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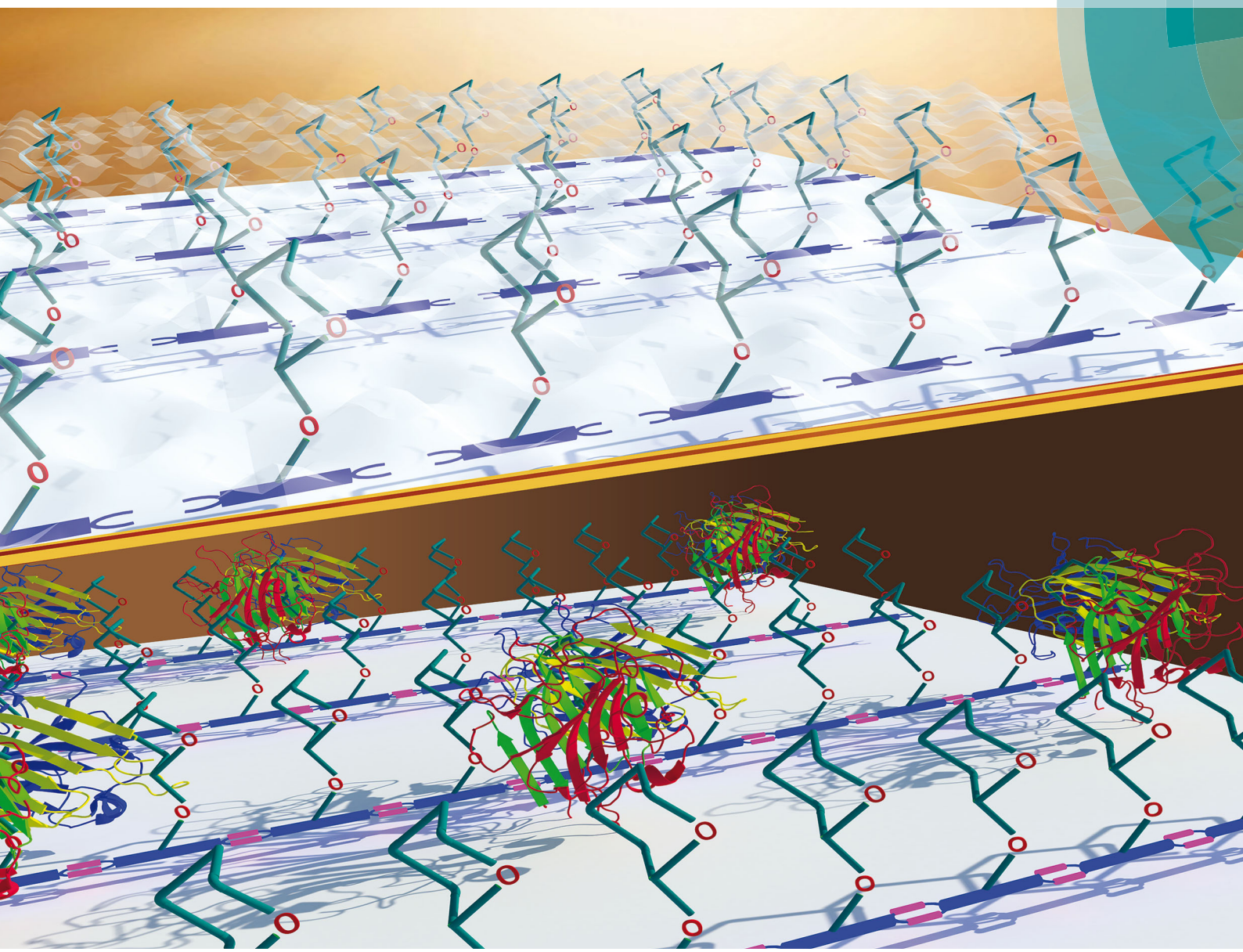

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Organogel-assisted topochemical synthesis of multivalent glyco-polymer for high-affinity lectin binding†

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An organogelator, 2,4-undeca-diynyl-4′,6′-*O*-benzylidene-β-D-galactopyranoside, which aligns its diacetylene upon gelation, has been synthesized. UV irradiation of its gel resulted in topochemical polymerization of the gelator forming polydiacetylene (PDA). We have used this gel-state reaction for the synthesis of surface-immobilized multi-valent glycoclusters, which showed 1000-fold enhanced binding, compared to monomers, with various galactose-binding lectins.

Gels formed by the self-assembly of low molecular weight gelators in solvents through various non-covalent interactions have attracted much attention[1] due to their potential applications in many fields such as sensors,[2] templated synthesis of materials,[3] oil-spill recovery,[4] stimuli-responsive materials,[5] electronics,[6] catalysis,[7] soft-optics,[8] drug delivery systems,[2,9] implants,[9] *etc.* The self-assembly of gelator molecules makes them proximally and spatially confined forming microstructures such as fibers, vesicles, *etc.*, which prevent the fluidity of the solvent leading to gelation. Topochemical reactions, the chemical reactions in crystals,[10] occur due to the proximal confinement of reactant molecules in crystal lattices, and there have been many elegant and successful endeavors in exploiting the proximal confinement of molecules in gels[11] and other ordered media[12] for topochemical reactions. The major challenge in this field is the design of a gelator, which can self-assemble in such a way that its reacting motifs are arranged at proximity for them to undergo topochemical reactions. Herein, we report the design and synthesis of a carbohydrate-based organogelator containing a diyne motif which aligns its diyne motifs at proximity in its gel and its exploitation for the gel-assisted topochemical synthesis of a multivalent galactopolymer having 1000-fold enhanced binding affinity compared to monomeric ligands for galactose-binding lectins (Fig. 1).

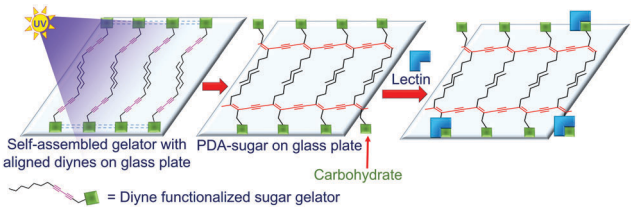

Fig. 1 Proposal for surface-immobilization of glycoclusters *via* topochemical polymerization of diyne-functionalized carbohydrate gelators to PDA–sugars in the gel state.

Carbohydrate–protein binding interactions are involved in many cellular recognition processes such as cell–cell adhesion, cell–cell communication, fertilization, antigen–antibody interactions, inflammation, pathogen infection, cancer metastasis, *etc.*[13] However, the binding affinity of a single carbohydrate unit with protein is rather weak and in order to circumvent this, nature uses multivalent glycoconjugates for efficient interaction.[14] There is great interest in synthetic multivalent glycoconjugates[15] as they not only serve as tools to study the effect of multivalency but also are important to develop biosensors based on carbohydrate–protein binding.[16] Pathogens such as bacteria, viruses, and protozoa express a large number of lectins or carbohydrate-binding proteins on their cell surface and use their interaction with host–cell glycoforms for infection and virulence.[17] Also, many lectins are over-expressed in cancer cell surfaces and are involved in tumor cell differentiation, metastasis, tumor progression and adhesion of cancer cells.[17] Thus easy-to-process multivalent synthetic glycoforms that can be immobilized on surfaces (*e.g.* glass plates) are important for the development of diagnostic tools.[18] However, apart from the difficulties associated with the synthesis of glycopolymers due to the involvement of air-sensitive reactions, the use of catalyst, high temperature, difficult purification, non-uniform and incomplete sugar-loading, *etc.*, their immobilization on solid surfaces is cumbersome and often requires functionalized surfaces.

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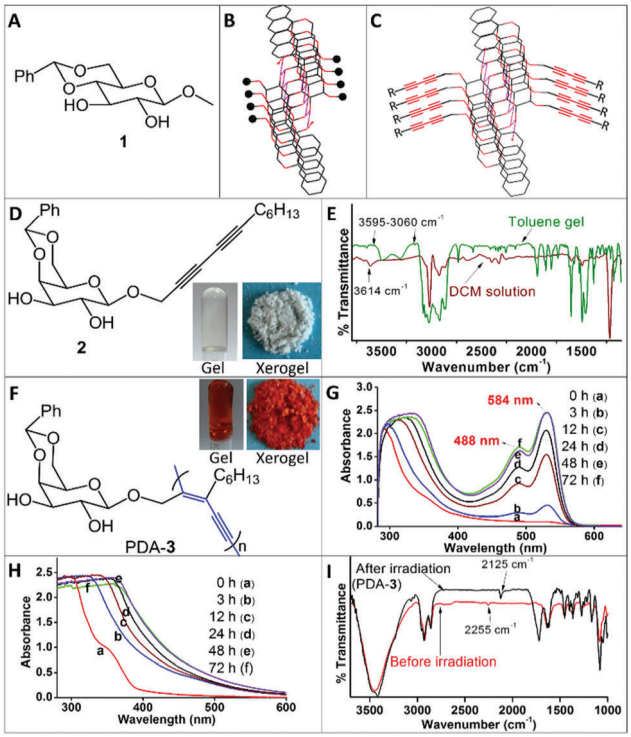

Fig. 2 (A) Chemical structure and (B) packing of 4,6-*O*-benzylidene methyl β-D-glucopyranoside (**1**) in its gel showing a stacked arrangement of methyl groups; (C) proposed packing arrangement of the diacetylene functionalized gelator; (D) chemical structure of a photo-polymerizable organogelator **2**. An inverted test-tube image of toluene gel is also shown; (E) IR spectral comparison of DCM solution with the toluene gel of diyne **2**; (F) chemical structure of PDA-**3** obtained by topochemical polymerization of diyne **2** in the gel state. A photograph of the polymerized gel is also shown; (G) and (H) time-dependent UV-visible spectra of **2** in toluene gel and in DCM solution, respectively; and (I) an overlay of FT-IR spectra of xerogels made before (**2**) and after UV-irradiation of toluene gel (PDA-**3**).

We envisioned that a diacetylene-modified carbohydrate gelator, which aligns its diacetylene motifs coaxially in its gel, would undergo topochemical polymerization to provide PDA with pendant carbohydrate units and such a solution processable gelator would be suitable for easy and direct immobilization on an untreated surface. Shinkai *et al.* developed various organogelators having a 4,6-*O*-benzylidene glycopyranoside skeleton, which undergo 1D hydrogen-bonded self-assembly leading to fibrils with a stacked arrangement of the anomeric substituent (Fig. 2A and B).[19] We have adopted this scaffold for designing a gelator for the generation of semi-conducting fabrics.[11a] In order to have an appropriate mimic of cell–surface glycoforms which are attached to either lipids or hydrophobic membrane-spanning proteins, we have chosen a diyne flanked by a sugar unit and a long alkyl chain which upon polymerization would give multivalent sugars protruding from a hydrophobic environment. Also the van der Waals interactions between alkyl chains of adjacent polymer strands make them associated even after polymerization. Thus we have synthesized diyne **2** (Fig. 2D) and to our satisfaction, it congealed various non-polar solvents and oils at low critical gelation concentrations (CGCs, Table S1, ESI†).

The involvement of hydrogen bonding in gelation was evidenced from a comparison of IR spectra of **2** in its dissociated state in dichloromethane (DCM) solution and its self-assembled state (toluene gel); the OH stretching appeared as a sharp signal at 3614 cm^{-1} in DCM, suggesting the presence of free hydroxyl groups, and as a broad signal in the range of 3595–3060 cm^{-1} in toluene gel, suggesting that hydroxyl groups are hydrogen bonded in the gel (Fig. 2E). A concentration dependent ^{1}H NMR study in the gelling solvent (C_6D_6) showed broadening and downfield shifting of OH proton signals with an increase in the concentration of diyne **2** (Fig. S2, ESI†). These experiments prove that intermolecular hydrogen bonding is the major driving force for self-assembly and gelation. However, the role of van der Waals attraction in gelation cannot be ruled out. Scanning electron microscopy (SEM) of the xerogel of diyne **2** showed a globular morphology (Fig. S3, ESI†). The Powder XRD (PXRD) pattern of the xerogel of **2** (made from toluene gel), though broad, showed some similarity with the PXRD pattern of diol **1**, suggestive of their similar packing, supporting our design (Fig. S4, ESI†).

To check the feasibility of the topochemical reaction of **2** in the gel state, we have irradiated its colorless transparent toluene gel with UV light of wavelength 300 nm for 2 days. The gel became orange-red in color, suggestive of the topochemical polymerization of diacetylene to PDA (Fig. 2F). T_{gel} at the CGC of this irradiated gel was increased by 5 °C, indicative of strengthening of the gel presumably due to the formation of PDA *via* topochemical polymerization of diacetylene (ESI†). Time-dependent UV-Vis spectroscopy also provided evidence for the topochemical polymerization of diyne **2** in its gel (self-assembled state). Prior to irradiation, the absorption spectra of toluene gel had only one absorption peak at 291 nm. Upon irradiation, two new absorption bands characteristic of PDA were observed at 488 nm and 584 nm (Fig. 2G). Intensities of these bands gradually increased with the duration of irradiation and reached a steady state in three days of irradiation. On the other hand, the absorption spectrum of a DCM solution (dissociated state) of diyne **2** after irradiation for a similar duration did not show any absorption band characteristic of PDA (Fig. 2H), suggesting that the hydrogen-bonded self-assembly is essential for the formation of PDA.

The topochemical reaction was further probed by FT-IR spectroscopic studies. The disappearance of the IR band at 2255 cm^{-1} due to the C≡C bond stretching of diyne **2** and the appearance of a new IR band at 2125 cm^{-1} characteristic of the C≡C stretching of PDA after irradiation (Fig. 2I) gave additional evidence for the photo-induced topochemical polymerization. Time-dependent Raman spectroscopy was also used to investigate the diacetylene polymerization of diyne **2**. During irradiation (λ 300 nm) of a toluene gel of **2**, small portions of the gel were removed at different intervals, freeze-dried to xerogels and analyzed using Raman spectroscopy. The intensity of the Raman band at 2257 cm^{-1} due to the C≡C bond stretching of diyne **2** diminished with the concomitant development of a new band at 2119 cm^{-1}, characteristic of PDA (Fig. 3A and Fig. S7, ESI†), with the duration of irradiation.

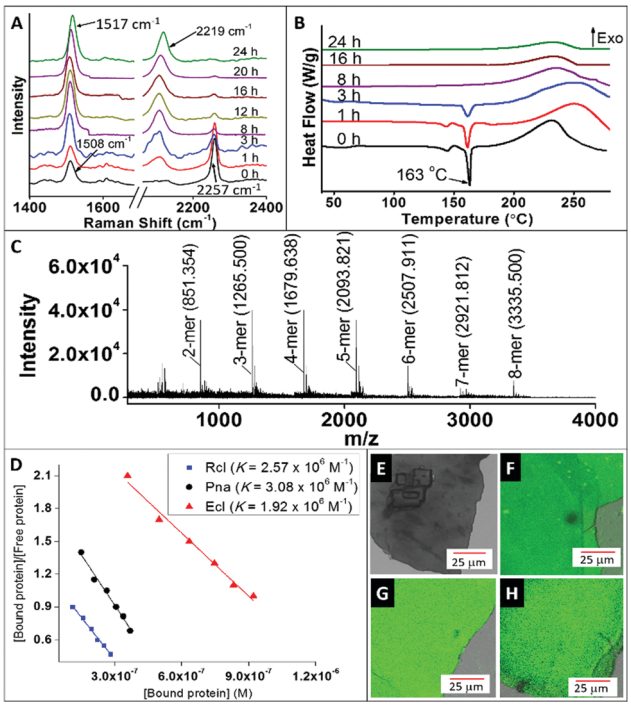

Fig. 3 (A) Time-dependent Raman spectra of xerogels made from the toluene gel of **2** irradiated with 300 nm UV light for different durations; (B) time-dependent DSC analyses of xerogels made from the toluene gel of **2** irradiated with UV light; (C) MALDI-TOF spectrum of PDA-**3** in DMSO showing the presence of oligomers ranging from 2-mer to 8-mer; (D) Scatchard plot for Pna (circle), Ecl (triangle) and Rcl (square) binding to PDA-**4**; confocal fluorescence microscopy images of PDA-**4** before (E) and after incubation with (F) Pna, (G) Ecl and (H) Rcl.

Differential scanning calorimetry (DSC) analysis of the xerogel of **2** showed an endothermic peak at 163 °C (due to melting) and an exothermic broad peak at 180–250 °C due to the uncontrolled thermal cross-polymerization reactions of diacetylene. DSC analysis of the xerogels of the gel irradiated for different durations showed a gradual decrease in the intensity of the exothermic peak (due to thermal reaction) and the disappearance of the endothermic peak due to melting with irradiation time (Fig. 3B). These observations are suggestive of time-dependent consumption of diyne and consequent formation of non-melting PDA, *via* topochemical polymerization. The globular morphology was intact even after polymerization, as evidenced from SEM analysis of the xerogel of the irradiated gel (Fig. S8, ESI†). PXRD analyses of xerogels of the fresh toluene-gel (parent phase) and the irradiated toluene-gel (daughter phase) of **2** provided additional evidence for the topochemical nature of polymerization. Apart from retaining the sharp peaks of the parent phase, additional sharp peaks appeared in the daughter phase (Fig. S9, ESI†), suggesting the preservation of the ordered nature during and after the reaction. The MALDI-TOF spectrum of the soluble fraction of PDA-**3** in DMSO showed the presence of oligomers up to 8-mers (Fig. 3C). However, it is to be noted that most of the sample is insoluble even in DMSO, suggesting that higher oligomers/polymers are formed in the reaction.

To test the proposed hypothesis, we have coated a toluene gel of **2** on a glass slide (1.2 cm × 1.2 cm) and irradiated it with 300 nm UV light for 7 days, by which time the colorless gel turned into a red film. While benzylidene is necessary for self-assembly, it has to be removed before investigation of the binding ability of this PDA based glycopolymer with lectins. Thus, the PDA-**3**-coated glass slide was dipped in dil. HCl solution for 3 h and then washed many times with water and finally with phosphate-buffered saline (PBS, pH = 7.4). The de-protected polymer PDA-**4** was characterized by Raman, FTIR and solid-state UV spectroscopy techniques. The FTIR spectrum of PDA-**4** showed an increase in the intensity of the peaks due to OH stretching and also the shifting of peaks due to C≡C bond stretching from 2125 cm^{-1} to 2133 cm^{-1} (Fig. S11A, ESI†). A comparison of Raman spectra of PDA–sugar before (PDA-**3**) and after HCl washing (PDA-**4**) showed a shift of the C≡C bond stretching band from 2116 cm^{-1} to 2154 cm^{-1} (Fig. S11B, ESI†), suggesting the deprotection of the benzylidene group. A comparison of the solid-state UV-Vis spectra of PDA-**3** and PDA-**4** showed a drop in the intensity of the absorption at λ below 280 nm (band due to the phenyl ring) in the latter, suggesting the removal of the benzylidene group (Fig. S12, ESI†).

For the binding study, we have chosen three fluorescein-labelled plant lectins *viz.* peanut agglutinin (Pna), *Erythrina crista-galli* lectin (Ecl) and *Ricinus communis* lectin (Rcl) which are known to bind β-D-galactosides selectively. As the glycopolymer (PDA-**4**) was immobilized, the Scatchard plot method[20] was employed for the determination of the association constants of interaction between the glycopolymer and lectins (ESI†). In this method, the ratio of concentrations of the bound ligand to the unbound ligand (*Y*-axis) against the concentration of the bound ligand (*X*-axis) is plotted. If the binding is not co-operative, the plot will be a straight line, the slope of which will give the association constant and its intercept at the *X*-axis would give the maximum binding.[20]

Similar amounts of PDA-**4** were immobilized on glass slides by drop-casting equal volumes of the monomer solution in toluene (gelling solvent) followed by photoirradiation of the gel formed on the glass slides to polymerize it to PDA-**3** and final deprotection of the benzylidene protecting groups by a mild acid. These PDA-**4**-coated glass slides were placed in wells of a 24-well cell culture plate and incubated with protein solutions of different concentrations. Concentrations of the unbound proteins in each well were measured from absorbance of the supernatant solution at λ = 494 nm, which is λ_{max} of the fluorescein-conjugated protein. Scatchard plots, plots of the ratio of concentrations of bound protein to unbound protein against the concentration of bound protein, were found to be straight lines in all the cases (Fig. 3D). Association constants (the slope of the lines) of PDA-**4** towards Pna, Ecl and Rcl were found to be $3.08 \times 10^6\ M^{-1}$, $1.92 \times 10^6\ M^{-1}$ and $2.57 \times 10^6\ M^{-1}$, respectively. It is to be noted that Ecl, Pna and Rcl bind to methyl-β-D-galactoside (monosaccharide) with affinities of $0.88 \times 10^3\ M^{-1}$, $1.00 \times 10^3\ M^{-1}$ and $7.70 \times 10^3\ M^{-1}$, respectively.[21] This suggests that PDA-**4** shows 1000-fold higher affinities as compared to the monomeric ligands. Like in the

case of other multivalent ligands, PDA-**4** having several pendant sugar units might be increasing the effective local concentration (statistical rebinding) near the binding site of lectin.[16] Lower oligomers formed by oligomerization of sugar diynes in solution bind lectins with much lower affinity.[22]

Confocal fluorescence microscopy of the glass slides (λ_{Ex} = 493 nm and λ_{Em} at 510–525 nm) clearly showed uniform distribution of proteins over PDA-**4**-coated glass plates for all the three cases (Fig. 3E–H). From the Scatchard plot, the maximum amounts of PNA, Ecl and Rcl that can be bound on PDA-**4** were estimated to be 144 mg g^{-1}, 168 mg g^{-1} and 122 mg g^{-1}, respectively.[20] To rule out non-specific binding, we have also synthesized PDA-**5** by cleaving the galactose units from PDA-**4** upon treatment with conc. HCl for 3 h, followed by washing with water. The formation of PDA-**5** was confirmed by FTIR, Raman and solid-state UV-Vis spectroscopy (Fig. S20–S22, ESI†). The concentration of protein solution before and after incubation with PDA-**5** was the same, suggesting that there is no interaction between lectins and this PDA. Also the confocal microscopy of the slides did not show any fluorescence due to the lectins, ruling out the non-specific binding (Fig. S23, ESI†). This confirms that multiple carbohydrate ligands are responsible for the superior binding capability of PDA-**4**.

In summary, we have designed a diyne-functionalized carbohydrate gelator which upon gelation aligns its diyne motifs at proximity as a result of which the gelator can undergo topochemical polymerization to PDA in the gel state. Using this method, we have developed a PDA-based glycopolymer having many galactose units on a simple glass plate, which was found to bind different galactose-binding lectins with 1000-fold higher affinity than monomeric ligands. The scalability and processability of the gel-based system make our method superior to other traditional methods of synthesis of multivalent ligands on surfaces.

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