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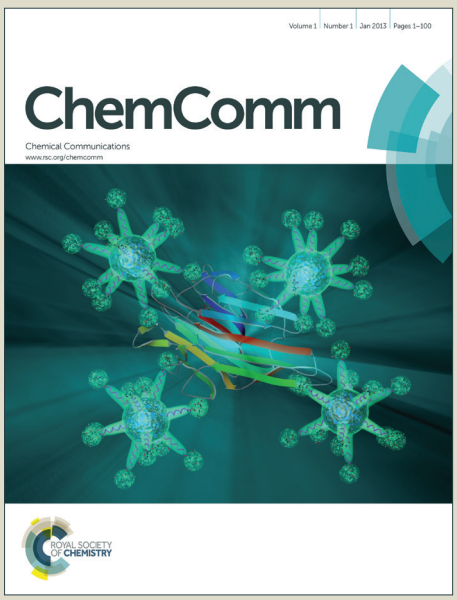

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Synthesis and Anticancer Properties of Fucoidan-Mimetic Glycopolymer Coated Gold Nanoparticles

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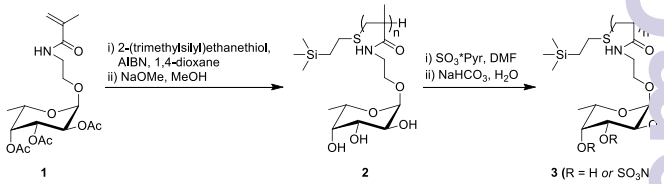
Gold nanoparticles coated with fucoidan-mimetic glycopolymers were synthesized that displayed good colloidal stability and promising anti-cancer properties. Fucoidan mimetic glycopolymers on their own were nontoxic, while glycopolymer coated gold nanoparticles displayed selective cytotoxicity to human colon cancer cell lines (HCT116) while it was non-toxic to mouse fibroblast cells (NIH3T3).

The Fucoidan is a fucose-rich sulfated polysaccharide obtained primarily from brown seaweeds. Structurally it resembles heparin, owing to the presence of L-fucose, with varying degrees of sulfation, in addition to the presence of D-xylose, D-galactose, D-mannose and glucuronic acid.[1] Fucoidan therefore possesses a range of biological activities that are similar to those of heparin, e.g. it has been report to be an anti-coagulant, anti-thrombotic, anti-angiogenic, anti-proliferative and anti-cancer etc.[2,3] The multivalent interactions of these polysaccharides with the cell surface receptors are essential for cellular response and bioactivity. Therefore, grafting these sulfated L-fucosides onto a polymeric network mimics the natural polysaccharide, facilitating multiple representation of the active molecule. Unlike the natural polysaccharide, the synthetic functional polymer can be synthesized, purified and scaled up in a controlled manner that can transform this natural product to into a medicinal product that can be advanced into clinical application. We recently reported the synthesis of fucoidan-mimetic glycopolymers (FM-glycopolymers) and showed that these synthetic polymers possess anti-viral properties similar to the natural polysaccharide.[4] Herein, we report the synthesis of FM glycopolymers via free-radical chain transfer polymerization reaction with improved polydispersity and higher yield compared to our previous report.[4]

An important criterion for the successful translation of these synthetic polymers into therapeutic molecules is their delivery to the target cells. The anionic polymer generally does not readily undergo endocytosis due to electrostatic repulsion and thermodynamic instability. However, transforming these glycopolymers into a nanoparticle formulation could promote efficient cellular delivery. Gold nanoparticles are versatile platforms for the delivery of therapeutic agents and are known to deliver large anionic polymers like glycosaminoglycans[5] and nucleic acids.[6] Apart from its carrier function,[7] gold nanoparticles have been reported to show anti-cancer properties[8] and also function as contrast agents, photothermal agents, and radiosensitizers.[9] In this study, we tested the hypothesis that coating gold nanoparticles with FM-glycopolymers, may result in a composite particle with synergistic anti-cancer effects. To validate the role of FM glycopolymer we also developed chondroitin sulfate-A (CS) coated gold nanoparticles (CS-Au-NP). CS is a natural glycosaminoglycan which is an integral part of extracellular matrix and is a structural component of human cartilage. Similar to Fucoidan, CS is a sulfated polysaccharide, however it is known to be non-toxic.



Scheme 1. Synthesis of fucoidan-mimetic glycopolymer **3**.

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To synthesize FM-glycopolymer, we utilized our previously reported fucoside monomer (2-methacrylamidoethyl 2,3,4-tri-O-acetyl-α-L-fucopyranoside)[4] 1 as a suitable starting material. Monomer 1 was polymerized in dioxane through a classical free-radical chain transfer polymerization with 2-(trimethylsilyl)ethanethiol as chain transfer agent and AIBN as the radical initiator (Scheme 1). The per-acetylated polymer was precipitated in ether, collected through filtration and de-protected in NaOMe/MeOH to give glycopolymer 2 (53% monomer conversion over two steps) upon purification by dialysis against deionized water. ^{1}H NMR spectroscopy (Figure 1) showed the presence of the trimethylsilyl protons at 0.0 ppm and was otherwise in good agreement with the results from our previously reported FM-glycopolymers.[4] Free-radical polymerization with thiols acting as chain transfer agents yields polymers with polydispersities ranging from 1.3 to 4.7.[10-12] GPC analysis of glycopolymer 2 (Figure 1B) showed a number-average molecular weight of 90600 Da and a satisfactory polydispersity of 1.32.

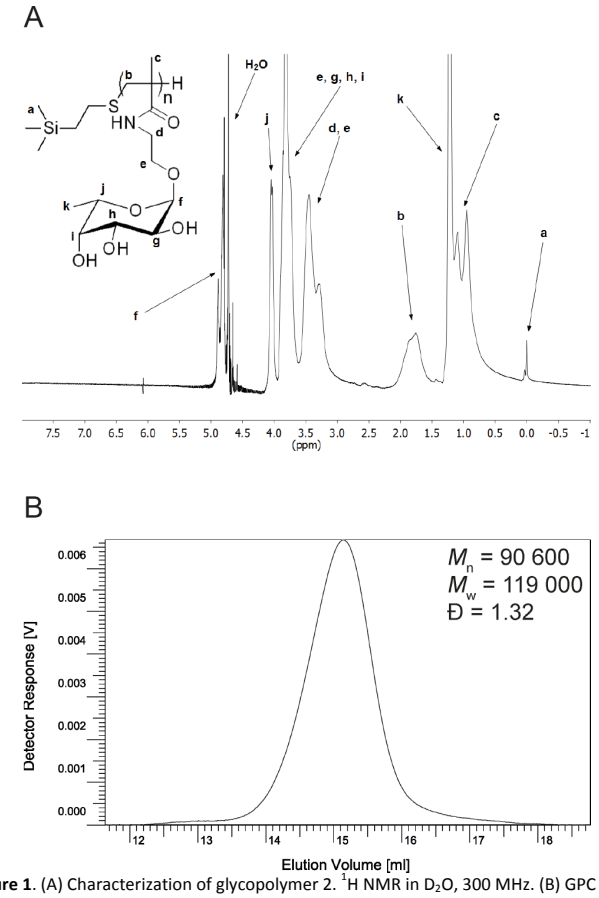
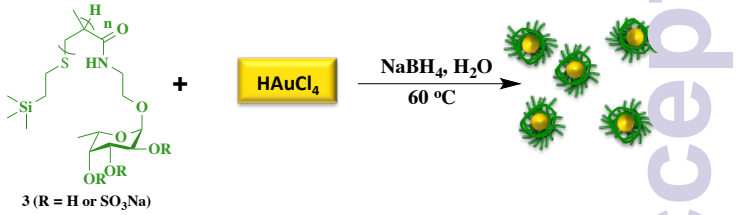


Figure 1. (A) Characterization of glycopolymer 2. ^{1}H NMR in D_2O, 300 MHz. (B) GPC elugram of glycopolymer **2**.

Glycopolymer 2 was subsequently partially O-sulfated using sulfur trioxide–pyridine complex in DMF and treated with sodium bicarbonate to furnish the sodium salt product FM-glycopolymer 3. The obtained product was purified by dialysis against deionized water. 1H NMR analysis showed a downfield shift of the fucoside protons neighboring the sulfate esters as described in our previous protocol4 thus confirming a successful O-sulfation. Elemental analysis showed carbon content to be 24.9 wt.% and sulfur content to be 14.4 wt.%, which corresponds to a hydroxyl group to sulfate ester conversion of 87%.

After successfully synthesizing the FM-glycopolymer, we synthesized gold nanoparticles using NaBH4 as the reducing agent and FM-glycopolymer as the capping agent (Scheme 2). The ratio of $HAuCl_4$, $NaBH_4$ and FM-glycopolymer was carefully optimized to deliver uniform particles with monomodal distribution. A molar ratio of 0.5 equivalents of $NaBH_4$, 1 equivalent of $HAuCl_4$ and 2 equivalents of glycopolymer (with respect to L-fucoside repeat units) was found to be efficient in producing spherical nanoparticle with average size of 44 nm as measured by dynamic light scattering (DLS) experiments (Figure 2A). As a control experiment we synthesized CS-Au-NP using $NaBH_4$ as the reducing agent, which formed the particles of 40 nm size (data not shown). Gold nanoparticles (Au-NPs) are generally stable as a colloidal suspension and a lyophilization procedure generally destroys the particle yielding macro-sized aggregates. However, for clinical translation nanoparticles that could be stored in dry state could be extremely advantageous, as this would prolong storage and would give control over particle dispersion/concentration.



Scheme 2. Schematic representation for the synthesis of FMG-Au-NP.

We anticipated that the gold particles coated with FM glycopolymer (FMG-Au-NPs) would possess good colloidal stability promoted by their inherent electrostatic repulsion. To test this hypothesis, we subjected the FMG-Au-NPs to lyophilization. Upon re-dispersion of the lyophilized sample in water we observed a change in particle size from 44 nm to 90 nm with monomodal distribution indicating re-assembly as a result of lyophilization (Figure 2C). Similarly, CS-Au-NP provided nanoparticles of 99 nm with good polydispersity (Figure S1 in supporting information or S). The UV-VIS analysis showed that the FMG-Au-NP possessed λmax at 530 nm, with single surface plasma resonance confirming that the particles were spherical in shape (Figure 2B). This was further corroborated by SEM analysis of FMG-Au-NP, which showed spherical nanoparticles ranging from 20-55 nm size distribution (Figure 2D). This difference in size distribution (as compared to DLS) is presumably due to hydration of the hygroscopic FMG coating of particles during DLS measurements, which is absent in dry samples used for SEM.

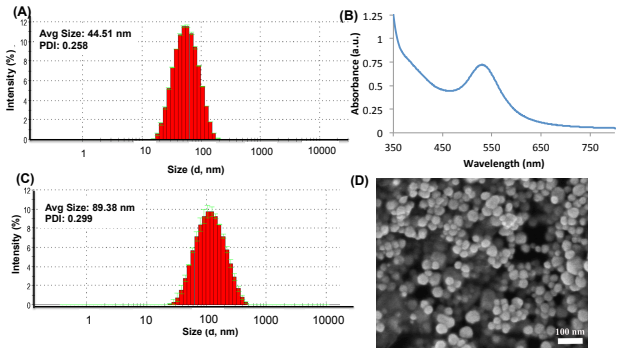


Figure 2. (A) Dynamic light scattering profile of FMG-Au-NP before lyophilization. (B) UV-VIS spectra of FMG-Au-NP. (C) Dynamic light scattering profile of FMG-Au-NP after lyophilization. (D) SEM image of FMG-Au-NP.

To evaluate the percentage of polymer presented on the gold nanoparticle surface of FMG-Au-NP and CS-Au-NP, we performed the thermogravimetric analysis (TGA). We observed that FMG-Au-NP and CS-Au-NP contained 65.55% and 83.16% of polymer respectively (Figure 3).

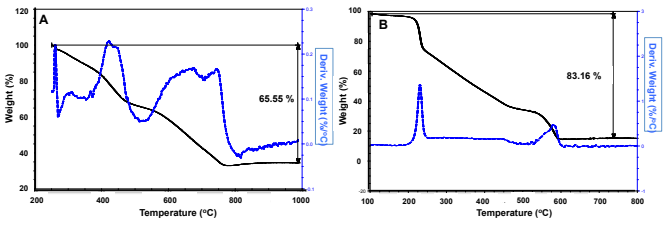


Figure 3. (A) **T**hermogravimetric analysis profile of FMG-Au-NP. (B) Thermogravimetric analysis profile of CS-Au-NP.

We then assessed the differential cytotoxic effects of FMG-Au-NPs and the FM-glycopolymer on a human colon cancer cell line (HCT116) and compared it with a non-cancer mouse fibroblast cell line (NIH3T3). We performed the cytotoxic evaluation using ApoTox-Glo™ Triplex Assay kit that assays for viability, cytotoxicity and apoptosis, following the manufacturer's protocol. We measured the cell viability and estimated the inhibition coefficient with 50% cell death (IC_{50}) for FMG-Au-NPs and FM-glycopolymer by logarithmic curve fitting of cell viability (%) using Graphpad Prism software (Figure 4). Dose dependent cytotoxicity for FMG-Au-NP was observed with the HCT116 cells while FG-glycopolymer alone had no effect (Figure 4). The IC_{50} value for FMG-Au-NPs against HCT116 cells was found to be 457.08 µg/mL, which is lower than fucoidan polysaccharides derived from *Ascophyllum Nodosum*[13] and *Saccharina Cichorioides*[14] indicating higher cytotoxicity.

Neither FM-glycopolymer nor FMG-Au-NP was cytotoxic to NIH3T3 cells. This clearly indicates that FMG-Au-NPs preferentially triggered apoptosis in the HCT116 cells. Unmodified Au-NP (citrate coated) is known to inhibit cancer cell proliferation[8] however, it is known to be nontoxic to HCT116 cells up to 1000 µg/mL concentration.[15] In order to decipher the role of FMG in FMG-Au-NP we investigate the cytotoxicity of analogous NP, namely CS-Au-NP. Interestingly, CS-Au-NP showed significantly lower cytotoxicity to cancer cells (HCT116), as compared to the non-cancerous fibroblast cells (NIH3T3; IC_{50} = 494.31 µg/mL). This clearly demonstrates that FMG-Au-NP possess selective cytotoxicity to cancer cells over normal cells.

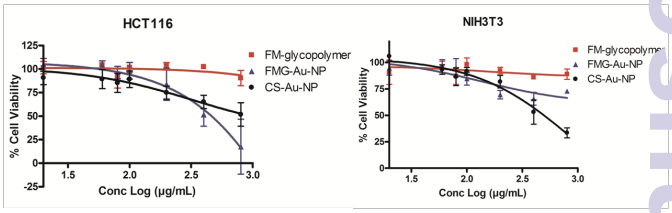


Figure 4. Dose dependent cytotoxicity profile with HCT116 and NIH3T3 cell lines

Kim et al. has earlier reported that fucoidan derived from *Fucus Vesiculosus* inhibited the growth of HCT116 cells and induces apoptosis via death-receptor-mediated and mitochondria-mediated apoptotic pathways.[16] However, Chen et al. recently concluded that the anti-tumor activity of fucoidan on HCT116 cells is exerted by modulation of the ER stress cascades.[17] Fucoidan from *Ascophyllum Nodosum* was shown to induce 57% apoptosis in 48 h at doses of 1000 µg/mL,[13] whereas fucoidan from *Saccharina Cichorioides* showed a modest 15% reduction in HCT116 cell numbers at 800 µg/mL in 24 h.[14] To further understand the mechanism by which FMG-Au-NPs exerted cytotoxicity to the HCT116 cells, we measured the activity of caspase 3/7, an endoprotease responsible for cell disassembly during apoptosis using the Caspase-Glo® Assay which provides a luminescent signal proportional to the level of caspase activity in each cell line (Figure S2 in SI). Interestingly, neither FM-glycopolymer nor FMG-Au-NPs triggered any significant caspase activity in HCT116 and NIH3T3 cell lines, suggesting that FMG-Au-NPs likely acted through caspase 3/7-independent mechanisms of apoptosis.[18]

In conclusion, we have described an efficient synthesis of a fucoidan-mimetic glycopolymer with good polydispersity and high yield. We exploited this glycopolymer for tailoring FM-glycopolymer coated gold nanoparticles. These nanoparticles when fully hydrated had an average size of 90 nm by DLS and displayed 20-55 nm when dried for SEM analysis. FMG-Au-NPs possessed good colloidal stability even after lyophilization due to electrostatic repulsion. FMG-Au-NPs showed differential cytotoxicity toward colon cancer cells and was non-toxic to a fibroblast cell line. FM-glycopolymer alone, however, was non-cytotoxic. This versatile biomimetic nanoparticle can be further explored for other biomedical applications where fucoidan are known to exhibit interesting bioactivity.

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