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Electrophoretic detection of alginate by Hematoxylin and Eosin fluorescence: Implications in cell encapsulation/tissue engineering

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ABSTRACT

Alginate is a biocompatible polymer commonly used for cell encapsulation and synthesis of biomaterials in pure or modified form. In this study we have developed a fluorescence based method to visualize Hematoxylin and Eosin (H&E) stained alginate (pure/protein modified) by electrophoresing it in agarose gel, followed by its UV exposure. The shift in the migration pattern of H&E stain indicates presence of alginate, whereas the conjugated protein (fibronectin) could be detected by UV spectroscopy. H&E stain was found to specifically and permanently stain alginate and the fluorescence and migration pattern of H&E was not affected in the presence of other dye (Rhodamine β Isothiocyanate - RBITC) conjugated to alginate. We found that the presence of all the ingredients of H&E is mandatory to influence the migration pattern of this dye (bound to alginate) upon agarose gel electrophoresis. The main advantage of this method is that it is cheaper and faster than the other methods for alginate visualization and can be accomplished with standard lab chemicals and equipment.

1. Introduction

Alginate is an unbranched polysaccharide consisting of 1–4 linked β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G) [1]. The divalent ion (Ca^{+}) forms cross-links in alginate by binding with the G residues, inducing a sol-gel transition in the material. Cross-linked alginate forms a stiff egg-box structure with relatively inert aqueous environment within the matrix. The affinity of alginate towards divalent ions decreases in the following order Pb > Cu > Cd > Ba > Sr > Ca > Co,Ni,Zn > Mn [2]. Presently, alginate is used for *in vitro* cell culture, for tissue engineering applications, for the generation of tissue in bioartificial matrix and for fundamental studies on entrapped cell, such as chondrocytes [3]. In order to enhance the chemical interactions of alginate matrix with cells, alginate is being functionalized with cell specific ligands/extracellular signaling molecules (for e.g., glycosylated peptide and graft polymerization), which also play a role in controlling the growth, differentiation and behavior of cells in culture. Alginate modification with many peptide sequences, including YIGSR and IKVAV (from laminin), REDV and RGDS (from fibronectin) and VAPG (from elastin), has also been done using carbodiimide chemistry to make it more biocompatible [4].

The conjugation of peptide to alginate could be verified by using techniques such as FTIR and UV spectroscopy. In FTIR, an amide peak at 638 cm^{-1} indicates peptide conjugation to alginate whereas in UV spectroscopy the protein/peptide is detected by absorbance at 280 nm [5]. NMR spectroscopy is used for the detection of alginate in a sample, which could reveal the ratio of M/G contents. Owing to its chemical inertness, only alcian blue [6], toluidine blue [7] and Stains all solution [8] have been used to stain and visualize alginate under white light in polyacrylamide gel and agarose gel upon electrophoresis. Nevertheless, staining with these dyes requires long processing time including overnight incubation.

H&E is the most widely used permanent stain in medical diagnosis. The staining method involves application of hemalum, which is a complex of aluminum ions and hematein (oxidized product of hematoxylin obtained by exposure to air/light, mercuric oxide or sodium iodate) [9]. Hemalum has an overall positive charge, so it binds to negatively charged molecules in cells and tissues (such as nuclei, keratohyalin granules and calcified material) and imparts blue color. It is followed by counterstaining with an aqueous or alcoholic solution of negatively charged Eosin Y dye, which colors other eosinophilic structures such as proteins (arginine, lysine and histidine at low pH) in various shades of red, pink and orange [10].

When exposed to UV light, H&E fluoresces green color due to its Eosin Y component [11–12]. Although non-microscopic application of Eosin Y for the staining of proteins on polyacrylamide gel electrophoresis (PAGE) has been reported but alginate detection by H&E staining has never been described earlier. We discovered that a solution containing both Hematoxylin and Eosin Y could detect alginate in agarose gel after electrophoresis when exposed to UV. This procedure requires only mixing of H&E with alginate, followed by gel electrophoresis and its visualization on UV illumination, therefore circumventing long staining and processing time. It was found that the samples having

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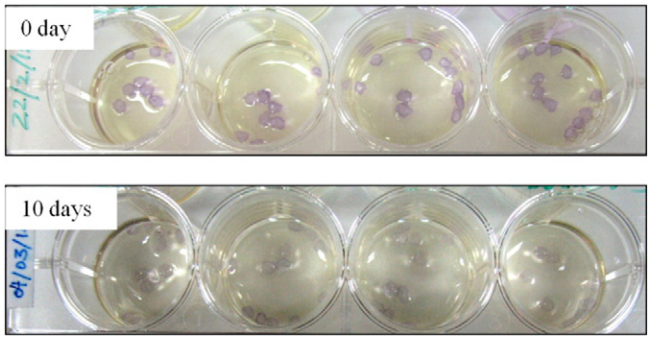

Fig. 1. Alginate capsules stained with H&E. The violet color of H&E stain does not leach out even after 10 days of incubation in human plasma.

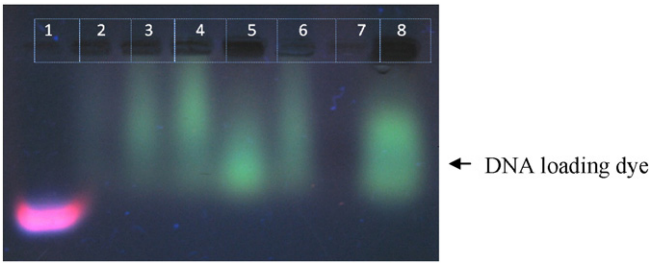

Fig. 3. UV exposed 0.8% agarose gel displaying different ratio of alginate (0.1%) with H&E. In L1, the DNA ladder constituents were observed to fluoresce. No smear was seen in L8, which contains only H&E, only distinct band was spotted. The H&E in L8 appears to move as two bands because of the presence of DNA loading dye. L1: DNA ladder; L2: Alginate + H&E (4:1); L3: Alginate + H&E (1.5:1); L4: Alginate + H&E (1:1.5); L5: Alginate + H&E (1:4); L6: Alginate + H&E (1:1); L7: Alginate only; L8: H&E only.

different concentration of alginate could be detected on the basis of migration pattern of H&E (bound to alginate) upon electrophoresis. The Hematoxylin or Eosin Y stain individually was not responsible for this phenomenon, notwithstanding, the presence of both the dyes and all other ingredients is requisite for affecting migration of H&E bound to alginate during agarose gel electrophoresis. Moreover, this method effectively facilitated the detection of alginate in Alginate-Fibronectin (AF) and RBITC-Alginate-Fibronectin (RAF) complex. Meanwhile, we have also optimized a method for alginate conjugate/complex purification by desalting with Sephacryl S-100 HR, followed by size exclusion chromatography. This method would yield pure fraction of peptide modified alginate which is commonly used for microencapsulation or in preparing biocompatible scaffolds.

2. Experimental

2.1. Preparation of H&E and alginate solution

The Papanicolaous solution 1a, Harris hematoxylin solution for cytological cancer and cycle diagnosis, (#109253, Merck, Mumbai, India) was mixed with 1% alcoholic solution of Eosin Y (#230251-25G) in 10:1 ratio for staining. Or, Papanicolaous solution 2a Orange G solution mixed with light green SF-Eosin solution in 1:1 ratio was used for staining as provided in RAPID-PAP Papanicolaous stain kit (BioLab diagnostics, India). 0.1% alginate solution having pH: 5.1 (341 mOsm) and 0.5% alginate solution having pH: 8.4 (20 mOsm) was prepared in triple distilled water.

2.2. Staining of alginate beads with H&E and its stability measurement

Alginate (molecular weight: 216–360 kDa, Fluka, Norway; #71238) was dissolved in water to make 1% solution. Alginate capsules were prepared by dropping alginate solution in 100 mM $CaCl_2$ (Qualigens, India) with constant stirring. The capsules were washed with saline (Bio Basic Inc., Canada) twice, followed by 5 min incubation with 200 μl of H&E stain (Merck, Germany) at room temperature (RT). Stained capsules were again washed with saline. To check the stability of staining, freshly isolated human plasma [13] was added to the alginate capsules and incubated for 10 days at 37 °C and 5% CO_2 in humidified atmosphere.

2.3. Preparation of activated alginic acid by cynogen bromide (CNBr)

For cynogen bromide activation, 1% alginate solution was mixed with 1 ml of $NaHCO_3/Na_2CO_3$ (pH 10.7) (SRL, India). This was followed by addition of 5 mg solid CNBr (or CNBr solution) (SRL, India). After mixing for 30 min at RT, the solution was dialyzed (cutoff of dialysis membrane = 12–14 kDa, Himedia, India) for 2 h against water (pH 9.0) to yield activated alginic acid [14].

2.4. Preparation of antibody-alginate conjugate

The alginate-antibody conjugate was prepared as described earlier with some modification [15]. 0.5–1.0 mg/ml of RRAD-anti-human mouse IgG antibody (Santa Cruz, USA) was added to 1 ml of CNBr activated alginate. After allowing the solution to stand at 4 °C for

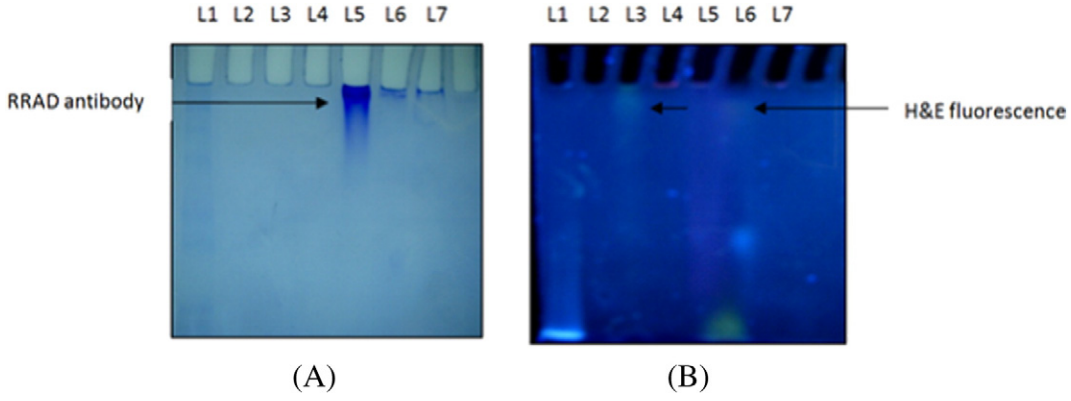

Fig. 2. (A) 10% Native (continuous) PAGE showing protein staining due to alginate-antibody complex in L5 and L6. (B) Same gel (as in Fig. 2A) exposed to UV prior to staining with Coomassie brilliant blue shows green fluorescence of H&E only in the lanes where alginate was present (L3 and L6). L1: Protein ladder (5 μl); L2: 1% alginate (25 μg); L3: 0.1% alginate (0.25 μg) + H&E (5 μl); L4: 0.1% alginate (0.25 μg) + PI (5 μl); L5: RRAD antibody (4 μg/20 μl); L6: Alginate-RRAD antibody conjugate (20 μl) + H&E (5 μl); L7: Alginate-RRAD antibody conjugate + PI (0.05 μg).