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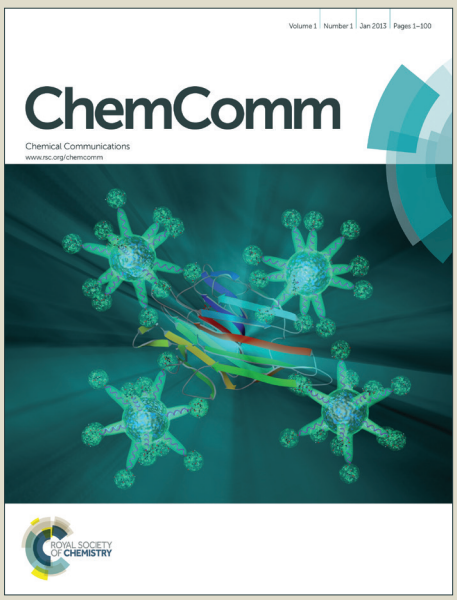

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Assembly, postsynthetic modification and hepatocyte targeting by multiantennary, galactosylated soft structures

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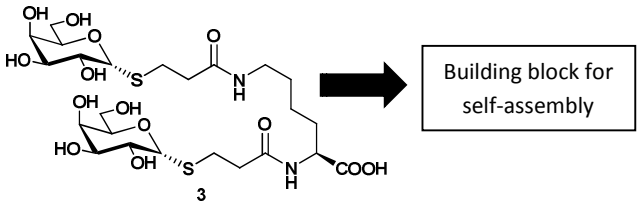
Enzyme modifiable, hollow self-assembled structures offer excellent scope for multiantennary delivery vectors. Herein, we report synthesis and applications of bis-galactose lysine based supramolecular ensembles, which possess surface galactose moieties amenable to enzymatic modifications. This post-synthetic modification generates reactive aldehyde groups, which could possibly serve as dynamic anchors for crosslinking and cell adhesion.

Autonomous self-assembly, also prevalent in nature, is a powerful tool to produce a range of hierarchical structures of varying morphologies,[1,2] where additive stabilization of non-covalent interactions affords stable structures. Bio-inspired approaches emulate naturally occurring systems to arrive at synthetic systems that are crafted to perform pre-designed activities and respond to external stimuli.[3-5] In recent time, enzymatic reactions on self-assembled substrates are considered to offer an additional level of control not achieved with light, temperature, pH variations.[6,7] Sugar based systems can be modified for therapeutic and biomedical applications.[8,9] Notably synthesis of several oligolysine-based saccharide clusters[10-12] and their interaction with a variety of cell lines and cell surface lectins[13-16] are reported. These strategies try to emulate multivalent recognition between carbohydrate ligands and cognate binding proteins, which is prevalent in biological processes[17], such as cancer metastatis[18], cellular trafficking, cell–cell interactions, and bacterial infections.

Common chemical approaches for the development multivalent saccharide display relies on carbohydrate grafting polymeric scaffolds, to afford clusters having higher affinity due to 'glycoside cluster effect'.[19,20] Another approach pertains to supramolecular glycoclusters that relies on autonomous organization of building blocks to reveal 'minicluster effect' for applications ranging from supramolecular arrangements capable of binding microorganisms[21-23] and guest molecule triggered self-organization[24], binding of specific lectins[25], study of carbohydrate-carbohydrate interactions.[26] Given our long-standing interest in peptide-based self-assembled structures,[27-34] we decided to explore supramolecular organization to achieve glycocluster synthesis based on a sugar amino acid. Herein, we report synthesis of a sugar amino acid, bis galactosylated lysine (**3**), for self-assembly followed by post-synthetic enzymatic processing of saccharide residues by galactose oxidase. We surmised that reactive galactose-6-aldehyde could also act as a potential cross-linker, through Schiff base/oxime formation with amines/hydroxyl amines.[35-37]

Sugar amino acid, **3**, was synthesized by appending two carbohydrate moieties to L-lysine amino groups, through mercaptopropionic acid linkers, using a simple protocol starting with conjugation of pentaacetylated D-galactose with 3-mercaptopropionic acid. It was followed by reaction with L-lysine to afford protected bis-L-galactose lysine (Scheme S1).[14] The acetyl groups were deprotected using NaOMe in methanol to afford **3**.



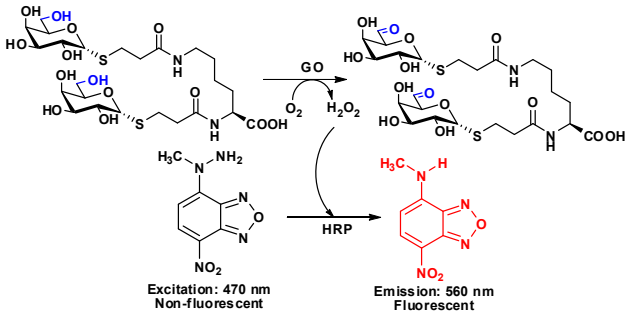
Scheme 1 Synthesis of sugar amino acid, bis-L-galactose lysine, **3.**

Notably, **3** readily afforded formation of soft spherical structures in water (Fig. S1) as characterized by multiple microscopic methods. An average cross-section of ~55 nm (Fig. S2) was observed for these structures from SEM and AFM micrographs (Fig. 1). This resulted in a facile supramolecular synthesis of glycoclusters, where galactose groups expectedly populate the exterior of these spherical structures. Carboxyl group provides two hydrogen bonds and helps create opportunity for stabilization of ordered soft structures, is implicated for soft matter formation as described recently for self-assembled monolayers and gel

formation.[38,39] While favourable interactions/conjugation of sugar residues groups were recently invoked for self-assembly and aggregation of modified graphene oxide sheets[40] and submicron-sized onion-like spherical vesicles.[41,42]

Fig. 1 a, b) AFM image and 3-D height profile of vesicles of **3**; **c, d)** SEM micrographs of vesicles of **3** (scale: 2 µm and 200 nm respectively).

We became interested in assessing accessibility and processing of galactose units in the self-assembled structures by galactose oxidase (GO),[43] which converts 6-OH group to corresponding aldehyde. The latter are useful cross linkers to interconnect proteins and peptides leading to interesting scaffolds,[44] as the aldehyde formed can undergo reactions with amino acids, similar to the Maillard reaction.[36] Formation of aldehyde on self-assembled **3** was studied spectrophotometrically using a non-fluorescent dye 4-(N-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole[45] (NBD), which reacts with hydrogen peroxide formed during the reaction,[46] in presence of horse radish peroxidase, to afford highly fluorescent N-methyl-4-amino-7-nitrobenzofurazan (MNBDA), having an emission wavelength of 560 nm.[45,47]



Scheme 2 Enzymatic conversion of **3** coupled to the conversion of NBD into its fluorescent deaminated form, MNBDA.[43]

This conversion was checked with several control reactions, which clearly suggested that the enzymatic reaction required all four components: **3**, oxidase, peroxidase, and dye, for post-synthetic modification. Reactions were also performed to verify concentration dependence by varying concentrations of one component, while keeping the others constant to reveal that increased concentration of **3** caused increased production of hydrogen peroxide, with a proportional increase in fluorescence. These experiments suggested ready accessibility of a number of galactose residues on self-assembled **3** for post-synthetic oxidation reaction. Moreover, there was no marked change in fluorescent intensity at 560 nm on changing the concentrations of GO or HRP, whereas a continuous increase in the fluorescent intensity was observed proportional to the addition of the dye NBD (Fig. S3).

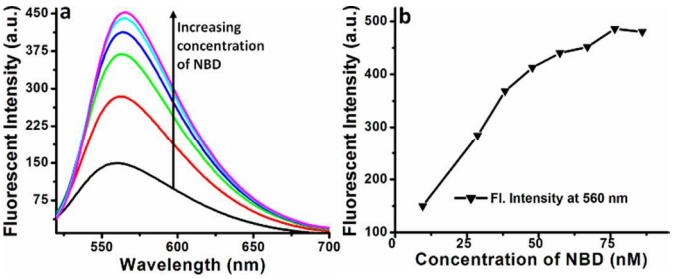


Fig. 2 a) Fluorescent intensity (initial conc. of NBD: 10 nM) after subsequent 10 nM additions of NBD (keeping concentrations of **3**, GO and HRP constant); **b)** Maximum fluorescent intensity at 560 nm with subsequent 10 nM additions of NBD.

Formation of reactive aldehyde and its ability to crosslink with amines, via Schiff base formation was studied using a few representative amines namely 1,4-diaminobutane (aliphatic diamino linker), melamine (aromatic triamino linker), and tren (aliphatic triamino linker). 6-OH group of galactose were oxidized to aldehyde using GO, which formed corresponding Schiff bases with di and tri amino linkers (Fig. 3). The reaction was monitored for increase in turbidity, owing to the formation of interconnecting fibrous networks (inset in Fig. 3).[25]

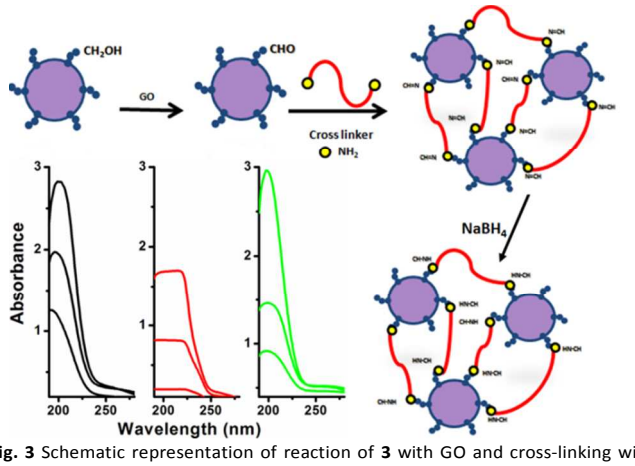


Fig. 3 Schematic representation of reaction of **3** with GO and cross-linking with amines. **Inset:** Graphs showing changes in UV spectra for the formation of Schiff base with increasing concentrations of amines 1,4-DAB (black), ii) melamine (red), iii) tren (green).

Given the reversible nature of imine equilibrium in presence of pH and temperature variation, one could envisage dynamic nature of interaction that has recently attracted much attention in self-assembled systems.[48-50] The resulting imine groups were reduced *in situ* with sodium borohydride or sodium cyanoborohydride, affording an interconnecting network, which brings the vesicles together with loss in gross morphology, as observed in dynamic transformation of vesicles to vesicle clusters and network, followed by gelation. A recent example of cationic bisaldehyde reported formation of Schiff bases in vesicle gels, which eventually lead to percolating networks (Fig. 4).[51]

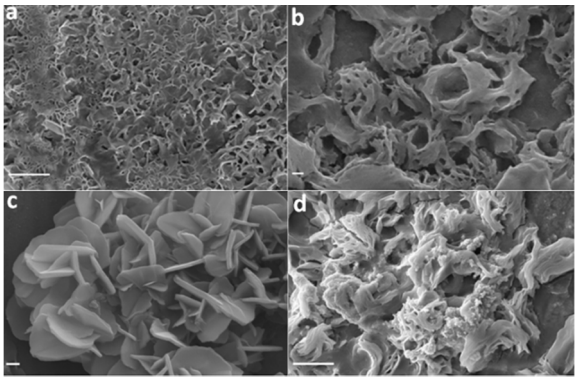
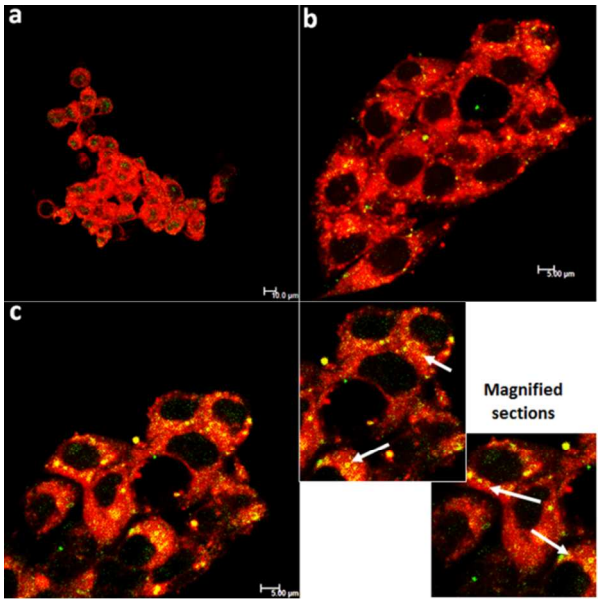


Fig. 4 SEM images of **a)** galactose-6-aldehyde (scale bar: 2 μm); **b, c, d)** Schiff bases of galactose-6- aldehyde formed using tren (scale bar: 200 nm), melamine (scale bar: 200 nm) and 1,4-diaminobutane (scale bar: 200 nm), respectively.

Galactosylated vesicles offer an attractive vehicle for liver specific delivery due to highly specific interaction between sugar residues and the asialoglycoprotein receptor present on hepatocytes, which binds galactose residues with variable affinity.[52] Transport occurs via receptor-mediated endocytosis through receptors present on hepatocytes, also providing a site for cell-cell interaction, eventually transiting to lysosomal compartments, which present degradative acidic and oxidative conditions. Needless to mention, such targeted delivery systems offer immense potential in nanomedicine of cancer, liver and other disease states.[53,54] Thus, we decided to assess potential of **3** as a delivery vehicle to transport green fluorescent gold nanoclusters (AuNCs).[55]

Human hepatocyte cell line (Hep G2) was used to study cellular uptake of **3**-AuNC complex (0.6mg/ml; Fig. S4), where the latter was incubated for 16 h to investigate the extent and location of cellular uptake. MTT studies performed with Hep G2 cells (Fig. S5), which showed that **3** lacked cytotoxicity. It was observed that **3**-AuNCs were internalized into cytoplasmic regions of the cell (Fig. 5c). It appears that the presence of **3** lead to a uniform distribution of AuNCs inside cells. Control experiments show that this vehicle also has effect on AuNC distribution in Hep G2 cells, suggesting benefits of targeted delivery by **3**. It is surmised that by further modifying galactose display to bi-, tri- or multi-antennary presentation,[14,55,56] selective stable delivery to hepatocytes, by self-assembling vehicles could be studied.

Fig. 5 Confocal laser scanning microscope images of Hep G2 cell lines (λ_{em} = 510 nm); cell membrane stain deep red plasma membrane dye was used as a reference and it exhibits red emission (λ_{em} = 633 nm); **a)** Hep G2 cells incubated with AuNCs alone for 16 h; **b, c)** Images of Hep G2 cells incubated with **3**-AuNC complex for 16 h. (Arrows showing the prominent green fluorescence of **3**-AuNC complex in cell cytoplasm).



In summary, we have presented a facile strategy to construct galactosylated soft structures and their enzymatic modification. In addition to being non-toxic to cells, we have explored its ability to act as a delivery vehicle by transporting fluorescent gold nanoclusters into hepatocytes, through a mechanism yet unclear. Further optimization of these multiantennary, galactose-presenting supramolecular structures is underway.

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