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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, photoirradiation (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).

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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a ubiquitous Gram negative bacterium that has become a real concern for hospital acquired infections.^{1,2} It is recognised as the main cause of morbidity and mortality in critically ill, immunocompromised

and cystic fibrosis (CF) patients.^{3,4} Moreover, *P. aeruginosa* has the ability to form biofilms on mucosal surfaces and medical implant devices,¹ which provide favourable and protective conditions for bacterial survival against host defence mechanisms and aggressive antibiotic treatment.^{1,5,6} 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.⁷ Therefore, *P. aeruginosa* is now considered as a major public health threat.^{8–10}

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)).^{11–15} 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).^{12,14,16–22} Researchers have continued to explore these platforms by incorporating additional antimicrobial metals such as copper (Cu) and silver (Ag).^{23–27} Within the field of material sciences, gold nanoparticles (AuNPs) are of particular interest owing to their unique multifunctional and biocompatible properties.^{11,28} The high surface area-to-volume ratio and ease of functionalisation of AuNPs has provided the capability to incorporate

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therapeutics or targeting ligand units (*i.e.* peptides, carbohydrates and genetic material) onto the surface of AuNPs through covalent/non-covalent interactions.^{28–31} As a result, AuNPs provide an attractive platform for the effective delivery of functional molecules through active and passive targeting.³² In conjunction, with their ability to selectively deliver therapeutic agents,^{30,33,34} AuNPs have been shown as effective phototherapeutics with the production of cytotoxic ROS (PDT) and/or heat (PTT).^{35–38} 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.^{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 toxicities³⁰ and could reduce the potential to develop MDR bacteria.

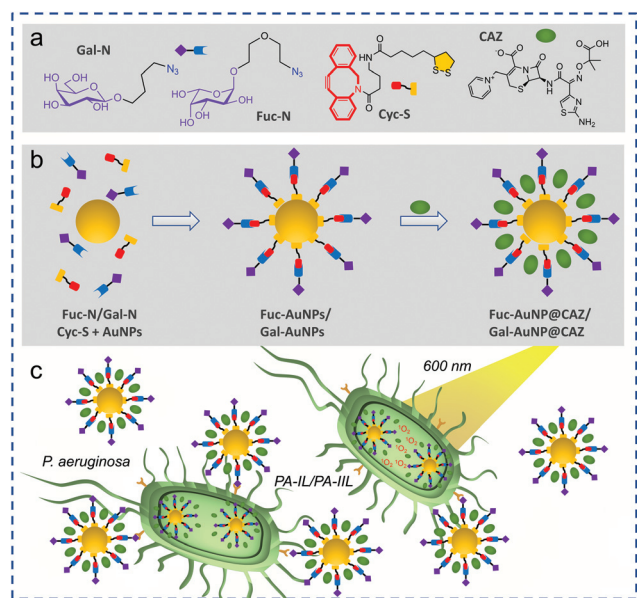
A key step in the pathogenesis of *P. aeruginosa* is the adhesion of bacterial cells to human tissue,⁴⁰ which is believed to occur through the targeted recognition of mammalian glycoproteins by virulence factors such as lectins (LecA and LecB).⁴¹ Exploiting the selective carbohydrate-based recognition 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 fluoro-

phore, dicyanomethylene-4*H*-pyran (DCM)⁴² loaded AuNPs (**Fuc-AuNP@DCM** and **Gal-AuNP@DCM**) afforded two highly selective imaging agents for the visualisation of *P. aeruginosa*. In addition, this result suggests binding with *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),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 *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.⁴⁴ Unfortunately, the use of Cu(I) ions for CuAAC chemistry can prove problematic for certain reaction substrates.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.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†).^{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.^{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 (a) Key building blocks for the construction of **Fuc-AuNPs** and **Gal-AuNPs**: carbohydrate functionalised azide (Fuc-N and Gal-N) and cyclooctyne tailed disulfide ring (Cyc-S). Antibiotic as co-loaded molecular cargo: Ceftazidime (CAZ). (b) Schematic illustration of the one-pot self-assembly procedure and co-loading of CAZ to form **Fuc-AuNP@CAZ**/**Gal-AuNP@CAZ**. (c) Schematic illustration of the targeted lectin (LecB/LecA, also called PA-III/PA-IL) approach of **Fuc-AuNP@CAZ**/**Gal-AuNP@CAZ** to selectively enter *P. aeruginosa* to simultaneously release CAZ and generate ROS/heat upon photoirradiation (600 nm).



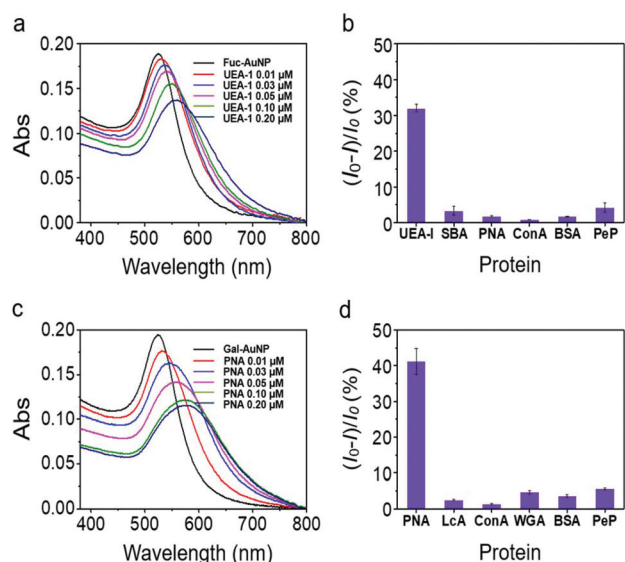


Fig. 1 (a) UV-vis spectra of **Fuc-AuNPs** (0.2 nM) with increasing concentrations of Ulex European Agglutinin 1 (UEA-1: 0.01–0.2 μM) in Tris-HCl buffer. (b) Change in UV-vis absorption at 525 nm of **Fuc-AuNPs** (0.2 nM) in the presence of UEA-1, SBA (Soybean Agglutinin), PNA, Con A (Concanavalin A), BSA (Bovine Serum Albumin), PeP (Pepsin) (protein concentrations: 0.05 μM). (c) UV-Vis spectra of **Gal-AuNPs** (0.2 nM) with increasing concentrations of Peanut Agglutinin (PNA: 0.01–0.2 μM) in Tris-HCl buffer. (d) Change in UV-Vis absorption at 525 nm of **Gal-AuNPs** (0.2 nM) in the presence of PNA, LcA (*Lens culinaris* Lectin), Con A (Concanavalin A), WGA (Wheat Germ Agglutinin), BSA (Bovine Serum Albumin), PeP (Pepsin) (Protein concentrations: 0.05 μM).

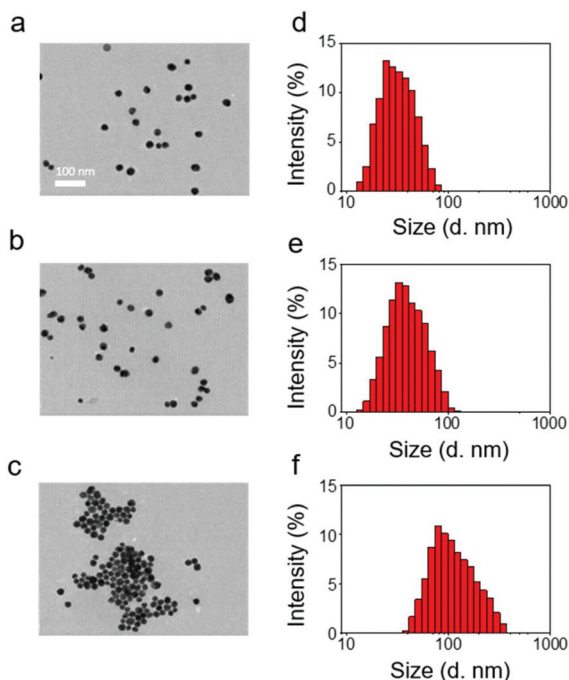


Fig. 2 High-Resolution Transmission Electron Microscope (HR-TEM) images: (a) AuNPs (0.02 nM) (b) **Fuc-AuNPs** (0.02 nM) (c) **Fuc-AuNPs** (0.02 nM) + UEA-1 (0.02 μM). Dynamic Light Scattering (DLS): (d) AuNPs (0.02 nM) (e) **Fuc-AuNPs** (0.02 nM) (f) **Fuc-AuNPs** (0.02 nM) + UEA-1 (0.05 μM).

agglutination (Fig. 2 and see ESI – Fig. S3 and S4†).⁵¹ 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 interactions⁵² 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/Gal-AuNPs**. The known ROS fluorescent probe dihydrorhodamine 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.⁵³ 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 $^1\text{O}_2$.^{33,54} 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 4*H*-pyran (DCM) (see ESI† for chemical structure)⁴² 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 D-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†).

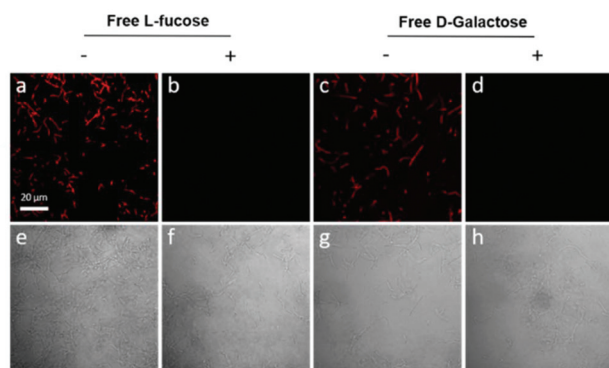


Fig. 3 Confocal Laser-Scanning Microscope (CLSM) images of *P. aeruginosa* (ATCC 27853): (a) **Fuc-AuNP@DCM** (0.2 nM and 1 μM) (b) pre-treatment with L-fucose (10 mM) followed by addition of **Fuc-AuNP@DCM** (0.2 nM and 1 μM) (c) **Gal-AuNP@DCM** (0.2 nM and 1 μM) (d) pre-treatment with D-galactose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

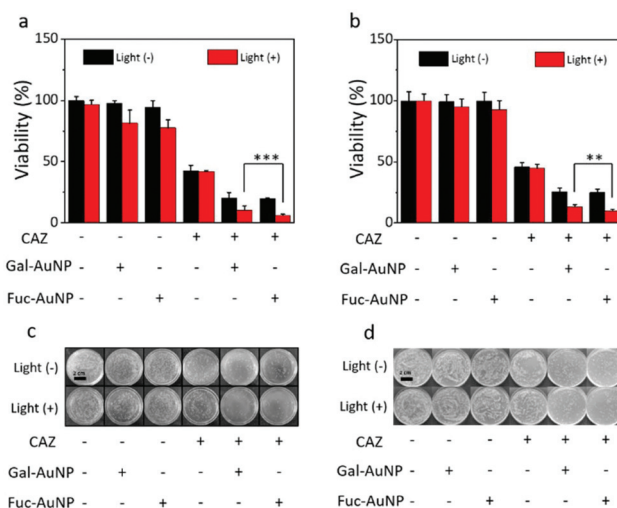


Fig. 4 (a) Relative viabilities of *P. aeruginosa* (ATCC 27853) treated with sterile water (control), **Fuc-AuNPs** (0.2 nM), **Gal-AuNPs** (0.2 nM), Ceftazidime (CAZ, 2 μg mL⁻¹), **Fuc-AuNP@CAZ** (0.2 nM and 2 μg mL⁻¹), and **Gal-AuNP@CAZ** (0.2 nM and 2 μg mL⁻¹) with and without light irradiation (30 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.

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 –



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.

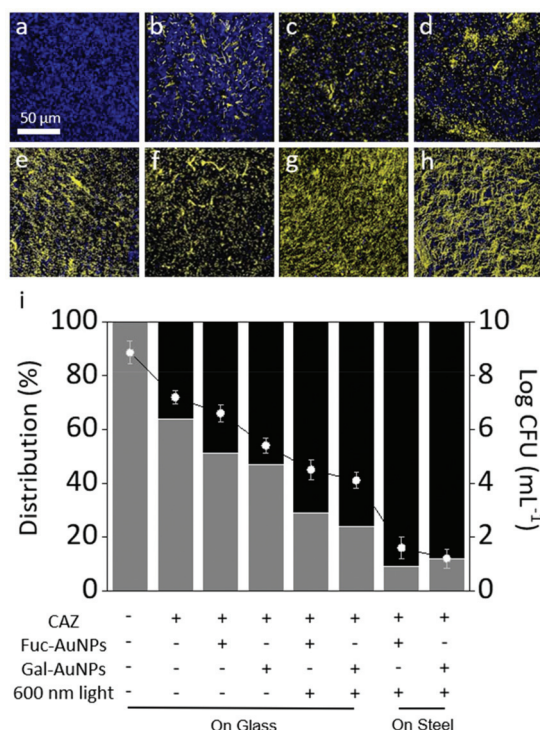


Fig. 5 The evaluation of **Fuc-AuNP@CAZ** and **Gal-AuNP@CAZ** for the treatment of biofilms. (a–f) Processed 3D images of *P. aeruginosa* biofilms on a glass-surface using the LIVE/DEAD biofilm viability assay. Dark experiments – (a) untreated (b) Cefazidime (CAZ) (32 µg mL⁻¹) (c) **Fuc-AuNP@CAZ** (0.2 nM and 32 µg mL⁻¹) (d) **Gal-AuNP@CAZ** (0.2 nM and 32 µg mL⁻¹) light experiments – (e) **Fuc-AuNP@CAZ** (0.2 nM and 32 µg mL⁻¹) (f) **Gal-AuNP@CAZ** (0.2 nM and 32 µg mL⁻¹) upon 30 min irradiation of 600 nm light (1 W cm⁻²). (g and h) Processed 3D images of *P. aeruginosa* biofilms on steel surfaces using the LIVE/DEAD biofilm viability assay. (g) **Fuc-AuNP@CAZ**-treated (0.2 nM and 32 µg mL⁻¹) and (h) **Gal-AuNP@CAZ**-treated (0.2 nM and 32 µg mL⁻¹) groups upon 30 min irradiation of 600 nm light (1 W cm⁻²). (i) Distributions of PI-stained (black, dead bacteria) and Syto9-stained (grey, live bacteria) *P. aeruginosa* biovolumes and CFU counts (white dots) of all above groups. Biovolume data are mean values of three independent replicates.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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