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Hyaluronic acid-g-poly(HEMA) copolymer with potential implications for lung tissue engineering

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ABSTRACT

Tissue engineering represents an attractive potential for regeneration of engineered functional pulmonary tissue. Hyaluronic acid, an extracellular matrix component promotes the growth and proliferation of most cells. The high water affinity of hyaluronic acid (HA) adversely affects its application in the field of tissue engineering. A copolymer of hyaluronic acid and poly(2-hydroxyethylmethacrylate), [poly(HEMA)] appeared as a good choice for the synthesis of a natural-synthetic polymer hybrid matrix with the synergistic properties of both the polymers like water stability and biocompatibility. The copolymer films were stable in water at both acidic and neutral pH in contrast to that of virgin HA films. Grafting significantly alters the mechanical properties of hyaluronic acid. The HA-g-poly(HEMA) is found to be non-cytotoxic to mammalian cells. Further, the polymer was analysed for supporting alveolar cell adhesion and growth and were found suitable for supporting multiple cell types with specific culture requirements. Thus, grafting with poly(HEMA) is a suitable method for the fabrication of stable, cytocompatible natural-synthetic polymer hybrid matrices for varied biomedical applications such as tissue engineering, wound dressings, drug delivery and so forth.

1. Introduction

Diseases like pulmonary hypoplasia (PH) (found in neonates), and emphysema (a chronic lung disease) have a deficient alveolar epithelium, tissue loss or reduced alveolar surfactant synthesis. In all these instances, tissue engineering represents an attractive potential for regeneration or augmentation with engineered functional pulmonary tissue. Development of 3D architecture of the lung is extremely challenging due to the unique architectural structure of lung parenchyma containing connections of alveolar units to airways and pulmonary circulation. Several strategies are being adopted to evolve suitable scaffolds for lung tissue engineering. There is a growing interest in blending natural and synthetic polymers as biomaterials for creating complex structures, which will act as scaffolds for tissue engineering. Lelkes et al. (2007) demonstrates the usefulness of elastin-based fibrous scaffolds with conducting polymer polyaniline or with a mixture of poly lactic acid/poly glycolic acid for pulmonary tissue engineering. Andrade, Wong, Waddell, Kesavjee, and Liu (2007) used gel foam sponge as a scaffold material with fetal rat lung cells as progenitors for their *in vivo* experiments to study lung tissue regeneration. Turner, Kielty, Walker, and Canfield (2004) and Amarnath, Srinivas, and Ramamurthi (2006) have shown that a commercial benzyl ester of HA and laboratory cross linked Hylan as excellent biomaterials for promotion of adherence of vascular endothelial cells and vascular tissue engineering. Recently, several reports are there where modified hyaluronic acid appeared as a good choice for different applications in tissue engineering (Calderon et al., 2010; Demirdogen, Elcin, & Elcin, 2010; Fan et al., 2010; Rampichova et al., 2010).

Hyaluronic acid, an extracellular matrix component promotes the growth and proliferation of most cells. HA is a linear polysaccharide composed of two alternatively linked sugars, D-glucuronic acid and N-acetyl D-glucosamine (Scheme 1). Due to the presence of uronic acid, the hyaluronic acid possesses negative charge. Its molecular weight ranges from 70,000 to 2–4 million Daltons. They exist in nature as hydrated gel, usually closely associated with other tissue components such as chondroitin sulphate (Arkins & Sheehan, 1972).

The main sources of HA are rooster combs, bovine vitreous humor and human umbilical cord. Rooster combs contain higher concentrations of HA with respect to other animal tissues. Another source is from microorganisms through a fermentation process (Jensen & Carlsen, 1954; Swann, 1968). HA and its derivatives have

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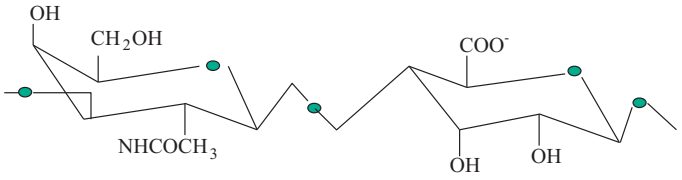

Scheme 1.

become important therapeutic agents in medicine. Several medical applications of HA exist; on account of its unusual rheological and lubricious properties, orthopedicians use it to lubricate joints, as a viscosupplement for patients with symptomatic knee osteoarthritis (Balazs, 2004; Soltes et al., 2006). HA is highly hydrophilic and lubricious and slippery when wet and is hence used to prevent surgical adhesions in the abdominal cavity and as coating for medical devices. It is also used to help the eye, retain its shape and protect tissues when removing cataracts and implanting intra ocular lenses and in the ophthalmic surgery as a vitreous humor substitute. Drug release is another interesting application and formulations of HA and its derivatives have been developed as topical, injectable and implantable vehicles for the controlled and localised delivery of biologically active molecules (Vasiliu, Popa, & Rinaudo, 2005). HA has also been shown to have an anti-platelet activity (Burns & Valeri, 1996), which is important in avoiding thrombus formation, hence HA is also used as a coating for blood contacting implants.

In spite of many potential applications of HA, it has some inherent drawbacks. The fabrication of new biomaterials is precluded by the poor biomechanical properties of HA. The coiled structure of HA can trap approximately 1000 times its weight in water. The high water affinity of this material adversely affects its extended application in the field of tissue engineering. These warrant various modifications, to improve its mechanical and physico-chemical properties to explore fully its applicability in the biomedical field. A variety of chemical modifications of native HA have been devised to provide mechanically and chemically robust materials (Abatangelo & Weigel, 2000; Luo, Kirker, & Prestwich, 2001; Prestwich, Marecak, Marecek, Vercruysse, & Zeibell, 1998; Vercruysse & Prestwich, 1998).

Chemical modification through grafting has received considerable attention in the area of biomedical applications. Poly(HEMA), is one of the most important hydrogels in the biomaterials world since it has many advantages over other hydrogels (Pescosolido et al., 2011). This include a water-content similar to living tissue, inertness to biological processes, resistance to degradation, permeability to metabolites, resistance to absorption by the body. Hence a graft copolymer of HA and poly(HEMA) appeared as a good choice for the synthesis of a natural-synthetic polymer hybrid matrix for use as a scaffold for lung tissue engineering.

2. Experimental

2.1. Materials

Hyaluronic acid sodium salt was procured from Sigma–Aldrich Chemicals, Pvt Ltd, Bangalore. 2-Hydroxyethylmethacrylate, ceric ammonium nitrate, acetic acid, methanol, and sodium hydroxide were of analytical grade and purchased from local sources. Culture medium, antibiotics, enzymes, growth factors, medium additives, all plastic wares were purchased from BD Falcon, and Percoll from GE health-care. Live/dead assay kit (L3224 Live/Dead viability/Cytotoxicity Kit for mammalian cells) was from molecular probes.

2.2. Preparation of HA-g-poly(HEMA) copolymer

1% (w/v) HA solution was prepared by dissolving 0.5 g sodium salt of HA in 50 mL distilled by overnight stirring at room temperature. The above solution was placed in a three-necked round bottomed flask, fitted with a condenser and stirrer. The flask was kept in a water bath and nitrogen was bubbled through it. 2.5 mL of 0.1 M ceric ammonium nitrate in 1 N nitric acid was added and the temperature of the water bath was increased to 60 °C. The reaction was allowed to take place for 3 h, under constant stirring. The contents were then poured into a disposable polystyrene mold and cured at 45–50 °C for 48 h. The film formed was extensively washed with distilled water to remove unreacted reactants and extracted with methanol for 3 h, frequently changing the solvent, to remove any homopolymer [poly(HEMA)], formed during the reaction. The purified film was kept in the refrigerator until use.

3. Characterizations

3.1. Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of the HA, poly(HEMA), and HA-g-poly(HEMA) samples were recorded using a Nicolet Impact 410 FT-IR Spectrophotometer with KBr pellet method. The analysis was done based on American Society for Testing and Materials (ASTM) international standards (ASTM E 573-01, Re-approved 2007).

3.2. Thermal analysis

Glass transition temperature (Tg) of the copolymer films was evaluated by differential scanning calorimetry (DSC 2920 Differential scanning calorimeter, TA Instruments Inc). Thermal stability of the copolymer films was studied on a SDT 2960 (simultaneous TGA-DTA, TA Instruments Inc). For DSC analysis, 5–8 mg of the samples were crimped inside aluminium sample pans and heated under nitrogen atmosphere at a rate of 10 °C/min from −50 to 70 °C. The second heat cycle is used for the calculation of glass transition temperature. The procedure used for the DSC analysis was based on (ASTM E-1356-08). For TGA analysis, 10–12 mg of the film samples were taken in a platinum cup and heated under nitrogen atmosphere at a rate of 10 °C/min from room temperature to 600 °C. The test method was derived from (ASTM E 1131-03).

3.3. X-ray diffraction analysis

X-ray diffraction analyses of the chitosan and the copolymers in the powder form were performed by a wide angle X-ray scattering using Siemens D5005 X-Ray Diffractometer.

3.4. Scanning electron microscopic (SEM) studies

The morphology of the HA-g-poly(HEMA) membranes was examined using scanning electron microscope, HITACHI 2400. The samples were gold sputtered and analysed.

3.5. Swelling studies

Square film samples of 100 mm^2 size (of known mass) were immersed in phosphate buffer of pH 7.4 and aqueous acetic acid of pH 1.98 solutions for known intervals of time. The strips were removed and carefully blotted between filter paper to remove excess fluid and weighed. Swelling Index = $[W/W_0 \times 100]$, where W is the (final weight − initial weight), and W_0 is the initial weight. A minimum of 6 readings were taken and averaged.

3.6. Contact angle

The water contact angles of both the HA and HA-g-poly(HEMA) films were measured using an OCA 15 Plus Video based contact angle measuring device (Data Physics, Germany) as per the instruction manual. The contact angle of the same drop was measured repeatedly after a definite interval to observe the change in contact angle resulting from intermolecular interaction between the polymer surface and water drop.

3.7. Mechanical properties

Tensile properties of the HA and HA-g-poly(HEMA) copolymer films were studied using Universal testing machine-Instron 1193 as per ASTM D 882 (2010). The films were conditioned in the testing atmosphere for 48 h. Rectangular strips of 10 mm width were elongated till break at a cross head speed of 10 mm/min. Stress at break and the percentage elongation of the films were calculated using the software. The computed values are the mean of 6 repeat measurements.

3.8. In vitro cytotoxicity tests

The *in vitro* cytotoxicity test using Direct Contact method was performed for test sample HA and HA-g-poly(HEMA) as per ISO 10993-5. Test sample, negative control (high density polyethylene) and positive controls (copper) in triplicate were placed on sub confluent monolayer of L-929 mouse fibroblast cells. Cells were incubated with test samples at $37 \pm 2\,^{\circ}C$ for 24 ± 1 h. Cell morphology was examined microscopically for cellular response like detachment, degeneration or lysis around test samples.

3.9. Growth of lung cells on HA-g-poly(HEMA) scaffold

3.9.1. Cell isolation

All animal procedures were carried out in accordance with the Institutional Animal Ethics Committee guidelines. Cell dissociation and culture were done according to the procedure detailed elsewhere with some modifications (Maya et al., 2002). Briefly, pathogen free Wistar rats 4–5 weeks of age were anaesthetized by ketamine/xylecine mixture given intra-muscularly. Lungs were excised free of bronchioles, subjected to enzymatic dissociation with porcine pancreatic elastase, collagenase type I, and DNAase mixture in DMEM at 37 °C shaker incubator with 80 rpm. The epithelial population was enriched by density gradient centrifugation with preformed percoll gradient. The epithelial band collected were counted with a haemocytometer and seeded at a density of 1×10^5 per scaffold.

3.9.2. Primary cell culture

The HA-g-poly(HEMA) copolymer was sterilized by ETO and pre-wetted with growth medium (DMEM:F12). Cells were seeded on to the material, the growth medium containing DMEM:HAM F12 (1:1) mixture with a cocktail of insulin, cortisol, cholera toxin, EGF, IGF, with 2% FBS was added and incubated in a CO_2 incubator at 37 °C in humidified atmosphere with 5% CO_2 for the duration of culture. The medium was changed every alternate day. Cells were monitored every day with a phase contrast microscope. Microscopic evaluation of lung cells was done by phase contrast microscopy, confocal microscopy and ESEM after 9 days of culture.

3.9.3. Cytotoxicity by live/dead assay

Viability of alveolar cells was assessed at various time points in culture using the Live Dead Assay kit from Molecular Probes. The assay was done according to the kit protocol supplied by the manufacturer. Briefly, the cells were seeded on to the co-polymer and cultured. The culture was terminated at different time points and assayed for viability. The samples were washed three times with Dulbecco's PBS to remove serum esterase activity. Unfixed samples were immersed in 2 μM ethidium homodimer and 4 μM calcein AM which were then incubated in dark at room temperature with gentle shaking. Images were taken with the help of a laser scanning confocal microscope (Carl Ziess LSM META 510).

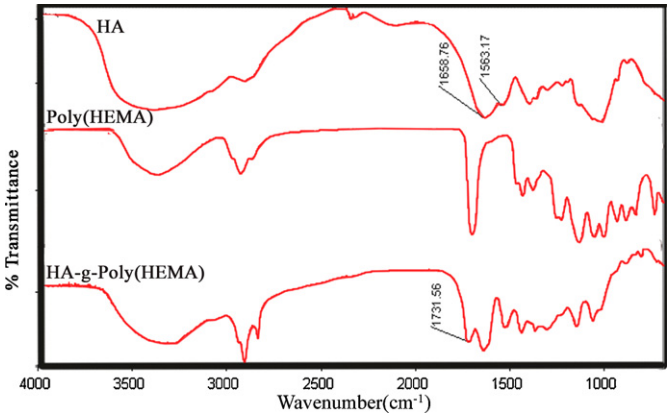

Fig. 1. The FTIR spectra of the HA, poly(HEMA), and HA-g-poly(HEMA).

3.9.4. Electron microscopy

Unfixed lung cells cultured on materials were observed with an environmental scanning electron microscope (ESEM Quanta 200 FEI, Netherlands) to evaluate the ability of the copolymer to support adhesion and growth of the different alveolar cells and also to assess distribution of cells on this novel scaffold.

4. Results and discussion

4.1. HA-g-poly(HEMA) polymer

The graft products of HA and HEMA showed good film forming property. The complete removal of homopolymer was confirmed from the FTIR spectra of the extract. The extract was evaporated on a sodium chloride window and the FTIR spectrum was taken. A blank spectrum of the bare NaCl window was the background. The absence of the carbonyl absorption peak in the sample spectra confirmed the complete removal of the poly(HEMA) homopolymer from the HA-g-poly(HEMA). The copolymer films were characterised using different physico-chemical and biological techniques as described below.

4.2. Fourier transforms infrared spectroscopic analysis

Infrared spectroscopy was used to analyse the chemical groups present in the sample. The spectrum of poly(HEMA) shows peaks at 3393 cm^{-1}, 2942 cm^{-1}, 2876 cm^{-1}, 1723 cm^{-1} and 1449 cm^{-1} for hydroxyl, alkyl and carbonyl groups. The HA-g-poly(HEMA) gave the peaks at 2924 cm^{-1}, 2853 cm^{-1}, 1731 cm^{-1} and 1658 cm^{-1} (Fig. 1). The carbonyl absorption band at 1731 cm^{-1} confirms the presence of poly(HEMA) pendant graft of the copolymer, thus confirming the grafting reaction between HA and HEMA.

4.3. Thermal analysis

TGA is used to study the thermal stability of the polymers. The information available consists of the number of stages of mass loss, a quantitative measure of mass-loss in any stage, etc. Thermal stability strongly depends on the compatibility of the polymers. The

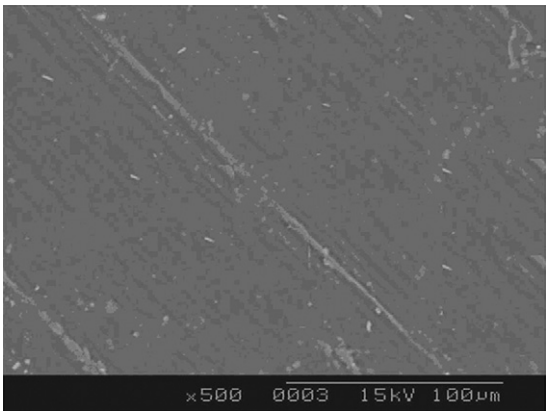

Fig. 2. The SEM image of HA-g-poly(HEMA).

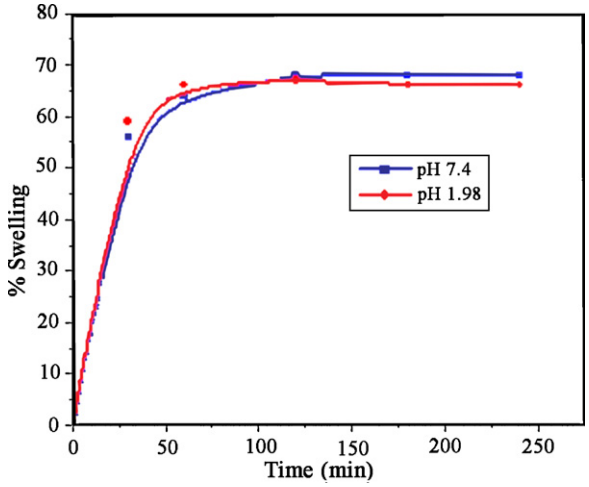

Fig. 3. The % swelling of HA-g-poly(HEMA) at different pH.

grafting of HEMA on hyaluronic acid has enhanced the thermal stability as seen from the TGA thermogram, Fig. S1 in the supporting information. The initial decomposition temperature of HA was not significantly affected due to grafting with poly(HEMA). However, the % residue remaining for HA-g-poly(HEMA) at the end temperature is only 14%. For virgin HA it is around 30%. The high temperature stability of HA is adversely affected due to grafting with HEMA, but this is irrelevant in the field of biomedical applications where one is only concerned with low temperature thermal stability.

DSC is used to characterise thermal properties of polymers like glass transition temperature, crystallisation temperature, melt temperature, etc. The temperature, at which abrupt cessation of all long-range segmental motion and polymer backbone rotation occurs, is called the glass transition temperature (Tg). The DSC scan of HA shows an exothermic peak at 17.9 °C and an endothermic transition at 95.6 °C along with small baseline shifts between these two temperatures (Fig. S2 in the supporting information). HA is highly hygroscopic and it absorbs moisture on storage. The exothermic transitions could be due to the uncoiling of the physical gels of HA molecules on interaction with the absorbed moisture. The endothermic transitions observed in the DSC measurements must be brought about by the release of the cooperative motion of water and the macromolecule (Takizawa & Nakata, 2000). Grafting of HEMA onto HA makes HA more amorphous. The copolymer manifests a glass transition temperature at 113 °C. The high transition temperature may be due to the possibility of formation of more hydrogen bonds with the –OH groups of HEMA and HA. The amorphous nature of HA is further confirmed from the XRD studies.

4.4. X-ray diffraction patterns

Wide angle X-ray diffraction patterns of powdered HA and the co-polymers are shown in Fig. S3 in the supporting information. Both the virgin hyaluronic acid and HA-g-PHEMA were found to be amorphous as evident from the XRD pattern. Malay, Yalcin, Batigun, and Bayraktar (2008) also reported similar observations for the amorphous nature of HA.

4.5. Scanning electron microscopic studies

The scanning electron micrographs of HA-g-poly(HEMA) showed a smooth surface without any phase separation indicating that homogeneous grafting has taken place between HEMA and HA. The SEM image also shows that the HA-g-poly(HEMA) film has a non porous surface. The micrographs are shown in Fig. 2.

4.6. Swelling studies

The swelling studies using HA-g-poly(HEMA) samples showed the water stability of the modified products. Virgin HA is very unsta-

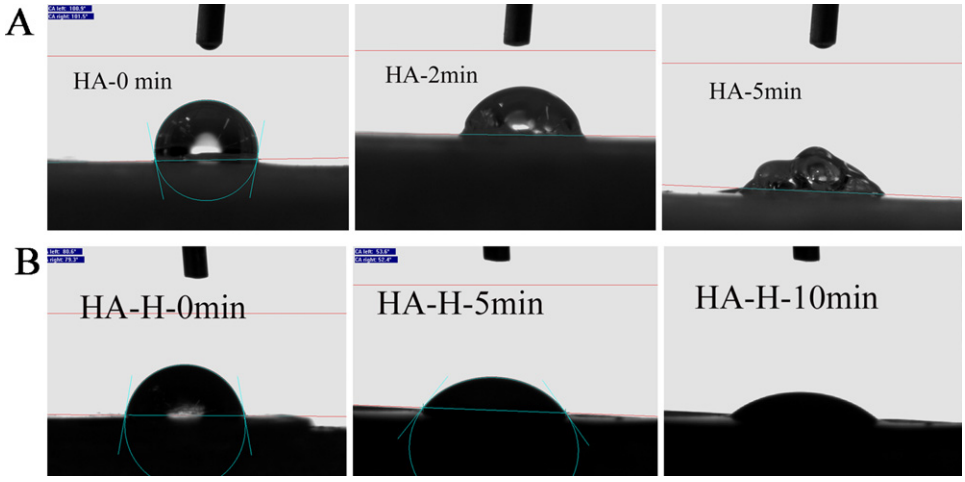

Fig. 4. Water-contact angles of HA and HA-g-poly(HEMA).

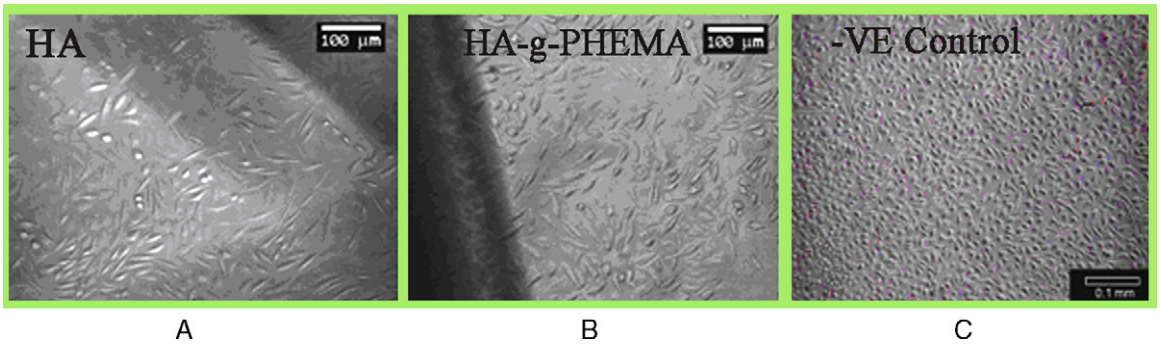

Fig. 5. L929 mouse fibroblast cells on contact with HA, HA-g-poly(HEMA) and negative control (high density polyethylene).

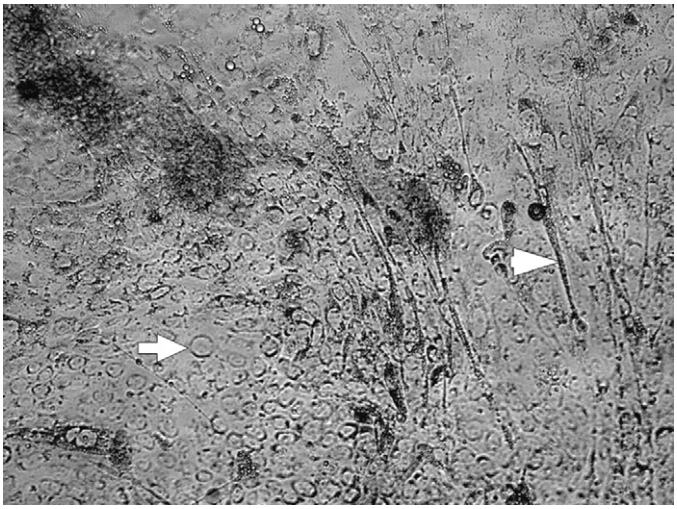

Fig. 6. Phase contrast microscopic image of both alveolar pneumocytes and fibroblasts on HA-g-poly(HEMA) scaffold. The elongated spindle shaped cells are the fibroblast (⇦) and the columnar cells (⇨) are the type II pneumocytes or alveolar pneumocytes.

ble in water at neutral pH as it gels out in a very short time. The HA-g-poly(HEMA), the uncross-linked film itself, is insoluble in water at neutral and acidic pH and swelled to around 66–68% at both the pH. The swelling index at neutral and acidic pH is depicted in Fig. 3. HA is more hydrophilic in nature. The poor biomechanical properties of this highly water soluble natural polymer currently preclude many direct applications in medicine. Jha et al. (2009) also report that in the absence of specific interactions like intra and inter particle cross linking, HA based hydrogel particles are simply colloidal suspensions that are not very useful for tissue engineering applications. The swelling studies confirm that grafting of HEMA onto HA can improve the water stability of HA significantly.

4.7. Water contact angle studies

It is already known that HA is highly hydrophilic. However, the high water-contact angle values obtained initially for HA and HA-g-poly(HEMA) films ((80–100°), indicate that the films are hydrophobic. They become hydrophilic after an equilibration of 5 min in water. The initial hydrophobic behavior of virgin HA films has been attributed to the peculiar arrangement of the molecular chains of large, polyanionic and linear glycosaminoglycan of the repeating disaccharide structure. Each monomer presents axial non-polar hydrogen atoms and equatorial more polar side chains, thus creating relatively hydrophobic and hydrophilic faces respectively. On immersion in water for 5 min, the hydrophilic faces of HA forms a gel due to interaction with water as shown in Fig. 4A. The HA-g-poly(HEMA) films, however, become highly hydrophilic (water contact angle value 28 °C) with an altered structure retaining its film morphology as depicted in Fig. 4B.

4.8. Mechanical properties

It is evident from Figs. S4 and S5 in the supporting information that grafting has effected in significant decrease in the tensile strength and substantial increase in the elongation of hyaluronic acid. However, the tensile strength of the HA-g-poly(HEMA) films, in the range of 2–3 MPa is sufficient for various biomedical appli-

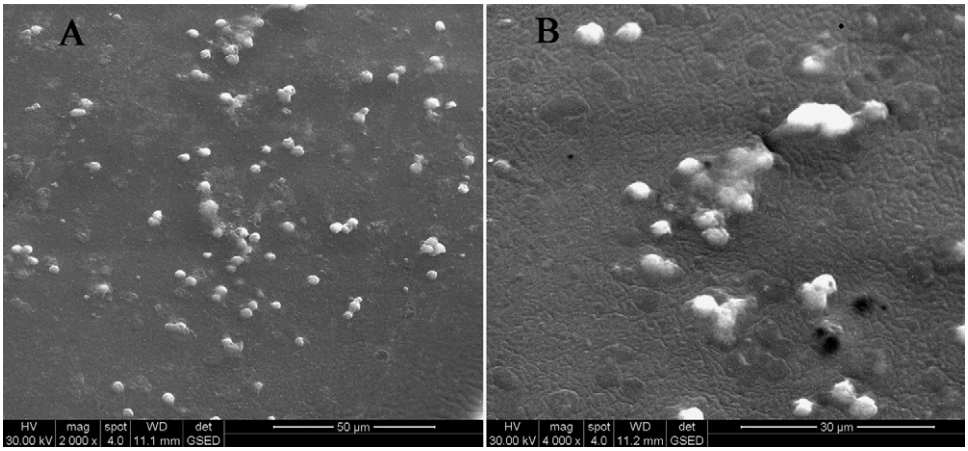

Fig. 7. Environmental scanning electron microscopic image showing that the alveolar epithelial cells are not spreading to form sqamous morphology. The cells maintain their columnar morphology, which will help in preservation of type II pneumocyte specific functions; B is magnified image of A.

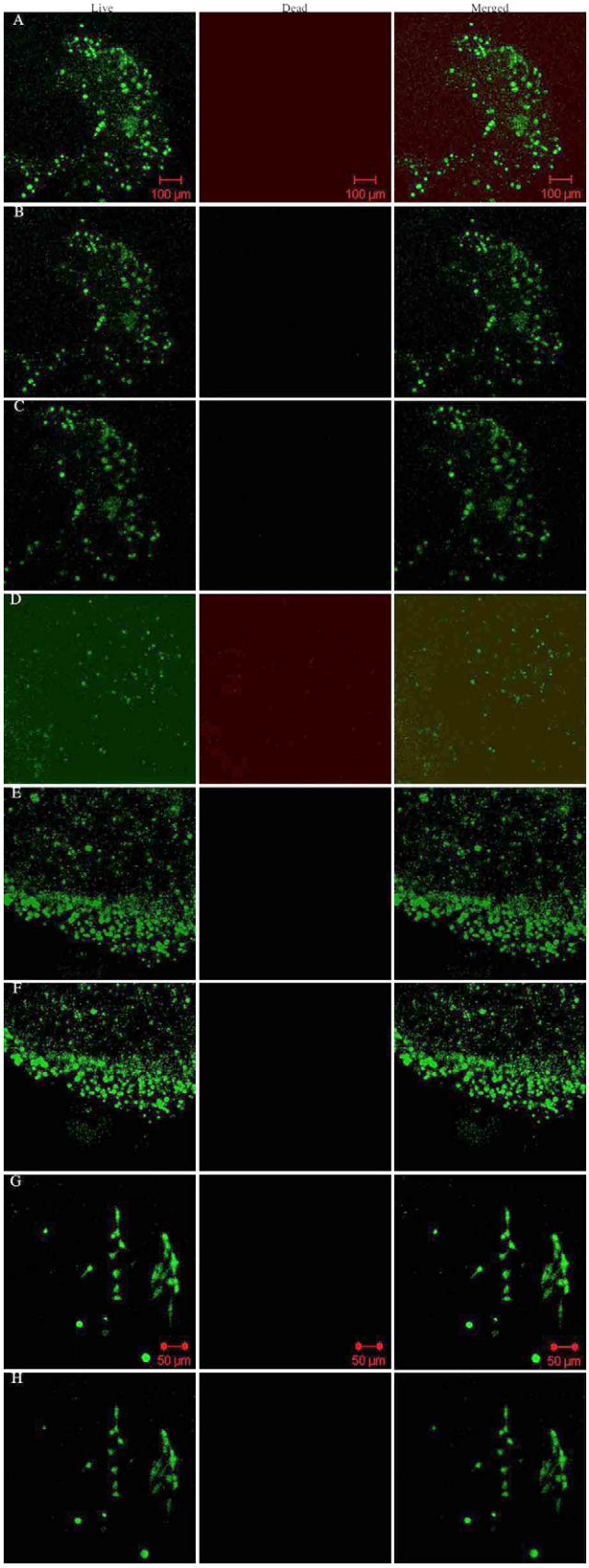

Fig. 8. Confocal analysis of the different lung cell survival on the HA-g-poly(HEMA) scaffold by viability assay. Lung cells on HA-g-poly(HEMA) scaffold on day 9 of culture. The green fluorescence shows live cells while red fluorescence shows dead cells. The cells were analysed to a depth of 50 μm of the scaffold.

cations. The elongation of the copolymer films around 230% of the original length is highly appreciable for tissue engineering applications. It is well known that cells are inherently sensitive to their surroundings, responding not only to the topographical features of the matrices but also to their viscoelasticity (Discher, Janmey, & Wang, 2005; Stevens & George, 2005).

4.9. In vitro cytotoxicity studies

Normal L929 Fibroblast cells are spindle shaped, healthy and viable, and with a glistening appearance as shown in Fig. 5A. Non toxic materials in contact with such cells would preserve the morphological aspects of the cells to larger extent, while the materials with cytotoxicity would cause the cells to undergo lysis or degeneration and loose the spindle shape and become more rounded. The HA-g-poly(HEMA) film in contact with L929 fibroblast cells do not cause any cell lysis, degeneration or loss of the spindle shape morphology of the cells. Fig. 5B and C show the morphology of the L929 cells after direct contact with virgin HA and HA-g-poly(HEMA) films. Hence, the grafting procedure did not affect the non-cytotoxic nature of the hyaluronic acid and were more suitable for interacting with viable cells.

4.10. Growth of lung cells on HA-g-poly(HEMA) scaffold

The functional unit of the lung is the alveoli with the gas exchanging surface consisting of the alveolar epithelial cells (the type I and type II pneumocytes) and the supporting cells (the alveolar fibroblasts). The type I pneumocytes are responsible for gas exchange and are terminally differentiated, while the type II pneumocytes are the alveolar surfactant secreting cells and also the alveolar stem cells which give rise to type I cells subsequent to injury during the repair mechanism. When alveolar type II pneumocytes are cultured in isolation they rapidly loose their specific properties and enter senescence. Several authors have reported that the selection of a suitable biomaterial as scaffold is important for cell adhesion and proliferation (Aigner et al., 1998; Hutmacher, 2000; Ishaug-Riley, Okun, Prado, Applegate, & Ratcliffe, 1999; Iwasaki et al., 2011; Jayakumar et al., 2009; LeBaron & Athanasiou, 2000; Madihally & Matthew, 1999; Sechriest et al., 2000; Suh & Matthew, 2000). Hence, in order to develop a tissue engineered lung model a combination of the different alveolar cell types in the optimal concentration on a suitable scaffold is a necessity. *This paper is a part of our quest for a suitable scaffold for lung tissue engineering.*

The phase contrast microscopic image shows the adhesion of all the different alveolar cell types uniformly over the material surface (Fig. 6). The spindle shaped cells are the fibroblast and the cobble stone morphology cells are the type II pneumocytes. The ESEM images shown in Fig. 7A and B also reaffirm the phase contrast microscopic observations.

Fig. 8 is viability assay done after lung cells were seeded on these scaffolds and placed in culture for 9 days. The green color of the calcein dye indicates the viable cells and the red color ethedium homodimer denotes the dead cells. The confocal images clearly attest to the fact that not only on the surface of the scaffold but the cells have penetrated into the depth of 50 μm of the scaffold and are also viable (Fig. 8).

5. Conclusion

Grafting with poly(HEMA) is a suitable method for the fabrication of stable, mechanically strong and cytocompatible matrices for all type of biomedical applications including tissue engineering. The HA-g-poly(HEMA) prepared is uncross-linked, however, it is stable in aqueous environment at neutral and acidic pH. This behavior is beneficial for the fabrication of matrices for drug delivery and

tissue engineering applications. The HA-g-poly(HEMA) could be fabricated into films and 3D scaffolds.

These studies clearly attest the fact that the copolymer of HA-g-poly(HEMA) is a suitable scaffold material for tissue engineering applications specifically for lung tissue engineering applications. They support adhesion and growth of the different lung cell types. Further studies are on to assess the functionality of the alveolar tissue construct.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.03.007.

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