Glu or (GM)L-FA can be determined from the following equation 5.

\[
C_L = \frac{(P_b - P_a)}{P_a \times M_w}
\]  

(5)

The P is the percent weight (%) of the (GM)L before (P_b) and after (P_a) ligand coupling at 700 °C. The M_w is the molecular weight of the organic residue (HEPES buffer) of the particles ~ 283.3 g/mol.

Then, the amount of ligand (β-Glucan) (mol) on one (GM)L (n_P) is found to be 0.0848 × 10^{-16} by combining equations 4 and 5.

\[
n_P = \frac{C_L}{N}
\]  

(6)

Ligand density (ρ_L) was calculated by using equations 2 and 6.

\[
ρ_L = \frac{n_P}{A}
\]  

(7)

The ligand density of β-Glucan on (GM)L was found to be 53.7 nmol/nm². Similarly, we have calculated ligand density of FA on (GM)L was found to be 12.9 nmol/nm².
B.4 Calculation of β-Glucan ligand density by phenol sulphuric acid assay.

Calibration curves were obtained for β-Glucan where various concentrations (10-100 nM) of β-Glucan in 1 mL of DMSO were placed in boiling tubes. Add 1 mL of 2.5 N HCL and hydrolyzed glucan by keeping it in a boiling water bath for 1 h. The mixture was cooled at room temperature and neutralized with sodium carbonate until effervescence ceased. Then centrifuged it at 15000 rpm for 10 min and collected the supernatant. 1 mL of supernatant was added in 24 well plates, following that 100 μL of 5% phenol solution and 500 μL of 96% H2SO4 were added. Then incubate this at room temperature for 30 min. Finally, UV-Vis spectra of the resulting solutions were recorded at 490 nm, and the data were plotted against the β-Glucan concentration.

During conjugation process, β-Glucan ligand 200 nM ~ 64 μg was added with (GM)L (0.5 mg). After purification and performing assay, we got 96.6 nM ~ 31 μg of β-Glucan ligand was obtained from the calibration curve of the assay (Figure B.5.). This
indicating that 0.5 mg of (GM)L have 96.6 nM ~ 31 μg of β-Glucan ligand. The molar concentration of ligand was calculated using equation 1.

\[
C = \frac{m}{V} \times \frac{1}{M_w}
\]  

(1)

Where \( C \) is the molar concentration (96.6 nM), \( V \) is the total volume 1.6 mL, \( M_w \) is the molecular weight of β-Glucan 2 x 10^5 gmol^{-1}, \( m \) is the mass of β-Glucan 31 μg.

**Calculation of the mass of one (GM)L-Glu**

\[
Density = \frac{Mass of \ (GM)L-Glu}{Volume of \ (GM)L-Glu}
\]  

(2)

Mass of (GM)L-Glu = 19 g cm^{-3} \times 1.79 \times 10^5 \ nm^3

= 35.54 \times 10^{-16} \ g

The density of (GM)L-Glu was approximated to be close to the density of GNR. From the literature, the value is 19.3 g cm^{-3}. The volume of ellipsoid shape (GM)L was 1.79 \times 10^5 \ nm^3. Therefore, the mass of one (GM)L-Glu was 34.54 \times 10^{-16} \ g.

**Total number of (GM)L-Glu**

\[
Number of \ (GM)L-Glu = \frac{Total \ mass \ of \ (GM)L-Glu}{Mass \ of \ one \ (GM)L-Glu}
\]  

(3)

\[
= \frac{0.5 \times 10^{-3} \ g}{34.54 \times 10^{-16} \ g}
\]

= 1447 \times 10^8

Therefore, 1.4 \times 10^{11} (GM)L-Glu are present in 0.5 mg mass of (GM)L-Glu.
Total number of β-glucan

\[ \text{Total number of } \beta - \text{glucan} \]

\[ = \frac{\text{Mass of } \beta - \text{glucan in } (GM)L - \text{Glu}}{M_w \text{ of } (GM)L - \text{Glu}} \times \text{Avogadro constant} \]

\[ = \frac{31 \times 10^{-6} \text{ g}}{2 \times 10^8 \text{ g mol}^{-1}} \times 6.022 \times 10^{23} \text{ mol}^{-1} \]

\[ = 9334.1 \times 10^{10} \]

Therefore, \(9.3 \times 10^{13}\) molecules of β-glucan are present.

Number of β-glucan per (GM)L-Glu

\[ \text{Number of } \beta - \text{glucan per } (GM)L - \text{Glu} = \frac{\text{Total number of } \beta - \text{glucan}}{\text{Total number of } (GM)L - \text{Glu}} \]

\[ = \frac{9334.1 \times 10^{10}}{1447 \times 10^8} \]

\[ = 645 \]

Therefore, 645 number of β-glucan per (GM)L-Glu.
Figure B.5. Typical nitrite standard calibration curve.
Figure B.6. Characterization of various nano-assemblies (a) zeta potential, (b) UV-Vis, (c) FT-IR analysis.
Figure B.7. Concentration dependent photothermal activity of (GM@BQ)L-Glu using 808 nm NIR laser, 500 mW, 15 min.
APPENDIX C

Figure C.1. Standard calibration curve of Doxorubicin.

Figure C.2. Standard calibration curve of IR 780.
Figure C.3. Standard calibration curve of Folic acid.

Figure C.4. Standard calibration curve of GE-11.
Table C.1. BET analysis of nano-assemblies

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<tr>
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<th>GM</th>
<th>GM@Dox</th>
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<tr>
<td>Surface area (Multipoint BET)</td>
<td>53.852 m²/g</td>
<td>42.060 m²/g</td>
</tr>
<tr>
<td>Pore volume (BJH method)</td>
<td>0.23 cc/g</td>
<td>0.171 cc/g</td>
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<tr>
<td>Pore size (BJH method)</td>
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<td>12.19 Å</td>
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Figure C.5. Colloidal stability analysis of GM@Dox and [(GM@Dox) LI]-PF. UV-vis spectra of (A) GM@Dox (B) [(GM@Dox) LI]-PF at 37 °C, in cell culture media. (C) Aggregation index (AI) of GM@Dox and [(GM@Dox) LI]-PF. (D) Zeta potential of GM@Dox and [(GM@Dox) LI]-PF in cell culture media at 37 °C.
Figure C.6. Cumulative drug release profile of Dox for [(GM@Dox) L] and [(GM@Dox) LI] nano-assemblies with laser irradiation (808 nm, 350 mW, 15 min).
Cell uptake mechanism.

The primary mechanism by which nanoparticles are transported into cells is endocytosis. At least four fundamental pathways are involved in endocytosis: caveolae-mediated endocytosis, clathrin-mediated endocytosis, macropinocytosis, and clathrin- and caveolae-independent endocytosis. Clathrin-mediated endocytosis is well-known for its function in the selective ingestion of molecules via unique receptors by all eukaryotic cells to internalize nutrients and degrade or recycle substances. Specific endocytosis inhibitors: chlorpromazine (an inhibitor of clathrin-mediated endocytosis), nystatin (an inhibitor for caveolin-dependent endocytosis), and wortmannin (a macropinocytosis inhibitor) were investigated to study the mechanism of cellular uptake. The mechanism for the internalization of the nano-assemblies into the lung cells was monitored by fluorescence microscopy (Figure C.7.a). The cellular uptake mechanism of RITC - GM@Dox and RITC – [(GM@Dox) LI]-PF in A549 cells was determined under different endocytosis-inhibited conditions. We have also quantified the cellular uptake of nano-assemblies using fluorescent assays. The fluorescence microscopy images reveal little or no visible red fluorescent RITC – [(GM@Dox) LI]-PF in chlorpromazine treated A549 cells (Figure C.7.b), which indicates chlorpromazine treated lung cells shows inhibited uptake of RITC – [(GM@Dox) LI]-PF. However, uptake of RITC – [(GM@Dox) LI]-PF into lung cells was not hindered when treated with wortmannin and nystatin (Figure C.7.b). These observations suggested that the trafficking of the nano-assemblies occurred preferably through a clathrin-mediated endocytosis.
To investigate the role of the liposome in cellular uptake, we quantified the cellular uptake of RITC-labelled nano-assemblies GM@Dox and [(GM@Dox) LI]-PF by measuring the intracellular fluorescence intensity using a fluorescence microplate reader. Liposome coating enhances the cellular uptake of nano-assemblies due to interaction with the lipophilic cell membrane that allows cellular entry of nanoparticles by various endocytosis pathways. In addition, the liposome was modified with the cationic peptide GE-11, so due to electrostatic interactions with the negatively charged cell membrane, the cellular uptake was higher for [(GM@Dox) LI]-PF as compared to GM@Dox. As shown in Figure C.7.b, chlorpromazine, an inhibitor of clathrin-mediated endocytosis, significantly inhibited both GM@Dox and [(GM@Dox) LI]-PF delivery into A549 cells. The quantified intracellular uptake of GM@Dox and [(GM@Dox) LI]-PF was 8.1 ± 1.2 % and 27.01 ± 1.5 %, respectively. Even in the presence of the inhibitor, a higher amount of [(GM@Dox) LI]-PF uptake was found in A549 cells, which indicates the inclusion of the liposome and the cationic peptide (GE-11) increases the ability of the cells to internalize the nano-assembly. GM@Dox has consistently shown a lesser amount of uptake (Figure C.7.b). These studies collectively suggested that the nano-assembly-mediated drug delivery occurred preferably through clathrin-mediated endocytosis, and nano-assembly internalization was enhanced due to the presence of the liposome and GE-11 cationic peptide.
Figure C.7. Mechanism of cellular uptake of nano-assemblies. (a) Evaluation of cell uptake mechanism of nano-assemblies by A549 cells under different endocytosis inhibitory conditions - fluorescence microscopy – A549 cells are stained with calcein dye (green), cell nucleus stained with DAPI (blue), [(GM@Dox) LI]-PF were labelled with red fluorescent RITC dye (red). Scale bar: 10 μm. (b) Shows quantitative intracellular uptake of GNR@MSNP@BDQ and GNR@MSNP@BDQ@TSL@NZX by A549 cells after inhibitor treatment.
Figure D.1. HAADF-STEM images and EDS spectra of SMNPs.
Figure D.2. SEM image of SMNP.
Table D.1. Parameters for fabricating SMNPs with different TEOS amounts

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<th>MNPs (mg)</th>
<th>IGEPAL (g)</th>
<th>Ammonia (µL)</th>
<th>TEOS (µL)</th>
<th>SIZE (nm)</th>
<th>Zeta (mV)</th>
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Table D.2. Parameters for fabricating SMNPs with different ammonia amounts

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<th>TEOS (µL)</th>
<th>SIZE (nm)</th>
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Table D.3. Parameters for fabricating SMNPs with different IGEPAL amounts

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<th>TEOS (µL)</th>
<th>SIZE (nm)</th>
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<td>100</td>
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Figure D.3. Fabricating SMNPs with varying concentrations of MNPs
Figure D.4. FTIR spectra of nanoparticles conjugated with control proteins.