A glycoconjugate-based gold nanoparticle approach for the targeted treatment of *Pseudomonas aeruginosa* biofilms†

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In this study, “core–shell” gold nanoparticles (AuNPs) have been functionalised using a simple one-pot approach to form fucose-based glycoconjugate AuNPs (Fuc-AuNPs) and galactose-based glycoconjugate AuNPs (Gal-AuNPs), respectively. Owing to the selective carbohydrate-based recognition of the key virulence factors of *P. aeruginosa*, LecB (fucose-specific lectin)/LecA (galactose-specific lectin), Fuc-AuNPs and Gal-AuNPs-based imaging and therapeutic strategies were evaluated towards *P. aeruginosa*. Both Fuc-AuNPs and Gal-AuNPs were non-covalently loaded with the fluorophore dicyanomethylene 4H-pyran (DCM) to afford two highly selective fluorescence imaging agents for the visualisation of *P. aeruginosa*. The loading of Fuc-AuNPs and Gal-AuNPs with the known antibiotic Ceftazidime (CAZ) exhibited an enhanced therapeutic effect, illustrating the significance of this targeted drug delivery strategy. Exploiting the phototherapeutic properties of AuNPs, photoradiation (600 nm) of Fuc-AuNP@CAZ/Gal-AuNP@CAZ provided both photothermal and photodynamic therapeutic (PTT/PDT) effects, which facilitated the release of CAZ. Fuc-AuNP@CAZ and Gal-AuNP@CAZ were shown to be effective photo/chemotherapeutics resulting in almost complete eradication of *P. aeruginosa* biofilms formed on clinically relevant surfaces (glass slides and steel surface).

**Introduction**

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a ubiquitous Gram-negative bacterium that has become a real concern for hospital acquired infections.†‡ It is recognised as the main cause of morbidity and mortality in critically ill, immunocompromised and cystic fibrosis (CF) patients.¶§ Additionally, *P. aeruginosa* has the ability to form biofilms on mucosal surfaces and medical implant devices,¶ which provide favourable and protective conditions for bacterial survival against host defence mechanisms and aggressive antibiotic treatment.†‡§ As a direct result, this has led to a continued increase in resistant and multidrug-resistant (MDR) *P. aeruginosa* and a restriction in treatment options.¶† Therefore, *P. aeruginosa* is now considered as a major public health threat.¶†–¶‡

Rapid advancements in material-based sciences has led to the identification of a range of drug delivery platforms with inherent diagnostic/therapeutic properties (i.e., photodynamic therapy/photothermal therapy (PDT/PTT)).¶†–¶‡ These systems have been demonstrated as effective anticancer and antimicrobial agents that can be activated by single or dual excitation wavelengths (e.g. 660 nm and 808 nm).¶†,¶‡,¶† Research has continued to explore these platforms by incorporating additional antimicrobial metals such as copper (Cu) and silver (Ag).¶†,¶‡ Within the field of material sciences, gold nanoparticles (AuNPs) are of particular interest owing to their unique multifunctional and biocompatible properties.¶†,¶‡ The high surface area-to-volume ratio and ease of functionalisation of AuNPs has provided the capability to incorporate...
therapeutics or targeting ligand units (i.e. peptides, carbohydrates and genetic material) onto the surface of AuNPs through covalent/non-covalent interactions.\textsuperscript{7,8–31} As a result, AuNPs provide an attractive platform for the effective delivery of functional molecules through active and passive targeting.\textsuperscript{12} In conjunction, with their ability to selectively deliver therapeutic agents,\textsuperscript{30,33,34} AuNPs have been shown as effective phototherapeutics with the production of cytotoxic ROS (PDT) and/or heat (PTT).\textsuperscript{15–26} Despite these promising attributes, the application of AuNPs have been mostly directed towards the development of anticancer agents with minimal attention on their use as antimicrobials.\textsuperscript{14,39} Therefore, we envisioned tailoring the design of AuNPs to target specific bacteria would allow the controlled and selective delivery of antibiotics. Moreover, in combination with PTT/PDT, we believed this would afford an effective antimicrobial platform with minimal off-target toxicities\textsuperscript{30} and could reduce the potential to develop MDR bacteria.

A key step in the pathogenesis of \textit{P. aeruginosa} is the adhesion of bacterial cells to human tissue,\textsuperscript{40} which is believed to occur through the targeted recognition of mammalian glycoproteins by virulence factors such as lectins (LecA and LecB).\textsuperscript{41} Exploiting the selective carbohydrate-based recognition of LecA (galactose specific) and LecB (fucose specific), we have developed two glycoconjugate-based AuNPs which were coated with the monosaccharides fucose (Fuc-AuNPs) and galactose (Gal-AuNPs), respectively (Scheme 1). NIR fluorophore, dicyanomethylene-4H-pyran (DCM)\textsuperscript{42} loaded AuNPs [Fuc-AuNP@DCM and Gal-AuNP@DCM] afforded two highly selective imaging agents for the visualisation of \textit{P. aeruginosa}. In addition, this result suggests binding with \textit{P. aeruginosa} facilitates the release of the molecular cargo from the AuNPs. As a result, both Fuc-AuNPs and Gal-AuNPs were subsequently loaded with the antibiotic Ceftazidime (CAZ),\textsuperscript{43} to afford the dual photo/chemotherapeutics, Fuc-AuNP@CAZ and Gal-AuNP@CAZ. This targeted lectin approach improved the therapeutic efficacy of CAZ and in combination with photoirradiation (600 nm), Fuc-AuNP@CAZ and Gal-AuNP@CAZ exhibited the effective destruction of the established \textit{P. aeruginosa} biofilms on clinically relevant surfaces – glass slides and steel surfaces (Scheme 1).

Copper-catalysed alkyne–azide cycloaddition (CuAAC) chemistry is routinely used for the synthesis of glycoconjugates.\textsuperscript{44} Unfortunately, the use of Cu(II) ions for CuAAC chemistry can prove problematic for certain reaction substrates.\textsuperscript{45} To overcome this limitation, strain-promoted “click” chemistry has been developed. In brief, a strained cyclooctyne unit covalently linked to a disulfide ring (Cyc-S) was used to form a self-assembled monolayer (SAM) on the surface of AuNPs.\textsuperscript{46} Subsequent “click” chemistry between AuNP-Cyc-S (on the gold surface) and azide Fuc-N (fucosyl) or Gal-N (galactosyl) afforded the desired Fuc-AuNPs and Gal-AuNPs (see ESI† for the one-pot procedure). This was confirmed by a clear signal enhancement in the Raman spectrum (see ESI – Fig. S1†).\textsuperscript{47,48} Moreover, zeta potential measurements demonstrated good aqueous stability for Fuc-AuNPs and Gal-AuNPs (see ESI – Fig. S1†).

With both Fuc-AuNPs and Gal-AuNPs in hand, the lectin binding properties of each AuNP was then evaluated. The addition of UEA-1 (Ulex European Agglutinin 1, fucose-specific lectin) to an aqueous solution of Fuc-AuNPs (0.2 nM) led to the broadening as well as a bathochromic shift of the UV-Vis absorption at 600 nm (Fig. 1a), This was attributed to aggregation of the AuNPs.\textsuperscript{49,50} Minimal changes to the UV-Vis absorption of Fuc-AuNPs were seen with the addition of other lectins/proteins (Fig. 1b), which was indicative of fucose selective binding. Similarly, the addition of PNA (Peanut Agglutinin, galactose-specific lectin) to a solution of Gal-AuNPs (0.2 nM) led to a gradual broadening as well as bathochromic shift of the UV-Vis absorption at 600 nm (Fig. 1c and d). Again, minimal changes were observed when in the presence of other lectins/proteins. In addition, both Fuc-AuNPs and Gal-AuNPs demonstrated a high sensitivity for each corresponding lectin with low limit of detection (LoD) Fuc-AuNPs – UEA-1 = 13.8 nM/Gal-AuNPs – PNA = 4.9 nM (see ESI – Fig. S2†).

HR-TEM (High-Resolution Transmission Electron Microscope) images and Dynamic Light Scattering (DLS) analysis revealed no obvious differences between unmodified AuNPs and Fuc-AuNPs/Gal-AuNPs (Fig. 2 and Fig. S3†). However, in the presence of their corresponding lectins, both Fuc-AuNPs and Gal-AuNPs were shown to form large clusters, which was indicative of lectin binding and lectin-mediated

![Scheme 1](image-url)
agglutination (Fig. 2 and see ESI – Fig. S3 and S4†). In addition, this further supported the lectin-mediated changes observed in the UV-Vis absorption of Fuc-AuNPs and Gal-AuNPs shown in Fig. 1a and c.

Upon identifying the lectin-binding properties of Fuc-AuNPs and Gal-AuNPs, the drug loading and phototherapeutic properties were then evaluated. Fuc-AuNPs or Gal-AuNPs (0.8 nM) were mixed in an aqueous solution containing CAZ for 4 h. Subsequent centrifugation (10 000 rpm) and washing (Tris-HCl buffer) afforded both Fuc-AuNP@CAZ and Gal-AuNP@CAZ. Both the loading of CAZ and corresponding AuNP@CAZ nanoparticle stability was confirmed by Raman spectroscopy and zeta potential measurements, respectively (see ESI – Fig. S1†). The non-covalent wrapping interactions between CAZ and Fuc-AuNPs and Gal-AuNPs were found to be stable over several days with no clear changes to both zeta potentials and the particle sizes (see ESI – Fig. S5†). Next, the phototherapeutic (PDT and PTT) properties of these systems were evaluated. 600 nm was chosen as the excitation wavelength for PDT/PTT experiments as this is the maximum absorption wavelength of Fuc-AuNPs and Gal-AuNPs. The known ROS fluorescent probe dihydroorhodamine was used to confirm ROS production upon light irradiation (600 nm, 1 W cm⁻²). The ROS colorimetric probe tetramethylbenzidine (TMB) was used to confirm the production of ROS using UV-Vis spectroscopy. Both Fuc-AuNPs and Gal-AuNPs were shown to produce ROS under light irradiation. No significant changes to ROS production were observed in the presence of lectins or by varying the pH and temperature (see ESI – Fig. S6†). Previous reports have shown the main ROS produced is ¹O₂. Interestingly, the light irradiation of UEA-1, AuNPs and Fuc-AuNP@CAZ displayed minimal PTT effects, however, upon lectin-binding a dramatic temperature increase was observed (see ESI – Fig. S7†). This observation is believed to be the result of the lectin-mediated formation of Fuc-AuNP@CAZ clusters allowing the generated heat to be localized, which enables an efficient increase in temperature. In contrast, when simple AuNPs are dispersed in solution, this results in a dissipation of the heat resulting in a poor PTT effect being observed. For Fuc-AuNPs and Gal-AuNPs in the presence of their corresponding lectins, a PTT conversion efficiency of 26.2% and 25.7% was observed respectively (see ESI – Fig. S8†). Fuc-AuNP@CAZ binding to P. aeruginosa (with LecA expression similar to UEA-1) was also shown to have a good PTT effect (see ESI – Fig. S9†). Demonstrating a potential mechanism of action, Fuc-AuNP@CAZ and Gal-AuNP@CAZ systems were shown to release CAZ gradually in the presence of either light irradiation or lectins (see ESI – Fig. S10 and S11†). No unwanted CAZ release was observed in their absence, which is in accordance with the stability data for Fuc-AuNP@CAZ and Gal-AuNP@CAZ systems (see ESI – Fig. S5†). According to these results, it is believed that the combined PDT and PTT effect will not only contribute to the overall therapeutic efficacy of CAZ towards P. aeruginosa, but also act as “triggers” to accelerate the release of co-loaded molecular cargos.
To confirm the LecB/LecA specificity and *P. aeruginosa* uptake of molecular cargos loaded on these glycoconjugate-based AuNPs, the fluorophore dicyanomethylene 4H-pyran (DCM) (see ESI† for chemical structure) was loaded onto **Fuc-AuNPs**/**Gal-AuNPs**. In addition, this study was used to illustrate lectin binding from *P. aeruginosa* facilitates the release of the molecular cargo. Using a similar loading protocol, **Fuc-AuNPs** or **Gal-AuNPs** (0.8 nM) were mixed in an aqueous solution containing DCM (10 μM). Subsequent centrifugation (10,000 rpm) and washing (Tris-HCl buffer) afforded both **Fuc-AuNP@DCM** and **Gal-AuNP@DCM**. As seen in Fig. 3, *P. aeruginosa* (ATCC 27853) treated with either **Fuc-AuNP@DCM** or **Gal-AuNP@DCM** displayed an obvious fluorescence emission signal, which suggested the uptake of the DCM. Confirming the LecB and LecA-mediated uptake of the NIR fluorophore, *P. aeruginosa* was pre-treated with either free L-fucose or α-galactose (10 mM). This led to a marked reduction in fluorescence emission intensity being observed. This was believed to be due to the pre-saturation of the lectin binding sites which prevented the binding and uptake of **Fuc-AuNP@DCM/ Gal-AuNP@DCM**. Overall, our approach demonstrates the targeted release of molecular cargos in *P. aeruginosa*.

Next, we turned our attention towards the evaluation of the antibacterial efficacy of each glycoconjugate-based AuNPs with and without light irradiation (30 min, 600 nm, 1 W cm\(^{-2}\)). In this study, *P. aeruginosa* (ATCC 27853) and drug-resistant *P. aeruginosa* (ATCC BAA2110) were treated with CAZ only, **Fuc-AuNPs**, **Gal-AuNPs**, **Fuc-AuNP@CAZ**, and **Gal-AuNP@CAZ**, respectively (Fig. 4). The sole use of CAZ (2 μg mL\(^{-1}\)) resulted in a moderate inhibition with no additional effect seen under light irradiation (30 min, 600 nm, 1 W cm\(^{-2}\)). **Fuc-AuNPs** and **Gal-AuNPs** were shown to have no “dark” therapeutic effect, whereas, the light irradiation of **Fuc-AuNPs**/**Gal-AuNPs** displayed a modest therapeutic effect with a slightly greater efficacy compared to unmodified AuNPs (see ESI – Fig. S12†).

The drug delivery capability of **Fuc-AuNPs** and **Gal-AuNPs** were confirmed with an enhanced therapeutic effect seen in comparison to CAZ. Remarkably, **Fuc-AuNP@CAZ** and **Gal-AuNP@CAZ** under light irradiation (30 min, 600 nm, 1 W cm\(^{-2}\)) demonstrated a significant cooperative therapeutic effect with CAZ, which almost resulted in full eradication (≥90%) (Fig. 4). This cooperative therapeutic effect was further confirmed using a LIVE/DEAD cell viability assay (see ESI – Fig. S13†). HR-TEM images were used to demonstrate the mode of action with **Fuc-AuNP@CAZ** initially interacting on the surface of *P. aeruginosa* and the subsequent light irradiation (30 min, 600 nm, 1 W cm\(^{-2}\)) resulting in a clear change to the morphology of the bacteria (see ESI – Fig. S14†). Confirming the selectivity for *P. aeruginosa*, the treatment of *E. coli* and methicillin-resistant *S. aureus* with **Fuc-AuNP@CAZ**/**Gal-AuNP@CAZ** were shown to have a significantly lower therapeutic outcome (see ESI – Fig. S15†). No toxicity was observed towards both 293T (human renal epithelial) and MDA-MB-231 (human breast cancer) cells (see ESI – Fig. S16†).

To demonstrate the full therapeutic potential of our glycoconjugate-based AuNPs strategy, *P. aeruginosa* biofilms were established on glass slides and steel surfaces. In these experiments, the performances were evaluated using the LIVE/DEAD biofilm viability assay (PI, dead – yellow and Syto9®, live –

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**Fig. 3** Confocal Laser-Scanning Microscope (CLSM) images of *P. aeruginosa* (ATCC 27853): (a) **Fuc-AuNP@DCM** (0.2 nM and 1 μM) (b) pre-treatment with L-fucose (10 mM) followed by addition of **Fuc-AuNP@DCM** (0.2 nM and 1 μM) (c) **Gal-AuNP@DCM** (0.2 nM and 1 μM) (d) pre-treatment with α-galactose (10 mM) followed by addition of **Gal-AuNP@DCM** (0.2 nM and 1 μM) (e–h) bright field images of (a–d).

**Fig. 4** (a) Relative viabilities of *P. aeruginosa* (ATCC 27853) treated with sterile water (control), **Fuc-AuNPs** (0.2 nM), **Gal-AuNPs** (0.2 nM), Ceftazidime (CAZ, 2 μg mL\(^{-1}\)), **Fuc-AuNP@CAZ** (0.2 nM and 2 μg mL\(^{-1}\)) and **Gal-AuNP@CAZ** (0.2 nM and 2 μg mL\(^{-1}\)) with and without light irradiation (30 min, 600 nm, 1 W cm\(^{-2}\)). (b) Relative viabilities of drug-resistant *P. aeruginosa* (ATCC BAA2110) treated with sterile water (control), **Fuc-AuNPs** (0.2 nM), **Gal-AuNPs** (0.2 nM), Ceftazidime (CAZ, 2 μg mL\(^{-1}\)), **Fuc-AuNP@CAZ** (0.2 nM and 2 μg mL\(^{-1}\)), and **Gal-AuNP@CAZ** (0.2 nM and 2 μg mL\(^{-1}\)) with and without light irradiation (30 min, 600 nm, 1 W cm\(^{-2}\)). (c) *P. aeruginosa* (ATCC 27853) colonies on Luria–Bertani (LB) agar plates treated with the respective systems with and without light irradiation (30 min, 600 nm, 1 W cm\(^{-2}\)). (d) *P. aeruginosa* (ATCC BAA2110) colonies on Luria–Bertani (LB) agar plates treated with the respective systems with and without light irradiation (30 min, 600 nm, 1 W cm\(^{-2}\)) ***P < 0.001; **P < 0.01.
blue). As shown in the processed 3D images of *P. aeruginosa* biofilms (Fig. 5), the use of just CAZ led to a partial antibiofilm effect, however, when co-loaded with Fuc-AuNPs or Gal-AuNPs, an enhanced antibiofilm effect was observed. Remarkably, light irradiation (600 nm, 30 min, 1 W cm⁻²) of Fuc-AuNP@CAZ/Gal-AuNP@CAZ resulted in almost complete biofilm destruction on both glass slides and on clinically relevant steel surfaces (Fig. 5). Overall, this illustrates the potential of Fuc-AuNP@CAZ/Gal-AuNP@CAZ as excellent light and chemo-based antimicrobials for the targeted treatment of *P. aeruginosa*.

In summary, fucose and galactose-based glycoconjugated AuNPs (Fuc-AuNPs and Gal-AuNPs) were developed using a simple one-pot self-assembly procedure. Both Fuc-AuNPs and Gal-AuNPs displayed selective lectin-binding properties as determined by UV-Vis, HR-TEM and DLS analysis. The Fuc-AuNPs and Gal-AuNPs targeting ligands were effective for the selective carbohydrate-based recognition of *P. aeruginosa* key virulence factors, LecB/LecA. DCM-loaded Fuc-AuNPs/Gal-AuNPs afforded a highly selective bacterial fluorescence imaging agent.⁵⁵ While, CAZ loaded Fuc-AuNPs/Gal-AuNPs were shown to have improved therapeutic efficacy towards *P. aeruginosa* when compared to CAZ alone. Exploiting the phototherapeutic properties of the AuNP core (PTT and PDT), light irradiation resulted in an enhanced therapeutic effect towards *P. aeruginosa* when combined with CAZ. Significantly our targeted photo/chemotherapeutics Fuc-AuNP@CAZ/Gal-AuNP@CAZ were able to eradicate *P. aeruginosa* biofilm formation on glass slides and clinically relevant steel surfaces, with biocompatibility fully confirmed among human cells. Therefore, we believe the ease of preparation (one-pot) and remarkable therapeutic effects observed using this AuNPs strategy, will encourage other groups to develop targeted therapeutic materials in order to combat the important and ever-worsening problem of antibiotic resistant bacteria.

**Conflicts of interest**

There are no conflicts to declare.

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