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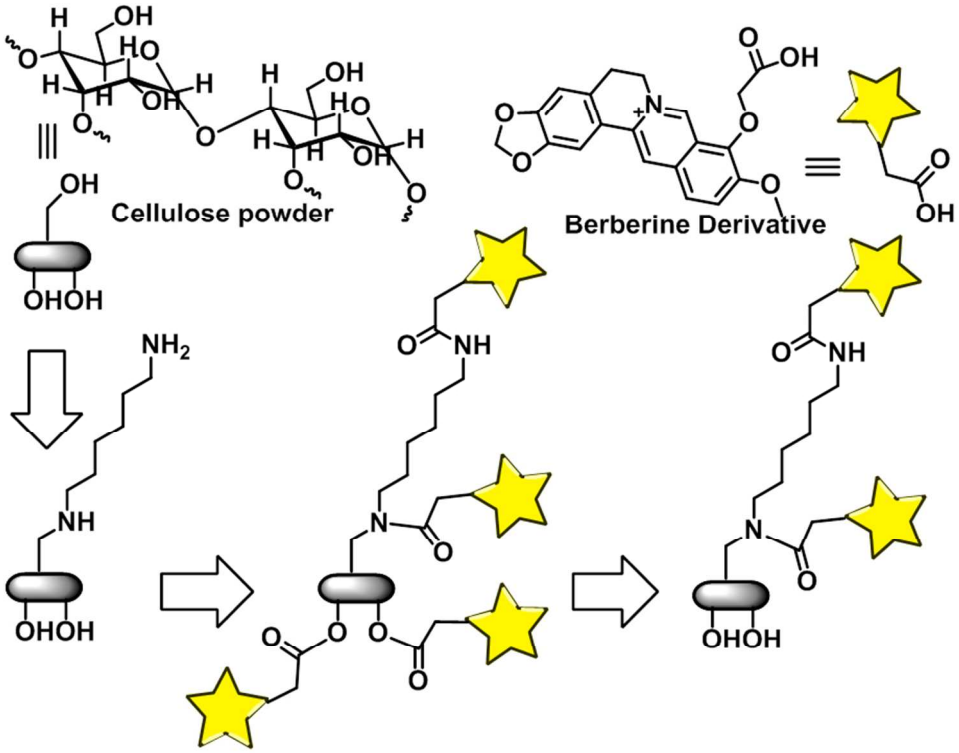

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Synthesis and spectroscopic studies of Berberine immobilized modified cellulose material

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This report describes the synthesis, characterization and spectroscopic studies of Berberine immobilized modified cellulose materials which would be new promisable biocompatible fluorescence material because Berberine is natural fluorescent molecule having important pharmacological aspects such as selective binding with DNA G-quadruplex. Thus Berberine immobilized cellulose materials would be applicable in screening of G-quadruplexing DNA sequence by bio-imaging techniques.

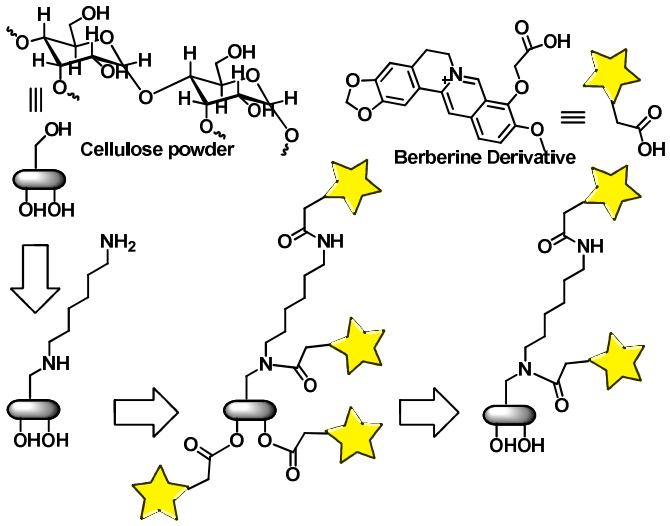
Cellulose is most abundant natural biopolymer containing long chain of anhydroglucose units which are linked together by β-1,4-glycosidic bond.[1, 2] Cellulose and related polymers are occurred in nature as major component of plants' cell-wall and woody-tissues where these polymers bind to proteoglycan materials. Cellulose contains multiple hydroxyl functional groups at surface. Thus its chemical relativities are almost like aliphatic alcohols, which are available for further transformation into other desired functional groups such as, amino and carboxylate group. By altering cellulose chemical functionality, many cellulose functional materials have been synthesized and studied in progress of new functional material.[3-5] Recently the morphologies of cellulose surface are easily achieved in control approach with gentle chemical reaction. For examples, surface properties such as aliphatic, aromatic and hydrophilic of cellulose are accomplished by incorporation of respective nature (aliphatic, aromatic and hydrophilic) of amino acids at cellulose *via* ester bond.[6,7] In spite of multiple hydroxyl groups, cellulose material are usually insoluble in most of the organic solvents and recently has been used as solid support for peptide synthesis.[8] Fascinatingly, cellulose supported peptides have been synthesized and used in development of peptide-array experiments for cell-adhesive peptides analysis.[9] Recently bioactive natural products as chalcones analogues have been attached with cellulose in expansion of microarrays for screening of antibacterial activities.[10]

As in progress, biocompatible fluorescence materials as fluorescent labeled cellulose materials are also generated efficiently.[11, 12] Due to its biocompatibility and hydroxyl functionality, the chemical syntheses of cellulose functional materials are becoming an emerging area of research and have been exploited for industrial and medical applications including drug delivery agent.[13] In repertoire of new cellulose functional materials, we planned to immobilize bioactive fluorescent natural products such as Berberine on cellulose material. As it is known, Berberine is potential *anti*-tumor and *anti*-cancer drug candidates and has been considered as potential therapeutic agent.[14-16] In addition Berberine and related compounds have high color index and are being used as dyeing agent in textile and leather industries. Moreover Berberine and related derivatives are small fluorescent molecules which can easily be visualized, even at low concentration, by fluorescence spectrophotometer and other available bioimaging techniques. Herein we report the synthesis; characterization; and photophysical properties of Berberine immobilized modified cellulose material *via* hexamethyl linker with amide bond which would be potential fluorescent functional material to find DNA G-quadruplex forming DNA sequences in presence of other secondary structures (duplex and triplex) forming DNA sequences. By doing this, off target effect of berberine related pharmalogical properties can be minimized.

Results and Discussions

The stepwise synthesis of modified cellulose materials are given in Scheme 1. The hydroxyl functional group of cellulose powder (**1**) was derived into *O*-tosylate derivative of cellulose (**2**) by following reported procedure[10,11] and then treated with hexamethylenediamine under basic condition by following other literature procedure.[10,12] This amino functionalized cellulose materials were characterized by FT-IR and powder X-ray diffraction methods (Fig 1). Comparative studies are given in below. In further amine derived cellulose material (**3**) was verified with positive Kaiser'Test or ninhydrin test where amino functionalized cellulose material **3** was turned into dark blue colour after gentle heating in presence of Ninhydrin. To immobilize berberine on amino functionalized cellulose material, Berberine hydrochloride salt was functionlized into carboxylate derivative (**4**) by following literature procedur,[17] where Berberine was treated with ethylbromoacetate at high temperature under

Scheme 1. Synthesis of Berberine Immobilized cellulose material



Reaction and condition: a. $TsCl/Et_3N$; b. Hesamethyldiamine/DMF/; c. **4**, EDC/DMF/ DIEA; d. Aq. NaOH (1N).

vacuum and then with aq. NaOH. Synthetic route is provided in ESI (S3). The characterization data (^{1}H-NMR & Mass) of **4** are also provided in ESI (Fig S2-S3). This carboxylic acid functionalized Berberine derivative (**4**), in excess, was treated with amino functionalized modified cellulose material (**3**) in presence of peptide coupling reagent EDC/DIEA/DMF under anhydrous condition and the reaction was monitored by Ninhydrin test. After completion of reaction, reaction mixture was washed thoroughly with following solvents order- water, ethanol and ether, to remove unreacted Berberine carboxylate derivative and excess coupling agents. As resultant yellow color powder was obtained which could be Berberine immobilized cellulose material *via* ester and amide bond. To remove ester bonded berberine, this material was further treated with aq. NaOH (0.1N) for 0.5h which exclusively hydrolyzed ester bond in presence of amide bond. Then this hydrolyzed yellow cellulose powder was washed with following solvents: water, ethanol and ether and then separated out pale yellow color material as conceivably amide bonded Berberine immobilized cellulose materials (**6**). To ensure the immobilization of Berberine at cellulose surface, powder X-ray diffraction pattern of modified cellulose/Berberine materials (**1-6**) were recorded, which are depicted Fig. 1. The diffracted X-ray intensity peaks for native cellulose (**1**) are appeared at angle ($2\theta^o$) = 22.6^o, 16.3^o, and 14.8^o in diffraction plane (002), which are reportedly characteristic for native cellulose (**1**).[18] We have used these values as control sample in comparative studies. The X-ray diffractograms of modified cellulose materials **2&3** have shown similar patterns as of native cellulose (**1**) but their peak intensities are significantly depleted at angle 22.6^o, which evidently support the partial modification of native cellulose material. This hexamethylenediamine is immobilized on cellulose *via* substitution reactions by replacement tosyloxy group. However, the X-ray diffractograms of Berberine derivative **4** shows two peaks at angle (2θ) 40.1^o (strong) and 34.2^o (very weak) and that of berberine immobilized cellulose material (**5**) shows three peaks at angle (2θ) 45.2^o (strong), 31.5^o (strong), 22.5^o (weak). Thus the presence of small peak at angle (2θ) 22.5^o and broad hump at 14-16^o in difractograms of modified cellulose material **5** are equivalent to peak angle of cellulose materials (**1-3**) though peak intensity is low. These results support the immobilization of Berberine at cellulose surface *via* amide bond through linker.

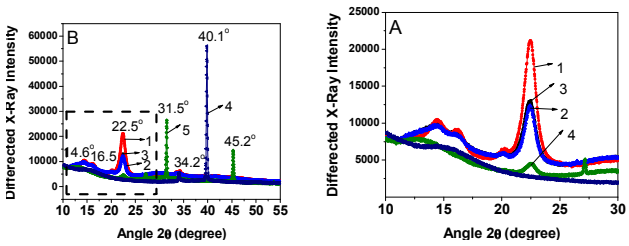


Fig 1. X-Ray diffraction pattern of native cellulose (**1**), tosylate derivative **(2)**; amine derivative **(3);** berberine derivative **(4)** and berberine immobilized cellulose material **(5)-** Full range (A) and Extended region (B).

In further, the FT-IR spectra of **1/2/3/4/5** were recorded from their freshly prepared pellets with KBr. These spectra are provided in Figure 2. In case of native cellulose (1), IR frequencies at 3000-3600 cm^{-1} are reportedly belong to the characteristic OH stretching band of cellulose while IR frequencies at 1650cm^{-1} support the bending of adsorbed water molecules at cellulose surface.[1] From a comparative studies, IR peak frequencies of amino functionalized derivative (**3**) are almost overlapped with that of native cellulose (**1**). However IR peak frequencies of Berberine derivative (**4**) appear at 3415 cm^{-1} (stong) which possibly belongs to O-H stretching of its carboxylic acid functional group. Nevertheless, IR frequencies of tosylate derivative (**2**) and Berberine immobilized modified cellulose (**5**) are almost overlapped with that of native cellulose with an exception at frequencies region 1200-1400cm^{-1}. The IR peak frequencies of modified cellulose material **5** are clearly disappearing at range of 1000-1450cm^{-1} region. Additionally, Beberine immobilized cellulose derivative (**5**) also exhibit two closed IR peaks 1600-1700cm^{-1} probably owing to amide bending. These IR results further support a partial the modification of native cellulose surface upto some extent. Finally we tried to characterize the modified cellulose derivative (5) by ^{1}H/^{13}C-NMR and ESI-mass methods. But ^{1}H/^{13}C-NMR and ESI-mass spectra of **5** are similar to native cellulose (**1**) (Fig S4-5). Possibly due to partial modification of Berberine at cellulose surface and high molecular weight of cellulose, characteristic NMR and mass peaks of Berberine are masked. At this stage we are unable to trace out exact amount of Berberine residue at surface of modified cellulose materials, **5** & **6** by NMR. Therefore we planned to study the surface morphology of modified cellulose and compared with control sample, unmodified native cellulose.

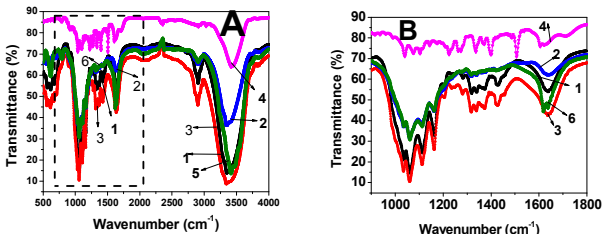


Fig. 2 FT-IR spectra (solid probe) of 2/3/4/5 full rage (A); extended range (B).

As it is known, surface morphology of cellulose are depends upon the physiochemical nature of covalently modified chemical entities. Without further characterization, the surface morphologies of modified cellulose materials were studied with well known SEM (Scanning electron microscope) techniques by comparing with control samples, native cellulose.[19] Thus thin layers film at silicon sheets were prepared from DMSO solution of materials **1**/**3**/**4**/5/**6**

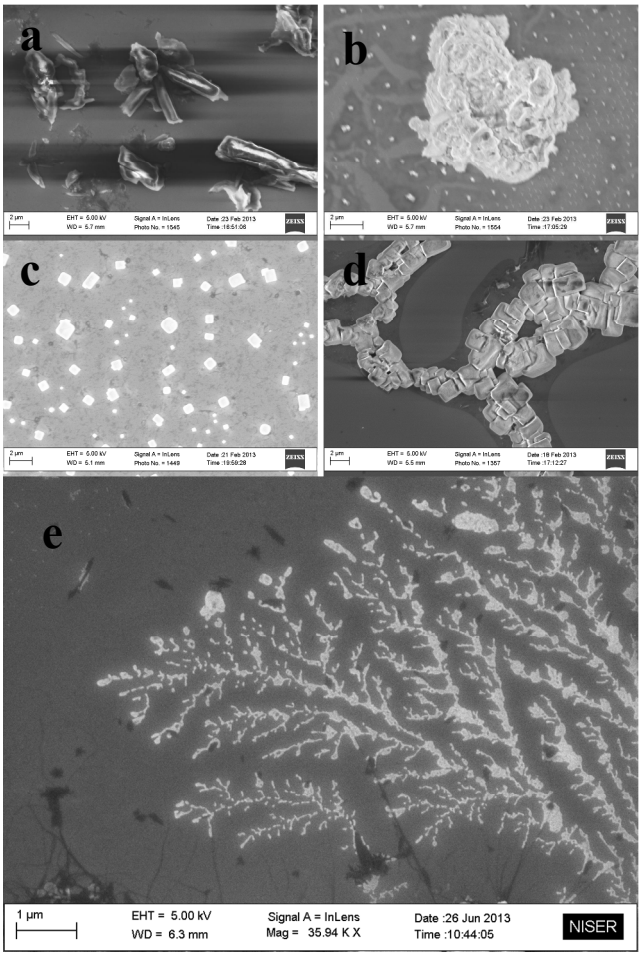


Fig. 3 SEM images: a. native cellulose (**1**) with 2µm size; b. amino derivatized cellulose (**3**); c. Berberine carboxylate (**4**); d berberine immobilized cellulose (**5**); e. NaOH treated berberine immobilized cellulose material (**6**).

and employed in scanning of cellulose surface images with SEM. For straightforward comparative studies, SEM images of **1**/**3**/**4**/5/6 at selective resolution, ~1-2µm, are given in Fig 3 (a-e), while their other SEM images at different resolution are provided in ESI (Fig S6-S16). By comparison with literature, the SEM images of native cellulose powder (1) reveal that unmodified cellulose surface has crystalline type of morphologies (Fig 3a and Fig S6). In other hand, the SEM images of amino functionalized cellulose material (**3**) clearly indicate that the cystalinality properties of cellulose surface have modified (Fig. 3b and Fig S9). However the SEM image of Berberine derivative (**4**) shows cubic crystalline type of surface morphology (Fig. 3c and Fig S10), Herein the SEM images of dark yellow color material **5** show Berberine type of cuboids crystalline units (Fig 3d and Fig. S13) at polymer surface which clearly indicate the immobilization of Berberine at native cellulose material. However the SEM images of pale yellow color material **6** are provided in Fig. 3e, & Fig S14, which show that fiber type of crystalline material morphologies containing fewer amounts of Berberine units in comparison to material **5**. Reduce amount of Berberine at material **6** are attributed owing to the hydrolysis of ester bonded Berberine residue. Thus only amide bonded Berberine residues are responsible for new physical properties including photophysical features at surface of modified cellulose fluorescent polymer (**6**). To ensure the immobilization of Berberine in modified cellulose fluorescent material (**6**), again we scanned the SEM image and extracted SEM EDX data to find percentage of N-element in new functional material and control samples (**1** & **4**). SEM EDX analysis results for native cellulose (**1**) are given in Fig S7-S9 (ESI) where no N-element contents are present, while EDX results of Berberine derivative (**4**) indicate are given in Fig S11-S12 (ESI) which show the presence of 9.0% N-atom by weight (%). However EDX results for new functionalized material (6) are given in Fig. S15-S16 (ESI) which indicate the presence of 4.9% N-atom by weight (%). Since Berberine derivative and hexamethylenediamine residues are N-atom containing chemical entities which can give N-element content, by weight (%), in material **6**, though its values low, but strongly support the immobilization of N-containing residue.

In next, the newly synthesized Berberine immobilized cellulose functional material (**6**) would be fluorescent material because the presence of Berberine derivative, natural fluorophore, *via* amide bond through linker. Thus we studied the photophysical behaviour of modified cellulose material (**6**) with UV and Flourescence spectroscopic methods by comparison with free berberine derivative (**4**). So UV and fluorescence spectra of free Berberine (**4**)/immobilized berberine (**6**) were recorded with respective spectrophotometers, UV-IR and fluorescence, by using solid probes. The absorption spectra of solid powder samples of **4**/**6** are depicted in Fig. 4A, while their emissions are given in Fig. 4B. Nevertheless, an UV absorption spectra of **6** exhibit an absorbance peaks at λ_{max} 442 nm as similar to control sample, free Berberine derivative **4**. Moreover, the fluorescence, spectra of **4**/**6** (powder form) show an emission peaks (λ_{em}) at ~530 nm (aprox) for both (4 & 6) by excitation with same wavelength (λ_{ex}) 342nm (Fig. 4B). The comparative UV studies support an immobilization of Berberine derivative (**4**) at native cellulose materials (**1**), which turned into

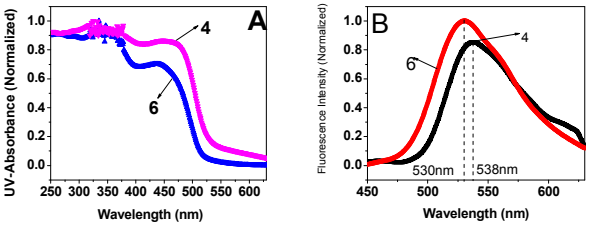


Fig. 4 UV spectra (with *solid probe*) berberine carboxylate derivative (**4**) and berberine immobilized modified cellulo(**6**); Fluorescence spectra (with solid

Probe) berberine carboxylate derivative (**4**) and berberine immobilized modified cellulose (**6**).

yellow colour fluorescent cellulose materials (**6**). Thereafter, UV and fluorescence spectra of Berberine immobilized cellulose material were also recorded in solution phase. Consequently, the dissolution of cellulose materials **1**/**6** along control sample Berberine derivative (**4**) were performed in aqueous solution of LiOH:Urea by following reported procedure.[20, 21] The Alkaline condition aq. LiOH:Urea may hydrolysed the ester bonds of **5**, that's' why only modified material **6** was employed for dissolution in LiOH:Urea. Afterwards, UV and fluorescence spectra of **1**/**4**/**6** were recorded with solution phase probe. The normalized UV spectra of materials **1**/**4**/**6** are given in Fig. 5A, while their normalized fluorescence spectra are depicted in Fig. 5B. The UV spectrum of **4** exhibits absorbance peaks at wavelengths (λ_{abs}) 267nm, 345nm, and 430nm (Fig 5A), which are also appeared in UV spectrum of **6** though their peak intensities are significantly gone down. However there is no absorbance peak, as per expectation for control sample, unmodified cellulose (**1**). These results support the presence of Berberine in modified cellulose material. In next, the fluorescence spectra of both Berberine (**4**) and Berberine immobilized material (**6**), exhibit emission peaks at wavelength 446 nm with same excitation wavelength (λ_{ex}) 345nm. The fluorescence spectra of **4** & **6** exhibit emission peaks at 517nm with same λ_{ex} 424nm, but nature of emission is slightly different in **6** compare to **4**. These studies further support the immobilization of Barbering at cellulose polymer.

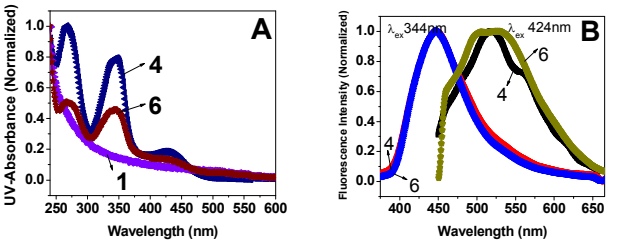


Fig. 5 UV spectra of native cellulose, berberine carboxylate derivative (**4**) and berberine immobilized modified cellulose (**6**) solution in LiOH:Urea; Fluorescence spectra (with solid Probe) berberine carboxylate derivative (**4**) and berberine immobilized modified cellulose (**6**) solution in LiOH:Urea

In understanding the nature of fluorescence, photoluminescence (PL) spectra of free Berberine derivative (**4**) and Berberine immobilized material (**6**), were recorded by using laser source at two different λ_{ex}. The PL spectra of **4**/**6** with λ_{ex} 342nm and 325nm are provided in respective Fig 6A and Fig 6B. The PL spectra of control sample **4** with λ_{ex} 342nm (Fig 6A), exhibits two strong emission peaks at wavelengths (λ_{em}) 433nm and 470nm, while PL spectra of modified cellulose material (**6**) show one extra shoulder peaks at λ_{em} 391nm along with strong peaks (λ_{em} 419nm), slightly blue shift (~25nm) with control sample (**4**). In Fig. 6B, the PL spectra of **4** with λ_{ex} 325nm demonstrate two types of emission peaks at wavelengths λ_{em} 454 nm and λ_{em} 478 nm with same peak intensity. Both λ_{em} peaks of

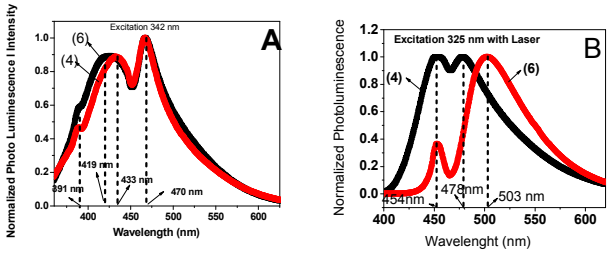


Fig. 6 Photoluminescence spectra of berberine acid (**4**) and berberine modified cellulose (**6**): without laser (A); and with laser (B)

control samples (**4**) are responsible for being florescent molecules Berberine as While PL spectra of berberine immobilized cellulose material (**6**) also exhibits two emission peaks at 454 nm and 503 nm but their intensities are quite different. From comparison studies, Berberine immobilized cellulose material (**6**) has shown red shift with ~25 nm over control sample (**4**) with significant dropped down in peak intensity at λ_{em} 503nm. These PL results reveal that the majority of fluorescence properties are exhibited from λ_{em} 503nm unlike to control sample (**4**). Interestingly, UV/Flourescence/PL studies results suggest that fluorescent cellulose material would be applicable in development of light sensitive functional materials. To extend the repertoire of biocompatible functional materials, the role of newly synthesized florescent material was examined in screening of Berberin interacting biomacromolecules by using bioimaging techniques such as fluorescence microscope and confocal. Free Beberine derivative (4) and immobilized Berberine (**6**) were visualized with fluorescence microscope within different colour channels. The fluorescence microscopic images of **4**/**5**/**6** within blue channel are given in Fig 7 (A-D), while their fluorescence images within other colour channels are provided in ESI (Fig S17-S18). Fluorescence intensity of control sample (4) in all colour channels is very low compare to that of new functionalized material (**5**) at same scale of visualization**.** Further the fluorescence microscopic images of control sample **4** & modified cellulose **6** with respect to size under blue channel were also measured at 50µm resolution (Fig 7C-D & Fig S19). The florescence intensity of control samples'images as oval shaped and that of modified cellulose (**6**) as rod shaped are observed; these studies further support strongly immobilization of Berberine at cellulose.

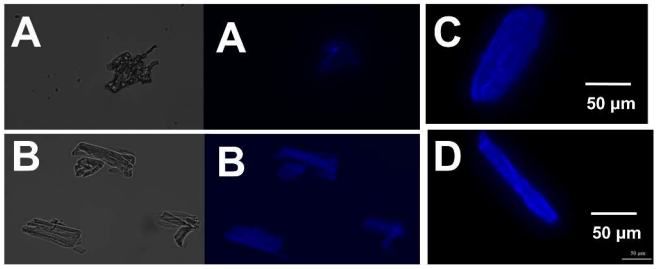


Fig. 7 Fluorescence microscope (of Olumpus) images: A. Free Berberine derivative (**4**); **B.** Berberine immobilized cellulose derivative **(5)**; and **C**. Free Berberine at resolution 50 µm and Berberine immobilized cellulose derivative **6** at resolution 50 µm.

In end, the confocal studies were performed with Berberine immobilized cellulose material (**6**) by using confocal microscope using green channel. The confocal images of Berberine immobilized cellulose (**6**) with resolution of 5µm are given in Fig. 8, while other confocal images at 30µm resolution are provided in ESI (Fig S20-S21). Finally these images strongly support synthesis of Berberine immobilized cellulose material (**6**).

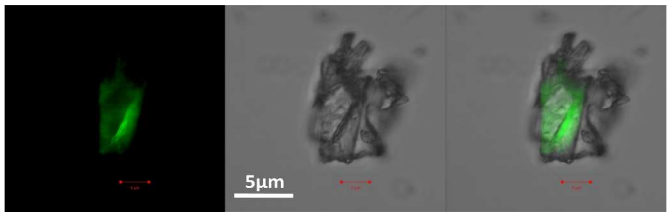


Fig. 8 Confocal images of immobilized Cellulose materials (**6**) at 5µm under green channel

In summary, the synthesis and photophysical characterization of Berberine immobilized cellulose materials are accomplished successfully from commercially available cellulose and Berberine. Then the surface morphologies of modified cellulose functional materials are studied with SEM technique, which strongly support the successful grafting of native cellulose material. The comparative UV, Fluorescence and PL studies further support the immobilization of native cellulose material with natural fluorescent molecule Berberine. The fluorescence and confocal images of synthesized colour cellulose material further support the immobilization of Berberine at cellulose surface cellulose. These labelled fluorescent cellulose material would be applicable in screening of Berberine interacting biomacromolecules such as the curbing of DNA G-quadruplex over other DNA secondary structures with bioimaging techniques. Imobilized therapeutics drug may reduced off target effect and become potential drug candidate

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Notes and references

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† Footnotes should appear here.

Electronic Supplementary Information (ESI) available: ^{1}H-/^{13}C-NMR spectra of berberine derivatives (2) are provided. SEM image of berberine, cellulose, and berberine immobilized cellulose at different resolutions are given. See DOI: 10.1039/c000000x/

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