Multifunctional Glyconanomaterials: Applications in Biorecognition and Drug Delivery

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献给我的家人

To my family

Abstract

This thesis consists of three parts, which revolve around the fabrication of multifunctional glyconanomaterials (cellulose nanocrystals, chitin nanocrystals, mesoporous silica nanoparticles) and the development of their applications in biorecognition and nanomedicine.

Part one presents a detailed introduction to glyconanomaterials, focusing on cellulose nanocrystals, chitin nanocrystals and mesoporous silica nanoparticles, including their general preparations, properties and applications.

Part two demonstrates dually modified cellulose and chitin nanocrystals. They are prepared by TEMPO-mediated oxidation, followed by conjugation with a fluorescent dye and carbohydrate ligands. The two functional nanocrystals are applied in carbohydrate-lectin recognition and bacterial imaging as new types of glyconanomaterials.

Part three describes two types of drug delivery systems based on carbohydrate-conjugated mesoporous silica nanoparticles: the first type is a trehalose-functionalized nanoparticle which can selectively recognize mycobacteria, release antimicrobial drugs and kill them; the second type is a lectin-gated drug container, which can perform controlled delivery of drugs to cancer cells in response to glutathione.

Keywords: glyconanomaterials, cellulose nanocrystals, chitin nanocrystals, mesoporous silica nanoparticles, lectin, biorecognition, drug delivery, bacteria, cytotoxicity, perfluorophenylazide, fluorescent dye, carbohydrate ligands.
This thesis is based on the following papers, referred to in the text by their Roman numerals I-VI:

I. **Synthesis of Multifunctional Cellulose Nanocrystals for Lectin Recognition and Bacterial Imaging**
   Juan Zhou, Núria Butchosa, H. Surangi N. Jayawardena, JaeHyeung Park, Qi Zhou, Mingdi Yan and Olof Ramström

II. **Glycan-Functionalized Fluorescent Chitin Nanocrystals for Biorecognition Applications**
    Juan Zhou*†, Núria Butchosa*†, H. Surangi N. Jayawardena, Qi Zhou, Olof Ramström and Mingdi Yan
    *† These authors contributed equally to this work

III. **Quantitative Fluorine NMR (**$^{19}$**F qNMR) to Determine Carbohydrate Density on Glyconanomaterials Synthesized from Perfluorophenyl Azide-Functionalized Silica Nanoparticles by Click Reaction**
    Na Kong, Juan Zhou, JaeHyeung Park, Sheng Xie, Olof Ramström and Mingdi Yan
    *Submitted for publication.*

IV. **Metal-Free Carbohydrate Immobilization on Nanoparticles Using Perfluorophenyl Azide-Based Azide-Aldehyde-Amine CycloadDITION**
    Na Kong, Sheng Xie, Juan Zhou, JaeHyeung Park, Olof Ramström and Mingdi Yan
    *Manuscript.*

V. **Trehalose-Conjugated Mesoporous Silica Nanoparticles for Efficient Delivery of Isoniazid into Mycobacteria**
    Juan Zhou, Kalana Jayawardana, Na Kong, Yansong Ren, Nanjing Hao, Mingdi Yan and Olof Ramström
    *Submitted for publication*

VI. **Lectin-Gated, Mesoporous, Photofunctionalized Glyconanoparticles for Glutathione-Responsive Drug Delivery**
    Juan Zhou, Nanjing Hao, Thareendra De Zoyza, Mingdi Yan and Olof Ramström
Papers not included in this thesis:

**VII. A Three-Component one-pot Azide-Amine-Aldehyde Cycloaddition for Surface and Nanomaterials Functionalization**
Sheng Xie, Juan Zhou, Xuan Chen, Na Kong, Olof Ramström and Mingdi Yan
*Manuscript.*

**VIII. Ciprofloxacin Derivatives with Pentafluorophenyl-Phenyl Moieties Displaying Aggregation-Induced Emission and Enhanced Antibacterial Activity**
Sheng Xie, Sesha Manuguri, Nanjing Hao, Yang Zhang, Andreas Fischer, Juan Zhou, Olof Ramström and Mingdi Yan
*Manuscript.*

**IX. Determination of Binding Affinity of Glyconanomaterials**
Juan Zhou, Sheng Xie, Kitjanit Neranon, Yang Zhang, Mingdi Yan and Olof Ramström
*Manuscript.*
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<tbody>
<tr>
<td>Ac</td>
<td>Acetyl</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BET</td>
<td>Brunauer Emmett Teller</td>
</tr>
<tr>
<td>BJH</td>
<td>Barrett Joyner Halenda</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>ChNCs</td>
<td>Chitin nanocrystals</td>
</tr>
<tr>
<td>CI</td>
<td>Crystalline index</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>CNCs</td>
<td>Cellulose nanocrystals</td>
</tr>
<tr>
<td>CNF</td>
<td>Cellulose nanofiber</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>DA</td>
<td>Degree of acetylation</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DP</td>
<td>Degree of polymerization</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>Et</td>
<td>Ethyl</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HPMOs</td>
<td>Hollow periodic mesoporous organosilicas</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>INH</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>Kₐ</td>
<td>Association constant</td>
</tr>
<tr>
<td>LCST</td>
<td>Lower critical solution temperature</td>
</tr>
</tbody>
</table>
min  minutes
MSNs  Mesoporous silica nanoparticles
MCM-41  Mobil composition of matter No. 41
MCC  Microcrystalline cellulose
NHS  N-hydroxysuccinimide
NMR  Nuclear magnetic resonance
PDB  Protein data bank
PEG  Polyethylene glycol
PMC  Polyelectrolyte macroion complex
PNIPAM  Poly(N-isopropylacrylamide)
PLE  Porcine liver esterase
PFPA  Perfluorophenylazide
PBS  Phosphate-buffered saline
PCC  Primary lung epithelial cell
QDs  Quantum dots
RBITC  Rhodamine isothiocyanate
RCA-120  *Ricinus communis* Agglutinin 120
STEM  Scanning transmission electron microscopy
SBA  Soybean agglutinin
TEMPO  2,2,6,6-tetramethylpiperidine-oxyl
TEOS  Tetraethyl orthosilicate
TMOS  Tetramethyl orthosilicate
TEM  Transmission electron microscopy
TGA  Thermogravimetric analysis
UV-Vis  Ultraviolet-visible
XRD  X-ray diffraction
α-CD  α-Cyclodextrin
1. Introduction

1.1. Glyconanomaterials

1.1.1. Carbohydrate-mediated recognition

Carbohydrates (also expressed as saccharides or sugars) are a large and diverse class of compounds and have a significant impact in chemistry, materials science, biology, and related fields.\(^1\) In terms of biological systems, carbohydrate-mediated recognitions play important roles.\(^2\) They are the communication media between cell and cell, cell and microbe, cell and protein (Figure 1). These recognitions are involved in many pathological changes including cancer growth and metastasis.\(^3\) Therefore, the study of carbohydrate-mediated recognition is essential for understanding these physiological processes as well as for further developing effective therapeutic and diagnostic tools for diseases.

![Figure 1. Illustration of carbohydrate-mediated recognition at the cell surface.](image)

Carbohydrate-binding proteins, lectins, have been utilized to study carbohydrate-mediated interactions. They can specifically bind mono- or oligosaccharides by hydrogen bonds or hydrophobic effects.\(^4\) Generally, lectins possess two or more carbohydrate binding sites, which can be cross-linked by carbohydrates. For example, Concanavalin A (Con A) is a tetramer above pH 5.5, containing four binding sites towards α-D-mannosyl residues (Figure 2).\(^5\) It
can primarily bind to $\alpha$-D-mannopyranosides with high selectivity in the presence of Ca$^{2+}$ and Mn$^{2+}$ ions, but with relatively low affinity ($K_a$ for D-mannopyranoside is $8.2 \times 10^3$ M$^{-1}$).\textsuperscript{6}

\textbf{Figure 2. Illustration of cross binding between Con A and mannose epitope (PDB id 3CNA)}\textsuperscript{5}.

The affinities can however be increased through multivalency, often leading to considerably stronger binding than the corresponding monovalent carbohydrate-protein interactions. The multivalency can be achieved by using solid scaffolds, because these scaffolds are able to provide platforms for presenting many copies of carbohydrate ligands. Nanomaterials as multivalent scaffolds for carbohydrate conjugation have been developed and the resulting glyconanomaterials have been used as new tools to analyze carbohydrate-lectin recognition and other biological activities.

\textbf{1.1.2. Properties of glyconanomaterials}

When using nanomaterials as scaffolds for carbohydrate display, the resulting products are called glyconanomaterials.\textsuperscript{7} The most common nanomaterials to display carbohydrates include micelles, carbon nanotubes, gold nanoparticles, quantum dots, polymer and silica nanoparticles (Figure 3A).\textsuperscript{8-12} Glyconanomaterials have attracted a great deal of attention. The physical and chemical properties of these nanomaterials contribute to their developments and applications in nanomedicine including drug delivery, bioimaging, biorecognition and biosensoring (Figure 3B).\textsuperscript{13-15} For example, silica glyconanoparticles have been used in several biomedical applications owing to their functionalities and biocompabilities. Wang et al. synthesized FITC doped silica nanoparticles conjugating with mono- or oligosaccharide \textit{via} photoirradiation. These silica glyconanoparticles were successfully used as fluorescence probes to detect and image bacteria as well as to determine the
carbohydrate-lectin interaction.\textsuperscript{16} In terms of drug delivery application, multifunctionalized CdSe/CdS quantum dots with high fluorescence were reported.\textsuperscript{17} The nanomaterials achieved high biocompatibility through PEG conjugation, and when covered by a galactose shell, allowed specific GLUT-1 recognition. Controlled attachment of dopamine through an ester bond also allowed hydrolysis by esterases, yielding a smart nanotool for specific biolabeling and controlled drug release.

\textbf{Figure 3.} (A) Representative scaffolds of glyconanomaterials. (B) Applications of glyconanomaterials in nanomedicine.

\subsection*{1.1.3. Preparation of glyconanomaterials}

There are two general protocols of conjugating carbohydrates onto nanomaterials: noncovalent- and covalent conjugation.\textsuperscript{7, 18} Normally, noncovalent attachment can be achieved \textit{via} hydrogen bonds and hydrophobic effects. For example, Chen et al. successfully coated carbon nanotubes with \textit{N}-acetylgalactosamine, and then decorated them onto polymethylvinylketone backbone using hydrophobic effects.\textsuperscript{19} Covalent binding is the most commonly used method to attach carbohydrate onto nanomaterials, because it can form a relatively more stable conjugation than noncovalent attachment.\textsuperscript{10} Yan and coworkers developed a simple method for immobilizing free carbohydrate onto nanomaterials using functionalized perfluorophenylazide (PFPA) (Figure 4). The azide group in PFPA can be converted to a reactive singlet nitrene that most significantly inserts into C-H bonds upon the activation with UV light, creating stable covalent linkages. Free carbohydrates are excellent substrates for this photocoupling. Wang et al. successfully attached mono-, oligo-, poly-
saccharides onto PFPA-thiol functionalized gold nanoparticles via this photocoupling method. 16, 20-22

![Photocoupling Diagram]

**Figure 4. Immobilization of un-derivatized carbohydrates onto nanomaterials using PFPA.**

Of the nanomaterial scaffolds, biocompatible nanomaterials are excellent platforms, and when functionalized with carbohydrates, have the potential to extend the utilities of glyconanomaterials for in vivo applications such as imaging, diagnosis, and therapeutics. In this thesis, cellulose nanocrystals, chitin nanocrystals and mesoporous silica nanoparticles have been employed as multivalent scaffolds for carbohydrates. In the following sections, they will be introduced in detail.

1.2. Cellulose nanocrystals (CNCs)

Cellulose is the most abundant renewable polymeric material on earth and a key constituent of all plants as well as various organisms. 23 It is a polymer of β-1,4-linked D-glucose, with cellobiose as the repeat unit (Figure 5). It has one reducing end of a hemiacetal and one nonreducing end with pendant hydroxyl groups. 24 Since the first separation and description of cellulose by Anselme Payen in 1838, 25 its structural features, chemical-physical properties and biosynthetic pathways have been extensively investigated. 26-27

![Cellulose Structure Diagram]

**Figure 5. Chemical structure of cellulose.**

Approximately 100 years later, colloidal suspensions of cellulose were prepared using sulfuric acid-catalyzed degradation of cellulose fibers. 28 These suspensions contained needle-shaped structures by TEM. Electron diffraction
analysis revealed that these particles were crystalline, which was consistent with the starting materials. Simultaneously, microcrystalline cellulose (MCC), discovered by Battista and coworkers, was synthesized through degradation of cellulose fibers with hydrochloric acid and ultrasonication.\textsuperscript{29-30} CNCs can be extracted from these MCC after optimization of acid hydrolysis and other mechanical and chemical treatments.\textsuperscript{31} CNCs are often referred to as nanowhiskers, nanoparticles, nanofibers, etc., because of their nanoscale dimensions.\textsuperscript{24, 32-33}

\subsection*{1.2.1. Preparation of CNCs}
Acid hydrolysis is a typical method of isolating CNCs from cellulosic materials.\textsuperscript{34} Sulfuric, hydrochloric, phosphoric and hydrobromic acid are normally used in hydrolysis protocols, depending on the source of cellulose materials.\textsuperscript{35-38} The procedures consist of subjecting pure cellulose fibers to the acid with strict control of temperature, reaction time and agitation; subsequently, the suspensions are diluted with water and washed by continuous centrifugation. Finally, CNCs are obtained after removing excess acid from the suspensions by dialysis.

The dimension, distributions and morphologies of CNCs depend on the origin of cellulose materials.\textsuperscript{39-41} Table 1 summarizes the typical sources of CNCs and the corresponding geometrical dimension distributions and shapes. The width of these nanocrystals is fairly monodisperse, while the distribution of the length is wide, which correlates with the degree of polymerization (DP).\textsuperscript{42-43}

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|l|c|}
\hline
Source & \textbf{Length (nm)} & \textbf{Width (nm)} & Morphology & Ref. \\
\hline
Bacterial & 100-1000 & 10-50 & Ribbon - like & 44 \\
Tunicate & 500-1000 & 10-30 & Ribbon - like & 45 \\
Wood & 100-200 & 3-5 & Coniferous & 37 \\
Ramie & 50-250 & 3-10 & Needle - like & 46 \\
Cotton & 100-300 & 5-15 & Rod - like & 47-48 \\
\hline
\end{tabular}
\caption{The geometrical dimension distributions and shapes of CNCs from different sources.}
\end{table}
1.2.2. Chemical modification of CNCs

Taking advantage of the abundance of hydroxyl groups on CNCs, a variety of chemical modifications have been performed on CNCs, such as noncovalent surface modification,\textsuperscript{49} polymer grafting,\textsuperscript{50} (2,2,6,6-tetramethylpiperidine-1-oxyl) (TEMPO)-mediated oxidation,\textsuperscript{51-52} The chemical modification not only introduces surface charges to improve the dispersion of CNCs, but also gives different properties to CNCs by conjugating different functional groups.

Noncovalent modification of CNCs is usually achieved by adsorption of surfactants or polymers. For example, xyloglucan oligosaccharide-based triblock copolymers were adsorbed on the surface of CNCs by noncovalent modification, and the resulting functionalized CNCs showed high dispersibility in nonpolar solvents.\textsuperscript{53}

Polymer grafting on CNCs involves two general approaches of “grafting-onto” and “grafting-from”.\textsuperscript{54} In the “grafting-onto” approach, CNC-polymers are formed by conjugation of hydroxyl groups or carboxyl groups on CNCs with the reactive part of a coupling agent. Using this method, PEG-NH$_2$ could be grafted to carboxylated CNCs through a carboxylation-amidation procedure, and the resulting copolymer showed excellent solubility in water.\textsuperscript{47} In “Grafting-from”, CNCs are firstly introduced the initiator of polymerization as active sites, then these active sites can be incorporated by other monomers through copolymerization or post polymer reaction. Habibi and coworkers applied this approach to covalently graft poly($\varepsilon$-caprolactone) on CNCs using Sn(Oct)$_2$-catalyzed ring-opening polymerization.\textsuperscript{55} The resulting product showed significant improvement in mechanical performance.

Another protocol of chemical modification is TEMPO-mediated oxidation (Figure 6). It was first introduced by de Nooy to oxidize polysaccharides, in which only the primary hydroxyl groups were oxidized.\textsuperscript{56} Later on, this method was widely utilized to oxidize the primary hydroxyl groups of cellulose to carboxyl groups.\textsuperscript{57} For example, Akira and others treated cellulose cotton linter with catalytic amount of TEMPO, NaBr and different concentrations of NaClO, and studied the properties in terms of crystallinity, morphology, carboxylate content, crystal size and degree of polymerization.\textsuperscript{58} The TEMPO-oxidized CNCs showed no change in morphology and crystallinity. They were able to
form homogeneous suspensions in water, owing to the contribution of negative charges from the installed carboxyl groups at CNCs surface.

![Diagram](image)

**Figure 6.** Selective oxidation of C6 primary hydroxyls of cellulose to carboxyls by TEMPO.

### 1.2.3. Application

CNCs have a great deal of value in many fields because of their unique morphologies, physicochemical properties, biocompabilities, low toxicities, etc. The typical applications are summarized in Figure 7.\textsuperscript{59-64} Owing to their outstanding mechanical features, CNCs are widely used as reinforcing filler in polymers,\textsuperscript{31} such as poly(caprolactone),\textsuperscript{36} and poly(vinyl alcohol).\textsuperscript{65} The resulting composite materials show significant improvements in thermal stability, mechanical strength, hardness, rigidity and flexibility owing to the formation of network structures in CNCs through hydrogen bonding. In addition, CNCs show significant improvement on the swelling performance of composite materials.\textsuperscript{66}
Since CNCs are also biocompatible, biodegradable and of low-toxicity, they have been applied in the area of biology, e.g., biosensors,\textsuperscript{67} fluorescence probes,\textsuperscript{68} drug delivery\textsuperscript{69} and antimicrobial materials.\textsuperscript{63} The hydroxyl groups on CNCs can serve as the recombination sites for loading metal nanoparticles. Liu et al. synthesized Ag/carboxylated CNCs by the reduction of Ag$^+$ in the carboxylated CNCs suspension. This nanocomposite was successfully used as a labeled DNA sensor for the identification of complementary DNA sequences.\textsuperscript{70} In addition, the hydroxyl groups can also be modified to bind different functional entities, enabling CNCs to carry out location analysis in cells. For example, CNCs conjugated with negatively charged FITC and positively charged RBITC have been used to analyze the influence of surface charge on cell uptake and cytotoxicity.\textsuperscript{71} CNCs are potential platforms for drug delivery after performing surface and charge modification. Wang et al. synthesized a multiparticulate drug delivery system which contained CNCs, chitosan and a polyelectrolyte macroion complex. This system could enter the affected area effectively and stay there for long time, thus improving the pharmacological effects.\textsuperscript{69}

1.3. Chitin nanocrystals (ChNCs)

Chitin, the second most abundant polysaccharide after cellulose, is a crystalline high molecular weight linear polysaccharide containing $\beta$-(1\textsuperscript{→}4)-2-acetamido-2-deoxy-D-glucopyranose repeating units (Figure 8).\textsuperscript{72} In 1823, A. Odier first identified chitin in demineralized crab carapace, and found that chitin was formed biosynthetically by numerous living organisms (e.g., yeasts, crustaceans,
Chitin was considered as an untreatable polymer for a long time because it is insoluble in almost any common solvent. Until the 1970s, it was recognized as the source of chitosan, a unique cationic polysaccharide. Years later, because of environmental requirements due to the organic solid wastes and byproducts generated by the food industry, part of the bio-waste was transformed into chitin and its derivatives.

Figure 8. Chemical structure of chitin.

One great interest in the use of chitin is from its nanostructured products, chitin nanocrystals (ChNCs). ChNCs have a variety of excellent properties such as non-toxicity, biodegradability, absorption properties and biocompatibility; hence they can be widely used in biomedical applications, cosmetics, agriculture, and sewage treatment. Moreover, ChNCs have high surface area, low density and active surface (-OH, -NHAc, and residual -NH₂) that facilitate surface modification.

1.3.1. Preparation of ChNCs

Based on the preparation of cellulose nanocrystal, Marchessault and coworkers initially reported a protocol of preparing chitin nanocrystal suspensions using acid hydrolysis. Since then, a variety of methods on preparation of ChNCs has been reported using similar procedures. The representative process was treating purified chitin with hydrochloric acid (HCl) under reflux. After removing the excess acid and diluting with water, a suspension of ChNCs was obtained. This type of acid-hydrolyzed chitin was able to disperse into rod-shaped particles spontaneously, which could further be concentrated to a liquid crystal phase. Later, Fan et al. synthesized ChNCs by mechanically treating partially deacetylated chitin. The protonation of the amino groups provides a large number of positive charges on the surface of the partially deacetylated chitin fibrils, which increase the repulsion between the fibrils. This repulsion facilitates the disintegration of chitin fibrils during the mechanical processing, resulting in chitin nanocrystals.
Similar to cellulose nanocrystals, the morphologies of ChNCs depend on the chitin origins. Normally, the obtained nanocrystals display a similar width of 10-50 nm regardless of the sources and hydrolysis time. In contrast, there is a wide length distribution from 150 to 2200 nm.77, 80-81

1.3.2. TEMPO-mediated oxidation
As with the chemical modifications of cellulose nanocrystals, ChNCs can undergo the reactions of etherification,82 esterification,83 and graft polymerization through the hydroxyl or amino groups.84 TEMPO-mediated oxidation has also been successfully applied in preparing and modifying ChNCs.85 In the presence of NaBr and sufficient amount of NaClO at pH 10, TEMPO-mediated oxidation can convert the water-insoluble particles into water-soluble products by selectively oxidizing the primary hydroxyl groups of chitin to carboxyl groups. When the resulting products are subjected to ultrasonic dispersion, mostly individual ChNCs can be obtained because the carboxyl groups on the surface enable to protect the nanocrystals from aggregation.

1.3.3. Application of ChNCs
As discussed above, chitin is the major source of chitosan, so ChNCs can be used as the crude material to form chitosan nanoscaffolds. For example, after three treatments of chitin whiskers in aqueous NaOH solution at 150 °C, the generated chitosan showed 98% of deacetylation and a porous structure was obtained.86

In addition, the nanoscale dimension, unique morphology and mechanical strength make chitin nanocrystals highly attractive as fiber reinforcement agents in tissue engineering when made into nanocomposites with natural and synthetic polymers.87 For example, in 2001 Paillet and coworkers used nanostructured chitin as the reinforcement agent in poly(styrene-co-butyl acrylate) matrix.88 Since then, ChNCs have been applied in different polymeric matrices (e.g., poly(caprolactone),78 chitosan89, and starch).90 The mechanical performances of the resulting nanocomposites is improved by adding ChNCs or chitin whiskers.
In terms of biological applications, nanocomposites with bactericidal activity against *Escherichia coli* (*E. coli*) were successfully prepared by introducing partially deacetylated ChNCs into bacterial cellulose networks. This green chemistry approach exploits new applications of ChNCs and provides a new species of nanocomposite materials with antibacterial effect.91 Additionally, Jayakumar and coworkers incorporated QDs into chitin nanogels and loaded BSA into the resulting nanomaterials. In response to pH, this system presented outstanding stability and reversible physical property. It was applied in cellular imaging and regulated protein delivery, which provided a potential platform for drug delivery, bioimaging and biosensing.92

1.4. Mesoporous silica nanoparticles (MSNs)

Mesoporous silica, the porous form of silica, was initially discovered by Kuroda and the scientists at Mobil Oil Company in the early 1990s.93-95 During the last 20 years, there has been a tremendous amount of research on perfecting the physical properties of MSNs, like uniformity and pore sizes control, high pore volume, chemical stability, large specific surface area, etc.96-97 MSNs are becoming one of the most popular nanomaterials in the applications of catalysis, sensing, bioimaging and drug delivery.

1.4.1. Synthesis of MSNs

The production of MSNs is usually based on a base-catalyzed sol-gel process.98 This process exploits the organosilane precursor tetramethyl orthosilicate (TMOS) or tetraethyl orthosilicate (TEOS) to form colloidal particles through co-condensation.99 For example, Grün and coworkers modified the Stöber synthesis of monodisperse silica spheres by adding a cationic surfactant to the reaction mixture and obtained submicrometer-sized particles.100 In the synthesis process, TEOS was used as the silica precursor, the surfactant cetyltrimethylammonium bromide (CTAB) as a template, and base as a catalyst. When the concentration is higher than the critical micelle concentration, CTAB would assemble into micelles. TEOS is then condensed at the surface of CTAB, giving rise to a silica wall around the polar head region of the micelles. After removing CTAB, MCM-41 type MSNs with well-ordered arrangement of uniform hexagonal mesopores can be obtained (Figure 9).101-102
1.4.2. Functionalization of MSNs

Due to the high specific surface area and easily modified surface, various organic functionalities can be introduced onto MSNs, providing a variety of mechanical features. Usually, these organic functional moieties include hydrophilic polymers, fluorescent dyes, carbohydrates, etc. These functionalizations give MSNs the ability to track, block, target and so on (Figure 10). There are two common pathways of attaching functional molecules: electrostatic interactions and covalent attachment. The electrostatic interactions use the negatively charged group SiO⁻ on the surface of MSNs to attach cationic polymers. The covalent conjugation usually involves co-condensation and post-synthetic grafting methods. The difference is the time point at which the functional molecules are added. The co-condensation method allows the addition of organic molecules in the form of silanes during the synthesis of particles. These silanes can be hydrolyzed and incorporated into silica particles. Conversely, post-synthetic grafting of functional moieties occurs after the formation of MSNs either before or after removal of the surfactant.
Owing to the mesoporous structure, MSNs have the ability to encapsulate a variety of guests in their pore channels. The cargo that can be stored in and released from MSNs are small molecules such as fluorescent dyes, drugs, or macromolecules such as nucleotides and proteins (Figure 10). The incorporation is achieved by dispersing particles in a solution of the cargo followed by adsorption of the cargo on the surface or in the pores of particles.

When the ability to carry cargo is combined with certain functional features like targeting, the nanocarrier MSNs can deliver drugs at the specific sites, such as cancer tumors. When gating is introduced, it can protect therapeutic agents from enzymatic degradation and premature release. Thus, the adverse side effect of certain drugs can be significantly reduced and the overall therapeutic efficacies can be improved.

1.4.3. Stimuli responsive MSNs for drug delivery

The functional moieties on the surface of MSNs make these particles promising platforms for nanomedicine, including probing, imaging, and drug delivery. In 2001, MSNs were reported as drug nanocarriers for the first time, but they still lacked control over cargo incorporation and release. Later on, great effort has been made in fabricating MSNs as stimuli-responsive nanocarriers. The functional molecules decorated on MSNs serve as protectors of the cargo, or act as target for receptors, which are sensitive to certain stimuli. When the multifunctional systems arrive at the specific sites, they can release cargo in a controlled fashion under the influence of stimuli. Depending on the different features, the triggers can be classified into two types: external and internal. The external can be light, temperature and some mechanical responses, while the internal stimuli include enzymes, redox events and other physical environmental factors. Some typical examples are summarized in Table 2. In addition, the research toward the development of multi-stimuli responsive nanocarrier has also been reported. These stimuli responses can further improve medical efficacy of drug delivery. Cui et al. prepared dually responsive nanoparticles based on a thermally sensitive polymer and pH sensitive deoxycholic acid. This system performed dissociation and deformation in response to both pH and temperature. Thus this system could release doxorubicin efficiently and exhibit comparable cytotoxicity to the free drug.
Table 2. Examples of stimuli responsive MSNs for controlled drug delivery.

<table>
<thead>
<tr>
<th>Type</th>
<th>Stimuli</th>
<th>System</th>
<th>Property</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>External</td>
<td>Light</td>
<td>Photo-responsive rotaxane functionalized MSNs</td>
<td>The back and forth movement of α-CD owing to light activated trans-cis photoisomerization of azobenzene result in the closing and opening of nanopores, allowing drug storage and release. The system has been successfully used for in vivo release of curcumin in zebrafish embryos.</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>PNIPAM-modified MSNs</td>
<td>Below LCST of polymer PNIPAM, the system can load and release cargo; above the LCST, it shows a low level of leakage.</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>Ultrasound</td>
<td>Organic-inorganic hybrid HPMOs</td>
<td>A good reservoir for loading cargo with high capacity and cargo release owing to the π-π stacking between the benzene group-bridged framework and doxorubicin, which is sensitive to ultrasound.</td>
<td>121</td>
</tr>
<tr>
<td>Internal</td>
<td>Redox</td>
<td>MSNs with CD gate that linked to the surface via disulfide units</td>
<td>An efficient system not only to uptake anticancer drugs into the pores reservoir but also to release the drug in response to GSH stimulation. It is useful for delivering drugs to target cancer cells.</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>Enzyme</td>
<td>PLE responsive snap-top MSNs</td>
<td>Following PLE-mediated hydrolysis of ester-linked stopper, the system efficiently enables the controlled release of cargo.</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>Polyadenine capped thymidine functionalized MSNs</td>
<td>The protonation-deprotonation interaction of the base paired thymine and adenine can open the polyadenine cap and release rhodamine B when the system is exposed to low pH. Under physiological pH, the system shows no premature release.</td>
<td>124</td>
</tr>
</tbody>
</table>

1.5. Aims of this thesis

The aims of the projects in this thesis are to:

1) Design and create platforms to introduce biorecognition capabilities to polysaccharides such as cellulose nanocrystals and chitin nanocrystals. TEMPO-mediated oxidation is applied to modify the surface of nanocrystals, providing reaction sites for conjugation with fluorescent dyes and carbohydrate ligands. The biorecognition property of the resulting multifunctional nanocrystals is analyzed using carbohydrate-binding proteins and bacteria. The
bindings are characterized by optical, confocal microscopy, and TEM (Chapter 2).

2) Develop targeted and controlled drug delivery systems for bacteria and cancer cells based on mesoporous silica glyconanoparticles. Two systems are developed according to the targeting carbohydrate of bacteria or corresponding proteins. They were applied in targeting and killing of *Mycobacteria* or controlled release of anticancer drugs into tumor cells (Chapter 3).
Carbohydrate-functionalized CNCs/ChNCs for biorecognition and bioimaging

(Paper I-II)

The two most abundant polysaccharides, cellulose and chitin, constitute renewable polymer resources available today. Their crystalline forms CNCs and ChNCs can be prepared after chemical and mechanical treatment of native cellulose and chitin, such as acid hydrolysis or TEMPO oxidation.\textsuperscript{125-126} These nanoscale materials have gained increasing interest not only due to their unique physical and chemical properties but also their light weight, high surface area, biocompatibility and biodegradability. Cellulose and chitin nanocrystals have been applied in nanocomposites and polymer reinforcements. The introduction of specific functionalities, especially biorecognition capabilities to these crystalline biopolymers through chemical modifications will further broaden the scope of their applications.

In this chapter, we functionalized cellulose and chitin nanocrystals with a fluorescence dye and carbohydrate ligands. They were applied to recognize and image lectin and bacteria. The detailed synthesis and characterization as well as subsequent biological experiments are presented.

2.1. Synthesis of multifunctional CNCs for lectin recognition and bacterial imaging

2.1.1. Overview

After TEMPO oxidation and acid hydrolysis of wood pulp, carboxylated cellulose nanocrystals (C-CNCs) were obtained. The carboxyl groups on the surface played an important role in the formation of multifunctional cellulose nanocrystals, because they provided sites to introduce the fluorescent dye and carbohydrate ligands (D-mannopyranoside, D-galactopyranoside). The bioaffinity of the resulting nanocrystals C-CNC-Man and C-CNC-Gal were confirmed by assessing their interactions with the corresponding cognate lectins.
These multifunctional nanocrystals were also used to image *E. coli* selectively by binding to the receptor on the bacteria surface (Figure 11).

![Figure 11. Multifunctional C-CNCs for biorecognition and bioimaging.](image)

2.1.2. Preparation

*Synthesis of C-CNCs*

C-CNCs were prepared following the synthetic route shown in Scheme 1. The suspension of wood pulp in water was oxidized with TEMPO and NaClO at pH 10 to form individualized carboxylated cellulose nanofibers (C-CNF). Subsequently, the freeze dried C-CNF was hydrolyzed to C-CNCs using a method developed by Salajková et al. C-CNF was dispersed in hydrochloric acid (HCl) solution, and then diluted with water. The resulting suspension was centrifuged to give a turbid supernatant, which was further dialyzed until it being neutral.

![Scheme 1. The synthetic route of C-CNCs.](image)

*Synthesis of fluorescent dye*

The fluorescent dye $R_1$-NH$_2$ was synthesized following the route in Scheme 2A. The reaction gave the fluorescent dye $R_1$-NH$_2$ and isomer $R_1'$-NH$_2$, but
the relative fluorescence intensity of $R_1$'-NH$_2$ was lower. Thus, we chose $R_1$-NH$_2$ as the fluorescence label for our research.

**Synthesis of carbohydrate ligands**

The carbohydrate ligands $R_2$-NH$_2$ and $R_3$-NH$_2$ were prepared using our previously reported method.$^{130}$ For $R_2$-NH$_2$, penta-$O$-acetate-$\alpha$-$D$-mannopyranoside reacted with 2-(2-(2-azidoethoxy)ethoxy)ethanol catalyzed by BF$_3$Et$_2$O to give compound 2, which was immediately deprotected by NaOMe to form 3. After reduction of the azide group in compound 3 by Pd/C, the ligand $R_2$-NH$_2$ was obtained (Scheme 2B). The synthesis of galactose ligand $R_3$-NH$_2$ was performed following the same procedure.

**Scheme 2. Synthesis of (A) fluorescent dye and (B) carbohydrate ligands.**

**Dual functionalization of C-CNCs**

The dually functionalized nanocrystals C-CNC-Man and C-CNC-Gal were prepared following Scheme 3. It was based on the condensation reaction between carboxyl groups on the C-CNCs and amino groups in the functional
moieties (R₁-NH₂, R₂-NH₂, and R₃-NH₂). Take the formation of C-CNC-Man as an example, EDC·HCl and NHS were added into the C-CNCs suspension and pH was adjusted to 5 to activate the carboxyl groups. The dye R₁-NH₂ and mannose ligand R₂-NH₂ were then added at pH 7.5–8.5. C-CNC-Man was obtained after dialysis in flowing deionized water. In the same manner, C-CNC-Gal was synthesized using galactose ligand R₃-NH₂ instead of R₂-NH₂.

Scheme 3. Synthesis of dually functionalized C-CNCs

2.1.3. Characterization

Morphology
The shape and size distribution of the C-CNCs were analyzed by STEM. From Figure 12A, the resulting C-CNCs possessed coniferous shape with the average size distributions of 265 ± 80 nm in length and 5.2 ± 0.3 nm in width.

X-ray diffraction pattern (XRD)
XRD measurement was used to characterize the crystalline structure of C-CNCs (Figure 12B). The wide peak around 15.3°, the intense peak at 22.4°, and a weaker peak at 34.6° corresponded to the crystal plains (110), (110̅), (200) and (004). The crystallinity index (CI) of C-CNCs was 64.7%, calculated by equation 1:

\[
CI = \frac{I_{200} - I_{am}}{I_{200}}
\]

Where \( I_{200} \) represents the intensity of the (200) lattice plane at 22.4° and \( I_{am} \) is the intensity of peak at 18°, which corresponds to the amorphous material in cellulose nanocrystals.

Carboxylate content
Conductometric titration was applied to measure the carboxylate content of C-CNCs (Figure 12C). Freeze dried C-CNCs were dispersed in HCl (0.01 M) and
titrated with NaOH (0.01 M). The carboxylate content reached to 1.56 mmol/g, calculated by equation 2:

\[ c_{\text{content}} = \frac{c (V_2 - V_1)}{w} \]  

where \( c \) is the concentration of NaOH, \( V_1 \) and \( V_2 \) are the end point volumes of NaOH (L), and \( w \) is the weight of the C-CNCs (g).

\[ \text{Figure 12. (A) STEM image, (B) X-ray diffraction pattern and (C) conductometric titration of C-CNCs.} \]

FT-IR spectroscopy
C-CNCs and carbohydrate-modified nanocrystals were characterized by FT-IR to confirm the functionalization. As shown in Figure 13, the common signals at 3345 cm\(^{-1}\), 2921 cm\(^{-1}\) and 1060 cm\(^{-1}\) were the typical cellulose characteristics, which were assigned to the stretching vibrations of O-H, C-H and C-O. Especially, both the O-H vibration absorption in the carboxyl group at \( \sim 3340 \) cm\(^{-1}\), which overlap with the O-H stretch vibrations in the CNCs, and the vibration absorption of C=O at 1736 cm\(^{-1}\) decreased owing to the reaction of carboxyl groups and amino groups after functionalization. The absorption peaks
at 1626 cm\(^{-1}\) increased due to the formation of amide bonds (CONH). All these changes indicated that the fluorescent dye and the ligands were successfully conjugated onto the cellulose nanocrystals.

**Figure 13.** Difference FT-IR spectra of C-CNCs, C-CNC-Man, and C-CNC-Gal (all spectra were baseline-corrected and normalized in relation to the absorption at 1060 cm\(^{-1}\)).

**Degree of functionalization**

The fluorescence characteristics were used to measure the amount of fluorescent dye attached to the nanocrystals. The dye content was 0.74 mmol/g for C-CNC-Man and 0.71 mmol/g for C-CNC-Gal, which was calculated by combining the standard calibration curve of the dye with fluorescence intensities of the nanocrystal suspensions (Figure 14A and 14B). The total carboxylate content of C-CNCs was 1.56 mmol/g, and the signal of the carboxyl group disappeared completely from the FT-IR spectra. Assuming the full conversion of the carboxyl group, the ratio of fluorescent dye versus carbohydrate ligands in C-CNC-Man and C-CNC-Gal was 0.91:1 and 0.84:1, respectively.
2.1.4. Lectin binding study

The biorecognition properties of the dually functionalized CNCs were investigated by lectin binding studies. Here, Concanavalin A (Con A) and *Ricinus communis* Agglutinin (RCA-120) were chosen for the binding experiments. Con A is a tetramer at physiological conditions. It has specific binding ability toward mannopyranoside, enabling potential crosslinking of C-CNC-Man and leading to aggregation. RCA-120 consists of two As-sB-type dimers that can bind to galactosyl residues ($K_a$ is $2.2 \times 10^3$ M$^{-1}$), but shows no binding to mannose. Indeed, when Con A was added to C-CNC-Man, aggregates formed in 30 min (Figure 15A). The TEM image of this agglomeration exhibited that the nanocrystals clustered together (Figure 15B). In contrast, the samples remained homogenous in the absence of Con A or in the presence of RCA-120. The difference in fluorescence intensities of each sample also confirmed this phenomenon: the intensity showed a significant decrease after treating C-CNC-Man with Con A for 30 min, whereas only a slight reduction was observed when C-CNC-Man was treated with RCA-120. This change was probably due to non-specific adsorption of RCA-120 to the nanocrystals (Figure 15C).

To further determine the binding specificity of carbohydrate-functionalized CNCs, C-CNC-Gal was treated with Con A and RCA-120. Aggregation occurred in the sample with RCA-120, owing to the multivalent interactions between the lectin and the galactosyl residues in the materials (Figure 15D). Analysis by TEM showed that nanocrystals were cross-linked with each other, but the aggregate level of this agglomeration was lower than that in C-CNC-
Man because of weaker binding affinity of RCA-120 toward galactopyranoside (Figure 15E). Conversely, the samples remained homogeneous in the presence of Con A, or lack of RCA-120. In addition, when treating C-CNC-Gal suspension with RCA-120, a drastic decrease in the fluorescence intensity occurred. However, the intensity decreased slightly in the sample treated with Con A as a result of non-specific protein adsorption. (Figure 15F).

**Figure 15.** (A) C–CNC-Man (left), C–CNC-Man treated with Con A (middle) and RCA-120 (right) in buffer solution (pH 7.2) under visible (top) and UV (bottom) light. (B) TEM images of C–CNC-Man treated with Con A. (C) Emission spectra of C–CNC-Man (blue) and after incubation with Con A (yellow) or RCA-120 (red). (D) C–CNC-Gal (left), C–CNC-Gal treated with Con A (middle) and RCA-120 (right) in buffer solution (pH 7.2) under visible (top) and UV (bottom) light. (E) TEM images of C–CNC-Gal after treatment with RCA-120. (F) Emission spectra of C–CNC-Gal (black) and after incubation with Con A (purple) and RCA-120 (green).

Dynamic light scattering (DLS) analysis was then carried out to measure the average size distributions of these precipitations. As shown in Figure 16, C-CNCs, C-CNC-Man and C-CNC-Gal had a similar size distribution from 190 nm to 220 nm, indicating that the functionalization had no effect on the size of CNCs. After binding with lectin, the average sizes of the resulting complexes increased to 460 nm for C-CNC-Gal and 540 nm for C-CNC-Man, supporting
the cross-linking between the dually functionalized nanomaterials and corresponding lectins.

Figure 16. DLS analysis of **C-CNCs** and functionalized nanocrystals before and after the addition of lectins.

2.1.5. Bacterial binding study

The biorecognition properties of these multifunctional nanocrystals were investigated by probing two strains of *E. coli* ORN 178 and ORN 208. ORN 178 expresses the α-D-mannoside selective FimH lectin on type 1 pili, whereas ORN 208 lacks this expression. Neither of them express β-D-galactose selective lectins. After incubating the two bacteria with **C-CNC-Man** for 2 h, TEM was used to detect the binding activities of the nanocrystals to the bacteria. As expected, a large amount of **C-CNC-Man** adhered to ORN 178 cells (Figure 17A), demonstrating that the mannose ligands on **C-CNC-Man** bound to the FimH lectin of ORN 178 successfully. On the contrary, there were no obvious nanocrystals at the surface of ORN 208 cells (Figure 17E). The observations were also confirmed by confocal fluorescence microscopy. The amount of green fluorescence presented in ORN 178 bacteria cells was significantly larger than that in ORN 208 (Figure 17B-17D; 17F-17H).
Figure 17. TEM images of E. coli (A) ORN178 and (E) ORN208 treated with C–CNC-Man; confocal fluorescence microscopy images of ORN 178 and ORN 208 incubated with C–CNC-Man: (B, F) bright field images, (C, G) fluorescence images, and (D, H) merged images.
The bioactivities of **C-CNC-Gal** towards *E. coli* were also tested. As shown in Figure 18A and 18B, no nanocrystals bound to either ORN 178 or ORN 208, owing to the lack of galactoside-selective lectins on their surface. These results were also confirmed by confocal fluorescence imaging (Figure 18C and 18D). It was demonstrated that our glycan-functionalized nanocrystals had the ability to selectively distinguish between bacteria.

![Figure 18. TEM images of E. coli (A) ORN 178 and (B) ORN 208 treated with C−CNC-Gal; confocal fluorescence microscopy images of ORN 178 incubated with C−CNC-Gal: (C) bright field image and (D) fluorescence image.](image)

2.2. Carbohydrate-functionalized fluorescent ChNCs for biorecognition applications

2.2.1. Overview

A simple protocol for the synthesis of ChNCs conjugated with fluorescent dye and carbohydrate ligands was developed. To demonstrate the utility of these dually functionalized chitin nanocrystals, the affinity of the resulting functionalized nanocrystals was confirmed by their interactions with the corresponding cognate proteins (Figure 19). The fluorescent labelling facilitates
the observation of these interactions by either fluorescence imaging or even with the naked eyes. Furthermore, the nanocrystals were successfully applied to image *E. coli* by taking advantage of their affinity with the carbohydrate receptor on the bacteria surface. Owing to the unique properties of chitin, such as biodegradability, biocompatibility, and non-toxicity, the new platform developed here may provide the opportunities for chitin-based glyconanomaterials in a wide range of bioanalytical and therapeutic applications.

![Image: The illustration of dually functionalized ChNCs for biorecognition.]

**Figure 19.** The illustration of dually functionalized ChNCs for biorecognition.

### 2.2.2. Synthesis

**TEMPO-oxidized chitin nanocrystals (TCNs)**

TEMPO-mediated oxidation was utilized to prepare carboxylated chitin nanocrystals from *α*-chitin of shrimp shells (Scheme 4).\(^{126}\) NaClO solution was dropped into the suspension of chitin, TEMPO and NaBr under magnetic stirring at pH 10. Then, the stable and transparent TCNs suspension was collected after washing with deionized water and homogenization.

![Scheme 4: Synthesis of TCNs by TEMPO-mediated oxidation.]

**Scheme 4.** Synthesis of TCNs by TEMPO-mediated oxidation.
Functionalization of TCNs

The functionalizations of TCNs with fluorescent dye ($R_1$) and carbohydrate ligands ($R_2$, $R_3$) were performed following Scheme 5. To a stirred TCNs suspension, EDC•HCl and NHS were added, and the pH was adjusted to 5.5. This condition was kept for 30 min to finish the activation of the carboxyl groups. $R_1$ and mannose derivative $R_2$ (or galactose derivative $R_3$) were added to the TCNs mixture at pH 7.5~8.5. After dialysis, TCN-dye-Man or TCN-dye-Gal was obtained.

![Scheme 5. Synthesis of dually functionalized TCNs.](image)

2.2.3. Characterization

Morphology

The morphologies of the TCNs were studied by AFM and STEM. As shown in Figure 20A and 20B, the TCNs were conifer-shaped and dispersed well. The size of TCNs was on the order of 4-8 nm in width and 140-360 nm in length.

X-ray diffraction pattern

XRD analysis was performed to investigate the crystalline structure of TCNs. As shown in Figure 20C, the peaks at 9.2°, 19.3°, 20.9° and 23.3° were the typical diffractions of chitin nanocrystals. The crystalline index (CI) of TCNs was calculated using equation 3:

$$\text{CI (\%)} = \frac{I_{110} - I_{am}}{I_{110}} \times 100 \quad (3)$$
where $I_{110}$ is the maximum intensity of the diffraction (110) at 20.9°, $I_{am}$ is the intensity of peak at 12.6°, which results from the diffraction of amorphous structure. The crystallinity of synthesized TCNs was 91.6%, which indicated that the chitin nanocrystals displayed a high degree of structural order.

**Carboxylate content**

The carboxylate content of the TCNs was measured by conductivity titration. NaCl was added to a diluted homogeneous suspension of TCNs, and then the pH was adjusted to 3.5. The resulting suspension was titrated with NaOH aqueous solution after stirring for 10 min, followed by measurements of the conductivity and pH (Figure 20D). The content of carboxyl and amine groups ($C_{ca}$) was 1.22 mmol/g, calculated by equation 4:

$$C_{ca} = \frac{C \times V}{W}$$

where $C$ is the concentration of NaOH (mol/L), $V$ is the volume of added NaOH (L), and $W$ is the weight of dried TCNs (g).

As measured by solid-state NMR spectroscopy, the degree of acetylation (DA) was 87%, which corresponds to an amine content of 0.66 mmol/g. Thus, the resulting value for the carboxylate content of TCNs amounted to 0.56 mmol/g.
Figure 20. Characterizations of TCNs: (A) AFM image, (B) TEM image, (C) X-ray diffraction pattern and (D) conductometric titration.

FT-IR spectroscopy

TCNs, **TCN-dye-Man** and **TCN-dye-Gal** were characterized by FT-IR spectroscopy. The main signals and their assignments are summarized in Table 3. Typically, a signal appeared at 3463 cm⁻¹ in the frequency range of OH groups, and the two absorptions at 1659 cm⁻¹ and 1628 cm⁻¹ corresponded to the vibration of amide I. The big difference in FT-IR signals between bare TCNs and functional TCNs is the shoulder at 1740 cm⁻¹, which was assigned to the vibration mode of C=O of the carboxyl group. The disappearance of this shoulder in **TCN-dye-Man** and **TCN-dye-Gal** indicated the successful reaction between TCNs and the dye or carbohydrate ligands.
Table 3. The major FT-IR signals common to TCNs, TCN-dye-Man and TCN-dye-Gal

<table>
<thead>
<tr>
<th>Absorption (cm(^{-1}))</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCNs</td>
<td>TCN-dye-Man</td>
</tr>
<tr>
<td>3463</td>
<td>(\gamma_{\text{OH}})</td>
</tr>
<tr>
<td>3276, 3127</td>
<td>(\gamma_{\text{NH}})</td>
</tr>
<tr>
<td>2889, 2914, 2950</td>
<td>(\gamma_{\text{CH}_3})</td>
</tr>
<tr>
<td>1659, 1628</td>
<td>(\gamma_{\text{C}=\text{O}}) (Amide I)</td>
</tr>
<tr>
<td>1557</td>
<td>(\gamma_{\text{C-N}}) (C-N-H) + (\delta_{\text{NH}}) (Amide II)</td>
</tr>
<tr>
<td>1422</td>
<td>(\delta_{\text{CH}_2})</td>
</tr>
<tr>
<td>1384</td>
<td>(\delta_{\text{OH}} + \delta_{\text{C-CH}_3})</td>
</tr>
<tr>
<td>1310</td>
<td>(\gamma_{\text{C-N}} + \delta_{\text{NH}}) (Amide III)</td>
</tr>
<tr>
<td>1250</td>
<td>(\delta_{\text{NH}})</td>
</tr>
<tr>
<td>1155</td>
<td>(\gamma_{\text{C-O-C}}) (ring)</td>
</tr>
<tr>
<td>1120, 1107, 1034, 992</td>
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</tr>
<tr>
<td>1740</td>
<td>(N/A)</td>
</tr>
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</table>

2.2.4. Lectin-TCNs binding study

The bioactivities of TCN-dye-Man and TCN-dye-Gal were investigated by treating these nanocrystals with lectins: concanavalin A (Con A) and soybean agglutinin (SBA). SBA is a carbohydrate-binding protein that preferentially binds to \(\beta\)-D-galactopyranoside \((K_a\) for methyl \(\beta\)-D-galactopyranoside = \(5.5 \times 10^2\) M\(^{-1}\)).\(^{135}\) After treating TCN-dye-Man with these lectins, aggregates were observed in the presence of Con A, whereas the nanocrystals remained homogenous without Con A or with SBA (Figure 21A I-III). The emission spectra of these samples were analyzed by fluorescence spectroscopy. As shown in Figure 21C, after incubating with Con A for 1 h, the fluorescence intensity of TCN-dye-Man solution decreased significantly. Meanwhile, only a slight reduction was observed in the SBA incubated sample, probably owing to nonspecific adsorption of SBA to nanocrystals.

The binding affinity of TCN-dye-Gal was investigated in the same manner. Precipitation was detected when incubating TCN-dye-Gal with SBA, but the formation of agglomerates was less significant, likely because the binding affinity of SBA towards \(\beta\)-D-galactopyranosides was weaker (Figure 21A VI). On the other hand, the samples remained suspended without SBA (Figure 21A IV), or in presence of Con A (Figure 21A V). The fluorescence intensity of
TCN-dye-Gal solution with SBA decreased dramatically, while it showed a slight reduction in the sample treated with Con A, owing to the nonspecific protein adsorption (Figure 21D).

Figure 21. TCN-dye-Man and TCN-dye-Gal in HEPES buffer (pH 7.2) under (A) visible light and (B) UV illumination. From left to right: TCN-dye-Man, TCN-dye-Man incubated with Con A, TCN-dye-Man incubated with SBA, TCN-dye-Gal, TCN-dye-Gal incubated with Con A, and TCN-dye-Gal incubated with SBA. Emission spectra of (C) TCN-dye-Man and (D) TCN-dye-Gal after lectin binding. $\lambda_{ex} = 450$ nm, $\lambda_{em} = 512$ nm.

These TCNs-lectin aggregates were characterized by STEM. In TCN-dye-Man, the nanocrystals were closely packed to each other because of strong multivalent binding of Con A with the multivalent mannose-presenting nanocrystal (Figure 22A). However, the level of aggregation of TCN-dye-Gal was less, owing to the lower binding affinity of SBA towards galactose (Figure 22B).
Figure 22. STEM images of (A) TCN-dye-Man after treating with Con A and (B) TCN-dye-Gal after treating with SBA.

2.2.5. Bacterial binding study

TCN-dye-Man was applied in bacteria binding studies to investigate its biorecognition property. The E. coli strains ORN 178 and ORN 208 were probed. As presented in the TEM and the confocal fluorescence images, nanocrystals adhered to the bacteria after incubating TCN-dye-Man with ORN 178 (Figure 23A and 23C), indicating that the binding occurred between the mannose ligands on the nanocrystals and the FimH protein on ORN 178. However, only few TCN-dye-Man nanocrystals were attached on the surface of ORN 208 (Figure 23B and 23D).
2.3. Conclusion

In the first part of this chapter, carbohydrate and fluorescent dye functionalized CNCs were studied. The biorecognition abilities of the dually modified CNCs were assessed through lectin interaction with the support of TEM and confocal microscopy. Furthermore, the resulting nanocrystals were used as bacterial sensors, presenting selective binding in response to the FimH expression on bacterial surface. These studies indicated that the carbohydrate-functionalized CNCs could be efficiently used as nanoplatforms for protein recognition and bacterial imaging.

In the second study of this chapter, the binding affinity of TCNs conjugated with both fluorophore and carbohydrate ligands were investigated. To demonstrate
the properties of the resulting chitin nanocrystals, the affinities were determined by measuring the binding of nanocrystals with corresponding cognate lectins. Furthermore, the multifunctional TCNs were successfully applied to image *E. coli* by using the interactions between carbohydrates on nanocrystals and lectin receptors on the bacteria surface. The glyconanocrystals developed here expand the utilities of chitin in biorecognition. The studies and results have determined the feasibility of these nanocrystals in bioanalytical and nanomedical applications.
In this chapter, the synthesis of PFPA-functionalized silica nanoparticles and subsequent conjugation of carbohydrates by several approaches, including photochemistry and cycloaddition are discussed. Two drug delivery systems based on glyconanoparticles were developed. The surface conjugated carbohydrates were designed to serve as targeting moieties for bacteria, and to introduce a capping agent to prevent premature release of encapsulated drug. After encapsulation of drugs, the drug release profile, antibacterial- and anticancer activity of the mesoporous silica glyconanoparticles were studied.

3.1. Approaches to immobilize carbohydrates on PFPA-SNPs

3.1.1. PFPA-functionalized silica nanoparticles

PFPA-functionalized silica nanoparticles were prepared by treating silica nanoparticles with PFPA-silane (Scheme 6). Carbohydrates were immobilized via copper-catalyzed azide-alkyne cycloaddition (CuAAC), perfluorophenyl azide-aldehyde-amine cycloaddition (AAAC) or PFPA photocoupling.

3.1.2. Copper-catalyzed azide-alkyne cycloaddition (CuAAC)

CuAAC is the most used ‘click’ reaction.\textsuperscript{136} It is easy to carry out, gives rise to the desired product in high yield with little or no byproduct. It also works well under various sources of copper catalysts and solvents.\textsuperscript{137} The formation of triazole is chemically inert to many reactive conditions, such as oxidation, reduction, and hydrolysis. The CuAAC reaction is widely applicable in organic synthesis, preparation and functionalization of polymers and dendrimers, and nanoparticle catalysis.\textsuperscript{136, 138} Here, this reaction was used for the synthesis of glyconanoparticles, using the reactivity of PFPA in the CuAAC reaction. In the experiments, acetone suspensions of PFPA-SNPs were mixed with aqueous solutions of glycosyl alkynes in the presence of sodium ascorbate and CuSO\textsubscript{4}·5H\textsubscript{2}O to give glyconanoparticles (Scheme 7).

TGA and \textsuperscript{19}F qNMR were used to determine the density of carbohydrates immobilized on SNPs. For TGA analysis, PFPA-SNPs had a weight loss of 14.5\%, an increase of 1.5\% compared to the weight loss of SNPs (Figure 24A). Based on this difference, the density of PFPA\textsubscript{5} on PFPA-SNPs was calculated to be of 15.2 ± 4.1 \times 10^{-16} \text{ nmol/nm}^2. The carbohydrate density, take mannose for example, was 13.0 ± 3.4 \times 10^{-16} \text{ nmol/nm}^2 from the weight loss difference between mannose-conjugated nanoparticles (Man-SNPs) and PFPA-SNPs.

For the \textsuperscript{19}F qNMR method, the glyconanoparticle Man-SNPs and a model compound were treated with HF (5\%) and the resulting solutions were lyophilized with in-line traps containing solid CaO. The obtained products were...
dissolved in methanol-\textit{d}_4 for $^{19}$F NMR analysis using methyl pentafluorobenzoate as the internal standard. As shown in Figure 24B, the F atom signals at -143.0 ppm (F$^{2,6}$) and -148.3 ppm (F$^{3,5}$) from the model compound were consistent with that from Man-SNPs. It further confirmed the successful conjugation of mannose. According to the ratio between the signal integral of F$^{4}$ in methyl pentafluorobenzoate (marked as "s") and F$^{2,6}$ in Man-SNPs (marked as "*"), the density of mannose was calculated to be $6.4 \pm 0.2 \times 10^{-16}$ nmol/nm$^2$, which was lower than that from TGA, since samples are heated up to high temperature in TGA, the residual solvents or organic contaminants trapped inside the particles will be vaporized together with the ligands, resulting in higher weight loss of the particles.

![Figure 24. TGA curves of SNPs, PFPA-SNPs and Man-SNPs.](image)

### 3.1.3. Perfluorophenyl azide-aldehyde-amine cycloaddition (AAAC)

Although CuAAC is an effective method of conjugating carbohydrates onto nanoparticles, the copper catalyst can be problematic due to its toxicity to cells. Various metal-free click reactions have been reported. In our laboratory, AAAC was developed. AAAC was developed. In this reaction, PFPA reacts with aldehydes and amines without metal catalysts under ambient conditions to give amidines. It was utilized to immobilize carbohydrates. To an acetone suspension of PFPA-SNPs, an aqueous solution of amine-derivatized carbohydrate and phenylacetaldehyde were added at room temperature. The carbohydrate-immobilized nanoparticles were obtained after stirring overnight (Scheme 8).
The disappearance of the azide absorption at 2135 cm\(^{-1}\) in the FT-IR spectra confirmed the successful conjugation of carbohydrate onto the nanoparticles by AAAC. The amount of coupled carbohydrate was determined by TGA, as shown in Figure 25A. Taking mannose as an example: the Man-SNPs had a 1.41% weight loss over the PFPA-SNPs, and the density of mannose on the nanoparticles was calculated to be 13.8 ± 2.9 \times 10^{-16} \text{ nmol/nm}^2. Furthermore, the bioaffinity of Man-SNPs was evaluated using FITC-labeled Con A (FITC-Con A). After incubating Man-SNPs with FITC-Con A for 1 h, aggregates appeared and the fluorescence intensity of the supernatant solution decreased significantly compared to the intensity of the initial FITC-Con A solution (Figure 25B). This is due to the multivalent mannose-presenting materials, inducing agglomeration of Con A by crosslinking. In contrast, the fluorescence intensity of FITC-Con A after incubating with galactose-conjugated nanoparticles (Gal-SNPs) only changed slightly, likely due to physical adsorption between Con A and galactose but no specific binding.
3.1.4. PFPA photocoupling

The CuAAC or AAAC reactions have been exploited to prepare carbohydrate-functionalized nanoparticles. However, the synthesis of carbohydrate derivatives can be considerably challenging, especially in the case of oligo- or poly-saccharides. As discussed earlier, PFPA is an excellent coupling agent to attach un-derivatized carbohydrates to nanoparticles via photoinitiation. The azide group on PFPA forms an electron-poor nitrene in the singlet state under UV-irradiation or by heat. The formed singlet nitrene can insert into C-H, N-H single bonds or add to C=C double bonds (Scheme 9).\[^{141}\] Insertion of the nitrene into carbohydrates lead to immobilization onto PFPA-functionalized nanoparticles. This approach avoids using metal catalysts, allows conjugation of complex carbohydrates onto the surface of nanoparticles and facilitates the process of surface modification.

In the next two projects, trehalose and mannose were conjugated on mesoporous silica nanoparticles by PFPA photocoupling. These carbohydrates were chosen to target bacteria and to introduce a capping agent for drug delivery.
3.2. Trehalose-conjugated mesoporous silica nanoparticles for targeted drug delivery to mycobacteria

3.2.1. Introduction

Tuberculosis (TB) is a chronic infectious disease primarily caused by *Mycobacterium tuberculosis* (*M. tuberculosis*).\(^{142}\) It is a global threat to millions of lives every year, and the situation is worsening owing to the advent of AIDS, drug abuse, population movements, etc.\(^{143-144}\) Eradicating TB is increasingly challenging due to the appearance of multi-drug resistant mycobacteria strains and the protective barrier of the cell wall which can prevent antituberculosis agents from permeating into the bacterial cytoplasm.\(^{145}\)

Isoniazid (INH), one of the first-line TB drugs,\(^{146}\) can be converted into an electrophilic species through the activation of *M. tuberculosis* KatG. It inhibits the biosynthesis of mycolic acid, an important component of the *M. tuberculosis* cell wall.\(^{147-148}\) However, a major problem that INH faces now is its toxic side effect on hepatocytes, which decreases the therapeutic efficiency and limits its utility.\(^{149}\) In mycobacteria, the disaccharide \(\alpha,\alpha\)-trehalose is an essential precursor for cell-wall glycolipids, which can translocate across the plasma

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**Scheme 9. Illustration of the formation of singlet nitrene from PFPA and respective insertions.**

\[
\begin{array}{c}
\begin{array}{c}
\text{F} \quad \text{N}_3 \quad \text{F} \\
\text{F} \quad \text{R} \quad \text{F}
\end{array}
\end{array}
\rightarrow
\begin{array}{c}
\begin{array}{c}
\text{F} \quad \text{F} \quad \text{N}^1 \quad \text{F} \\
\text{F} \quad \text{R} \quad \text{F}
\end{array}
\end{array}
\rightarrow
\begin{array}{c}
\begin{array}{c}
\text{F} \quad \text{F} \quad \text{N} \quad \text{H} \\
\text{F} \quad \text{R} \quad \text{F}
\end{array}
\end{array}
\rightarrow
\begin{array}{c}
\begin{array}{c}
\text{F} \quad \text{F} \quad \text{N} \quad \text{C} \quad \text{F} \\
\text{F} \quad \text{R} \quad \text{F}
\end{array}
\end{array}
\]

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membrane in monomycolate form. It has been reported that mycobacteria can selectively uptake trehalose or trehalose conjugates. 150-153

Here we designed a drug delivery system based on trehalose-functionalized MSNs, encapsulating high doses of INH. M. smegmatis mc² 651 was employed to verify the selectivity and bactericidal activity.

3.2.2. Synthesis

MSNs were prepared using TEOS as the silica precursor, CTAB as pore-forming template and NaOH as catalyst. 102 MSNs were then functionalized with PFPA-silane to introduce a surface layer of PFPA. The resulting-PFPA functionalized MSNs (M-PFPA) were subjected to a trehalose solution under UV irradiation, yielding trehalose-functionalized MSNs (M-PFPA-Tre). M-PFPA-Tre was suspended in a concentrated isoniazid (INH) solution for encapsulation of the drug and formation of the INH loaded MSNs (M-PFPA-Tre-INH) (Scheme 10).

Scheme 10. Synthesis of INH loaded, trehalose-conjugated MSNs.
3.2.3. Characterization

Morphology
As shown in Figure 26A, MSNs were spherically shaped having an average size of 120 nm. Their mesoporous structures were confirmed by the nitrogen adsorption and desorption isotherms Brunauer Emmett Teller (BET) and Barrett Joyner Halenda (BJH). The results demonstrated that MSNs were mesoporous with a surface area of 742 m$^2$/g and a pore size of 2.83 nm (Figure 26C). After surface functionalization and drug loading, the resulting particles **M-PFPA-Tre-INH** remained spherical, but the mesopores were less, possibly due to incorporation of large amount of INH (Figure 26B).

FT-IR spectroscopy
The surface modifications were analyzed by FT-IR spectroscopy (Figure 26D). In the MSNs, and the **M-PFPA** and **M-PFPA-Tre** particles, the peaks at 1100 cm$^{-1}$, and 824 cm$^{-1}$ were in agreement with Si-O-Si bonds and the peak around 3400 cm$^{-1}$ denoted hydroxyl groups. In addition, modification of MSNs with PFPA-silane resulted in an azide signal at 2135 cm$^{-1}$. The modification was further supported by the signals at 1500 cm$^{-1}$ and 1660 cm$^{-1}$ from the C=O group belonging to PFPA. Upon photocoupling, the azide signal disappeared.
Figure 26. TEM images of (A) MSNs and (B) M-PFPA-Tre-INH. (C) The nitrogen adsorption-desorption isotherms of MSNs and its pore size distributions (insert). (D) FT-IR spectra of MSNs, M-PFPA and M-PFPA-Tre.

Trehalose density on nanoparticles
TGA was applied to measure the amount of conjugated trehalose. As shown in Figure 27, M-PFPA-Tre displayed a weight loss of 7.6% compared to M-PFPA in the same time and temperature range. From this weight loss, we derived that the density of trehalose was $16.3 \times 10^{-15}$ nmol/nm$^2$. 
Figure 27. TGA graph of M-PFPA and M-PFPA-Tre.

The loading of INH
The encapsulation of INH was carried out by dispersing of M-PFPA-Tre in INH solution. M-PFPA-Tre-INH were obtained after centrifugation and the washing solution was collected for UV-Vis analysis. According to the absorption intensity of the original INH solution, the washing solution and the calibration curve, the amount of loaded INH was determined to be 170 µg/mg.

3.2.4. Release profile
Release of INH from the trehalose-conjugated MSNs was monitored by dispersing M-PFPA-Tre-INH in water under stirring at 37 °C, followed by centrifugation at pre-determined time intervals. As can be seen from Figure 28, the INH release followed a two-phase kinetic process: an initial burst release during the first 8 h, and a sustained release process over the following 40 h. These release processes can be determined by the loading states of INH into MSNs. The part of the INH that was directly adsorbed on the outer surface of the MSNs caused the initial burst release, because these drugs could quickly dissociate into the solution. Part of the INH was physically adsorbed/retained inside the pores. This portion of the drugs released slowly, resulting in a sustained release process. In principle, the two-phase release kinetics may be advantageous, where a localized, high initial concentration of INH leads to a more efficient bactericidal effect, following by continuous killing from the sustained release.
Figure 28. Percentage of INH released from M-PFPA-Tre-INH at fixed time points. The inset is the enlarged image of the percent INH released from particles in the first of 8 h.

3.2.5. Antibacterial activity

To evaluate the bactericidal activity of the trehalose-conjugated MSNs towards *M. smegmatis* mc² 651, different concentrations of particles were incubated with bacterial suspensions for 2 days, and the survival colonies were counted. MSNs loaded with INH (M-INH) were also tested to evaluate the mycobacterial targeting effect of trehalose. As displayed in Figure 29, MSNs, M-PFPA and M-PFPA-Tre at the tested concentrations from 0.5 mg/mL to 5 mg/mL, showed no obvious toxicity on *M. smegmatis* mc² 651. In contrast, the viability of *M. smegmatis* declined with increasing concentrations of M-PFPA-Tre-INH and M-INH. The trehalose-conjugated nanoparticles M-PFPA-Tre-INH produced a largest bactericidal effect at a concentration of 3-4 mg/mL, whereas M-INH was only effective at a higher concentration. These results indicated that the trehalose-conjugation at the surface of MSNs indeed played a targeting role on *Mycobacteria*. In addition, the free INH released from 3-4 mg of the M-PFPA-Tre-INH corresponded to 390-520 μg, which was calculated by the INH-release profile. While the minimum inhibitory concentration (MIC) of free INH towards *M. smegmatis* mc² 651 was 1.0-1.5 mg/mL, the combined targeting and localized release of INH lead to improved bactericidal activity.
Figure 29. Log CFU/mL vs. particle concentration for MSNs, M-PFPA, M-PFPA-Tre, M-INH and M-PFPA-Tre-INH with *M. smegmatis mc² 651*.

TEM images further showed the interaction between particles and bacteria. As shown in Figure 30A-30C, due to the specific targeted function of trehalose on the surface of particles, **M-PFPA-Tre** could bind to *M. smegmatis mc² 651* more efficiently as compared to MSNs and **M-PFPA**. Comparing the two antibacterial drug carriers **M-INH** and **M-PFPA-Tre-INH**, both of them had bactericidal effect on *M. smegmatis mc² 651* after incubation for 48 h (Figure 30D-30E), but **M-PFPA-Tre-INH** could gather around bacteria in large amount and kill the bacteria efficiently while **M-INH** only started to gradually destroy the wall of bacteria.
3.3. Lectin-gated mesoporous silica glyconanoparticles for glutathione-responsive drug delivery to cancer cells

3.3.1. Introduction

MSNs have proven excellent potential as drug delivery vehicles, owing to their tunable pore size, high surface area and good biocompatibility. For the development of efficient delivery systems based on nanomaterials, controlled drug release and no premature leakage are key features. MSNs can meet these requirements by attachment of targeting molecules and gatekeepers. When the latter are detached by triggers, such as light, pH, enzyme action, temperature and redox state, the encapsulated cargo would be released. Generally, non-proteinic structural elements such as synthetic polymers and supramolecular assemblies have been used as gatekeepers. Using proteins as protectors is still at its early stage, although they are inherently biocompatible, and suitable to a wide range of recognition systems.
We developed a Con A-gated, MSNs-based drug release system using carbohydrate-lectin specific recognition as a gating mechanism (Figure 31). Con A was chosen as the gatekeeper, because it is a homotetrameric protein at pH above 5.5 with a size of approximately 8 nm, which is larger than the nanoparticle pores, and it can bind primarily to α-D-mannopyranosides with high specificities. To achieve the aim of controlled release, a disulfide linker was engineered into the gate function. Thus, the release would be maintained by glutathione (GSH), which can specifically cleave disulfide bonds and remove the protection of the gate. The concentration of GSH in cancer cells is higher than in healthy cells, which provides a trigger for removing the gate, and also a control mechanism to selectively target cancer cells.

Figure 31. GSH-responsive drug delivery system based on mesoporous silica glyconanoparticles.

3.3.2. Synthesis

As shown in Scheme 11, FITC-doped MSNs were prepared to facilitate the visualization. The resulting FMSN particles were treated with 3-mercaptopropyltrimethoxysilane and 2-(pyridin-2-yldisulfanyl)ethyl 4-azido-2,3,5,6-tetrafluorobenzoate, yielding FMSN-PFPA particles with disulfide-linked PFPA. Subsequently, the particles were mannosylated under UV irradiation using the previously described photocoupling method. Finally, the glyconanoparticles (FMSN-Man) were suspended in a doxorubicin solution to load the drug. The gatekeeper protein (Con A) was added, capping the FMSN-
**Man** particles through multivalent Mannose-Con A interactions, yielding the final drug delivery system **FMSN-DOX-Con A**.

### Scheme 11. Synthesis of Con A-gated mesoporous silica glyconanoparticles for GSH-responsive drug delivery.

#### 3.3.3. Characterization

**Morphology**
The starting material **FMSN** and the final product **FMSN-DOX-Con A** both displayed average diameters of 130 nm and were spherically shaped. The ordered lattice array indicated a uniform, well-defined mesoporous structure (Figure 32A and 32B).

**Dynamic light scattering**
The particle synthesis, the loading and gating process were also followed by DLS (Figure 32C). **FMSN** displayed a hydrodynamic diameter of approximately 180 nm, which increased slightly to 200 nm after PFPA functionalization and mannosylation. After doxorubicin loading and Con A capping, the size of the particles increased to 300-600 nm, owing to partial crosslinking. The smaller particle population at around 30 nm was likely due to Con A aggregation under the experimental conditions used.
Nitrogen adsorption and desorption isotherm

The size of a Con A protomer is approximately 4 nm in one dimension, and the tetrameric protein is close to twice that size.\textsuperscript{5} To confirm the gating potential of the lectin, nitrogen adsorption and desorption analysis were used to measure the size distribution of the mesopores. As shown in Figure 32D, the properties of the original FMSN preparation were typical to mesoporous silica particles with approximately 1020 m\textsuperscript{2}/g surface area and 3.1 nm pore diameter (b), while FMSN-DOX-Con A showed significantly decreased surface area and no obvious mesopores (a). These results, owing to doxorubicin loading and lectin capping, are similar to those reported by Zhang and coworkers.\textsuperscript{109}

\textbf{Figure 32.} TEM images of (A) FMSN and (B) FMSN-DOX-Con A preparations. (C) Size distribution of nanoparticles by DLS. (D) Nitrogen adsorption-desorption isotherms of (a) FMSN-DOX-Con A and (b) FMSN samples; inset: corresponding pore size distribution of (a) FMSN-DOX-Con A and (b) FMSN.

Raman and FT-IR spectroscopy

Raman and FT-IR spectroscopy were also used to confirm the structures. FMSN-SH showed a Raman peak at 2560 cm\textsuperscript{-1}, which was attributed to free thiol groups (Figure 33A). The formation of disulfide link was supported by the
absorptions of azide groups at 2175 cm$^{-1}$ from FT-IR (Figure 33B), accompanied by the disappearance of the Raman thiol signal. After reaction with mannose, the azide peak disappeared.

![Image](image.png)

Figure 33. (A) Raman spectra of FMSN-SH and FMSN-PFPA. The arrow indicates the absorption of thiol groups on FMSN-SH around 2580 cm$^{-1}$. (B) FT-IR spectra of nanoparticles.

**Determination of mannose coupling yield and amount of DOX loaded**

The amount of mannose conjugated on the surface of particles was analyzed by a colorimetric assay using anthrone and sulfuric acid. Various concentrations of mannose solution, FMSN-PFPA or FMSN-Man suspensions were treated with freshly prepared anthrone/H$_2$SO$_4$ solution (0.5 wt%, 2 mL) under stirring at 0 °C, respectively. Then, the mixture was heated to 100 °C for 10 min. After cooling to room temperature, the absorbances at 620 nm of the resulting solutions were recorded by UV-Vis spectroscopy, giving rise to the corresponding calibration curve (Figure 34A).

Combined with the UV absorbances of the particles, the experimental D-mannose density was calculated to be $6.7 \times 10^{-5}$ mol/g (12 mg/g). The theoretical maximal D-mannose density was $1.4 \times 10^{-4}$ mol/g, which was calculated by equation 5:

$$\rho_{max} = \frac{6}{\rho d S_0 N_A} \quad (5)$$

where $\rho$ is the density of MSNs (2.2 g/cm$^3$), $d$ is particle diameter (130 $\times$ 10$^{-7}$ cm), $S_0$ stands for D-mannose space occupancy (0.24 $\times$ 10$^{-14}$ cm$^2$) and $N_A$ is Avogadro’s number. The coupling yield of D-mannose in this system was 48%.
In addition, the loading amount of DOX was 75 mg per gram of particles according to the calibration curve for DOX and the UV absorbance of the collected washing solution after centrifugation (Figure 34B).

![Figure 34](image.png)

**Figure 34.** (A) Calibration curve for mannose obtained by treating various concentrations of the mannose with anthrone/H$_2$SO$_4$ solution and measuring the absorption at 620 nm. (B) Calibration curve for DOX in water.

3.3.4. Drug release profile

The gating behavior of Con A in the presence of GSH was then evaluated. FMSN-Man were first incubated with FITC-labeled Con A for capping. After centrifugation, the resulting FMSN-Con A-fl was subsequently exposed to increasing concentrations of GSH in PBS buffer (pH 7.4). The residual fluorescence emanating from reduction of the disulfide bond and release of the FITC-labeled Con A was measured. As shown in Figure 35A, no detectable fluorescence signal was found when FMSN-Con A-fl was dispersed in PBS in the absence of GSH. In contrast, fluorescence appeared when adding GSH into FMSN-Con A-fl suspension. The fluorescence intensity became stronger as the concentrations of GSH increased after incubation for 12 h. The result indicated that GSH played an effective role in cleaving the disulfide linker of this system.

Absorbance spectroscopy was used to check the release of DOX from FMSN-DOX-Con A before and after exposure to GSH in vitro (Figure 35B). During the first 24 h, the absorbance of DOX was very low in the absence of GSH, indicating that the lectin-gated glyconanomaterial was intact. On the other hand, upon increasing concentrations of GSH from 0.04 mM to 10 mM, the absorbance increased significantly, indicating pore opening and DOX release.
resulting from the removal of Con A. At a GSH concentration of 10 mM, the system showed an initial burst phase within 7 h, reaching 61% of release; then it turned towards a prolonged period of slower release. These results demonstrated that the lectin gating system showed efficient sealing properties before exposure to GSH, and rapid opening and release of the nanoparticle cargo upon stimulation. Therefore, the Con A-gated glyconanoparticles would be a potential drug carrier against cancer cells without premature release of drugs into blood vessels and normal cells.

Figure 35. (A) Emission spectra of FMSN-Con A-fl in the presence of increasing concentrations of glutathione (λex = 490 nm, λem = 519 nm). (B) Release profiles of doxorubicin from FMSN-DOX-Con A in response to increasing concentrations of GSH.

3.3.5. Cell experiments
The intracellular killing efficiency of this drug delivery system was investigated using cytotoxicity assays and two mammalian cell lines: primary lung epithelial cells (PCC) and adenocarcinomic alveolar basal epithelial cells (A549). These cells were treated with different concentrations of FMSN and FMSN-DOX-Con A, and the viability was analyzed after 24 h. In both cells, FMSN did not show significant cytotoxicity at concentrations from 1.56 μg/mL to 200 μg/mL. About 90% of the cells were still alive, demonstrating the biocompatibility of the FMSN particles (Figure 36A).

The IC50 of FMSN-DOX-Con A against A549 cells was around 25 μg/mL, while the viability of PCC cells was still above 90% at this concentration (Figure 36B). Because of the high GSH concentration in A549 cells (~ 12 mM), the disulfide link could be reduced quickly and efficiently when incubating FMSN-DOX-Con A with A549 cells, and Con A would be removed. However, normal
cells such as PCC have lower concentration of GSH in PCC cells (~ 1 mM), resulting in considerably slower disulfide cleavage and doxorubicin release.\textsuperscript{162}

![Figure 36. Percent viability of A549 and PCC cells treated with (A) FMSN and (B) FMSN-DOX-Con A.](image)

**Confocal fluorescence microscopy**

The actions of the FMSN and FMSN-DOX-Con A towards cells were furthermore analyzed using confocal fluorescence microscopy. The green fluorescence of FITC in FMSN was visible in PCC (Figure 37A) and A549 cells (Figure 37B) after incubation for 6 h, indicating that mammalian cells can take up MSNs very well.

In Figure 38A, A549 cells gave rise to strong red fluorescence after incubation with FMSN-DOX-Con A for 6 h. This result reflected our expectation that the influence of GSH towards disulfide bonds would cause the removal of Con A gates in A549 cells, thereby leading to the release of DOX, which showed red fluorescence. In contrast, the weak red fluorescence in PCC cells demonstrated that DOX remained safely entrapped in the pores due to the low concentration of GSH. This system showed a good blocking effect with minimal premature release of drug in PCC cells (Figure 38B)
Figure 37. Confocal microscopic images of (A) PCC cells and (B) A549 cells incubated FMSN.

Figure 38. Confocal microscopy images of (A) PCC cells and (B) A549 cells incubated with FMSN-DOX-Con A; from top to bottom: emission measured at 500-530 nm (fluorescein), emission measured at 650-710 nm (doxorubicin), merged images showing both channels; scale bars 50 μm.
3.4. Conclusions

In the first study of this chapter, we described the applications of CuAAC, AAAC and PFPA photocoupling in immobilizing carbohydrates to PFPA-SNPs. TGA and lectin-binding were used to determine the ligand densities and bioaffinities of the resulting glyconanoparticles. In addition, $^{19}$F qNMR as a new tool to measure the ligands on fluorine-containing nanoparticles was introduced. It avoided further separating individual component and provided comparable results to TGA.

In the second project of this chapter, we applied PFPA photocoupling to synthesize isoniazid-loaded, trehalose-conjugated MSNs for selective targeting and killing of mycobacteria. These nanoantibiotics had ~8% trehalose and were encapsulated with approximate 170 μg/mg of INH. The release profile indicated that INH exhibited burst release in the initial 8 hours followed by slow, sustained release. The bactericidal efficacy of the resulting M-PFPA-Tre-INH towards *M. smegmatis* mc² 651 was confirmed by cytotoxicity studies. Compared with free INH and other particles lacking of complete functionalization, the mesoporous silica glyconanoparticles showed increased ability to kill bacteria, displaying the target function of trehalose to facilitate the localized release of INH. The glyconanomaterials not only show higher bactericidal activity towards mycobacteria, but in principle demonstrate high potential for reviving the antibacterial effects of common antibiotics, a venue we are currently pursuing.

In the third study of this chapter, the development of lectin-gated, FITC-doped mesoporous silica glyconanoparticles as delivery vehicles for controlled drug release was introduced. The particles were first conjugated with D-mannose using photocoupling. Subsequently, the resulting particles were encapsulated with the anticancer drug DOX and capped with the mannose-binding lectin Con A. When a disulfide linker was introduced, the lectin gatekeeper could be opened by GSH, showing good uncapping effects in cancer cells that have higher concentration of GSH, while good blocking efficiency were observed in normal cells having low levels of glutathione. This Con A-gated glyconanomaterial provide an efficient platform for controlled drug delivery, releasing the content at the site of disease.
4. Concluding remarks

To expand the utilities of glyconanomaterials in bioanalysis and nanomedicine, three types of carbohydrate-conjugated nanomaterials were developed and applied in biorecognition and drug delivery.

Chapter 2 describes two types of new glyconanomaterials based on cellulose nanocrystals and chitin nanocrystals synthesized from natural polysaccharides. TEMPO-mediated oxidation was successfully introduced to surface modification of these nanocrystals, selectively converting C6 primary hydroxyl group to carboxyl group. The resulting carboxylated nanocrystals were functionalized with a fluorescent dye and carbohydrate ligands via one-pot reactions. The biorecognition properties of these glyconanocrystals were demonstrated by treatment with lectins and bacteria, due to the specific binding of carbohydrate ligands and corresponding cognate proteins. The biodegradability, biocompatibility, low toxicity and biorecognition ability give these glyconanocrystals new opportunities for biomedical applications.

In chapter 3, we described the design and fabrication of two kinds of drug delivery systems based on MSNs. One is trehalose-modified nanoparticles carrying the anti-TB drug INH. Trehalose conjugated onto the surface of MSNs can lead to selective targeting towards mycobacteria, where the materials can bind mycobacteria, release the drug and kill the bacteria effectively. The other one is a controlled drug delivery system consisting of Con A-gated MSNs encapsulated with the anticancer drug DOX. The Con A-gating effect was achieved through the specific carbohydrate-protein binding with mannose. When incubating the nanoparticles with cells, obvious controlled release, triggered by GSH in cancer cells, was shown. In contrast, the system was less effective against normal cells due to the lower concentration of GSH in normal cells. The two projects expand the capability of using mesoporous silica glyconanoparticles as drug delivery vehicles to improve the antimicrobial and anticancer efficiencies.
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Appendix

The following is a description of my contribution to Publications I to VI, as requested by KTH.

Paper I: I contributed to the formulation of the research problems, performed the majority of the experimental work, and wrote the manuscript.

Paper II: I contributed to the formulation of the research problems, performed the majority of the experimental work, and wrote the manuscript.

Paper III: I contributed to the formulation of the research problems, performed part of the synthesis of the nanomaterials, and contributed in writing the manuscript.

Paper IV: I contributed to the formulation of the research problems, performed part of the synthesis of the materials, and contributed in writing the manuscript.

Paper V: I contributed to the formulation of the research problems, performed the majority of the experimental work, and wrote the manuscript.

Paper VI: I contributed to the formulation of the research problems, performed the majority of the experimental work, and wrote the manuscript.
References
