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Research Paper

Influence of physical and mechanical properties of amphiphilic biosynthetic hydrogels on long-term cell viability

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ABSTRACT

Maintaining the mechanical properties of biofunctional hydrogels of natural resources for tissue engineering and biomedical applications for an intended period of duration is a challenge. Though anionic polysaccharide alginate has been hailed for its excellent biomimetic characters for tissue engineering, it usually fails in load bearing and other dynamic mechanical environment. In this paper this issue was addressed by copolymerizing alginate with the biocompatible and mechanically robust synthetic biodegradable polyester and crosslinking with polyethylene glycol diacrylate (PEGDA) and vinyl co-monomers, 2-hydroxy ethyl methacrylate (HEMA), methyl methacrylate (MMA) and N N' methylene bis acrylamide (NMBA) to form three hydrogels. All three hydrogels were amphiphilic, hemocompatible and non-cytotoxic. These hydrogels exhibited appreciable water holding capacity. Comparatively, hydrogel prepared with PEGDA–NMBA crosslinkers displayed larger pore size, increased crosslinking, higher tensile strength and controlled degradation. With appreciable swelling and EWC, this hydrogel elicited better biological responses with long-term cell viability for cardiac tissue engineering.

1. Introduction

Even though the natural hydrogel materials exhibit better biocompatibility and cell affinity for tissue engineering applications than synthetic polymers, they are less versatile when considering properties like mechanical strength and variable degradation rate. Copolymerization of a mechanically robust synthetic polymer with a biocompatible natural polymer can overcome most of these difficulties. The scaffolds made from such a copolymer can maintain the cell phenotype and modulate its function because the synthetic materials can greatly enhance properties of natural polymer (Li and Guan, 2011). An ideal scaffold for tissue engineering should possess the structural characteristics of synthetic materials and the biofunctional characteristics of natural materials.

The advent of biosynthetic hybrid hydrogel is highly relevant for tissue engineering scaffolds, especially to that of heart, as it can solve several mechanical and biological

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issues arising during the individual use of synthetic or natural polymers. Scaffold materials of natural origin like alginate were reported to be effective for mediating cell-signaling mechanisms for cardiac repair and regeneration. Despite their poor mechanical properties, the inflammatory potential is high which depends on the type, chemistry and source. On the other hand, synthetic cardiac scaffold materials can be customized to get a wide range of tunable properties in terms of degradation, mechanical strength and durability. Still their inherent cellular interactions are limited; however this can be improved by functionalization with biological moieties Novakovic et al., 2010).

Among the natural biomaterials, the seaweed-derived polysaccharide alginate attracted the attention of many tissue engineers mainly due to its inert chemistry that allows interactions with mammalian cells. Despite their mechanical issues, many researchers explored alginate for tissue engineering especially for cardiac applications (Andersen et al., 2012; Rosellini et al., 2009). Alginate is composed of 1, 4-linked β-D-mannuronate and 1, 4-linked α-L-guluronate units that forms a linear polymer which can undergo gelling by chelating calcium ions. The calcium alginate gel is immunologically inert and non-digestible by mammalian cells. Calcium alginate gels can degrade in the biological systems by the gradual diffusion of calcium ions and exchange with monovalent ions from the medium. The degradation products are non-toxic and are excreted through the urine (Novikova et al., 2006). Moreover, alginate in its hydrogel form closely imitates native glycocomponents of ECM (Leor and Cohen, 2004).

The unsaturated linear polyester poly(propylene fumarate) (PPF) can be modified or crosslinked through its double bonds. Therefore, it is an ideal choice for various biomedical applications. PPF degrades by simple hydrolysis of the ester bonds; the degradation products primarily fumaric acid and propylene glycol are proven to be non-toxic and can enter the mitochondrial TCA cycle. The unsaturation present in the PPF allows the crosslinking of the polymer into a covalent polymer network. These networks can be designed with a wide range of controlled properties as needed for cardiac tissue engineering (Kasper et al., 2009; Finosh and Jayabalan, 2012).

Linking the biological polymer with structurally versatile synthetic polymers will pave a way for the construction of a crosslinked network with a balanced physiochemical and biomechanical properties and a controlled degradation profile, which is a requisite for the *in vitro* engineering of organ parts. Hydrogels are composed mainly of hydrophilic polymer network that offers a strong rapport for water while their physical or chemical crosslinking ensures the association of water by preventing the dissolution. Nevertheless, the water penetrates into the polymer network and allows swelling. The high water content offers them excellent biocompatibility by which hydrogels have become as an ideal choice for tissue engineering, drug delivery, and bio-nanotechnology applications (Peppas et al., 2006).

Based on the background we aimed at the synthesis of copolymer (AP) hydrogel scaffolds using alginate and PPF. In order to enhance the mechanical properties and amphiphilic character for cell growth, the AP copolymer was modified with polyethylene glycol diacrylate (PEGDA) and vinyl monomers to form hydrogels. The present paper deals with studies on the influence of physical and mechanical properties of amphiphilic biosynthetic hydrogels on long-term cell viability.

2. Methodology

2.1. *Materials*

Sodium alginate [guluronic acid (39%) and mannuronic acid (61%) from brown algae, medium viscosity, Product no. A2033], sodium chloride, maleic anhydride, calcium chloride, L-ascorbic acid, ammonium per sulfate, polyethylene glycol diacrylate (PEGDA), 2-hydroxy ethyl methacrylate (HEMA), methyl methacrylate (MMA) and N N' methylene bis acrylamide (NMBA) were obtained from Sigma-Aldrich, Spruce Street, St. Louis, USA. Sodium acetate, sodium hydroxide etc. were supplied by Merck specialties Pvt. Ltd, Mumbai, India. 1-2 propylene glycol and morpholine were provided by SD fine chemicals India Ltd. The mouse fibroblast cells (L929) were purchased from National Centre for Cell Science, Pune, India for cell culture studies.

2.2. *Preparation of chemically crsslinked and mechanically favorable biosynthetic Hydrogel scaffolds*

Hydroxyl terminated-poly (propylene fumarate) (HT-PPF) was synthesized by condensing maleic anhydride with 1–2,propylene glycol as described elsewhere (Jayabalan et al., 2009). The biosynthetic copolymer poly(propylene fumarate)-alginate, AP was prepared using HT-PPF and sodium alginate. 3.7 g HT-PPF was accurately weighed and warmed to 80–90 °C for 10 min. After cooling to 60 °C, 4 drops of conc. H_2SO_4 were added and stirred well. 7.4 g sodium alginate was immediately added under stirring condition and kept for more than 30 min to get poly(propylene fumarate)-alginate copolymer, AP. The entire mixture was then slowly dissolved in 150 ml distilled water under constant stirring and stored at room temperature for further studies. The molecular weight of AP was determined by HPLC using THF mobile phase at a flow rate of 1 ml/min and polystyrene standards (Mp-100,000, 9130, and 162). The biosynthetic copolymer hydrogels were prepared by crosslinking AP with PEGDA and vinyl monomer at 60 °C for overnight. 60 g AP was mixed with 3.75 g polyethylene glycol diacrylate (PEGDA) and 3.75 g each of 2-hydroxy ethyl methacrylate (HEMA). The reactants were mixed and casted at 60 °C for overnight. The cast sheets were treated for ionic crosslinking with Ca^{2+} and free radical induced polymerization with ascorbic acid and ammonium per sulfate. The hydrogel thus formed with 2-hydroxy ethyl methacrylate (HEMA) is coded as AP-PH. Similarly, methyl methacrylate (MMA) and N N' methylene bis acrylamide (NMBA) were used along with PEGDA to get AP-PM and AP-PN, respectively. The samples were cleaned and immersed in distilled water at room temperature to leach out the unreacted molecules. The samples were then freeze dried overnight, sterilized by ethylene oxide and used for further studies.

2.3. Characterization of hydrogels

2.3.1. Physiochemical and mechanical characterization

2.3.1.1. ATR spectral studies. ATR spectrum of biosynthetic copolymer hydrogels AP-PH, AP-PM and AP-PN were recorded by using Nicolet 5700 FTIR Spectrometer based on ASTME 1252-98 (re-approved 2007) and E 572-01 (re-approved 2007).

2.3.1.2. Dynamic water contact angle measurement. The surface properties of biosynthetic copolymer hydrogels were carried out by dynamic contact angle studies. Six clean samples of uniformly rectangular shape and known width were swelled in distilled water. The advancing and receding contact angle were determined using a Wilhelmy method on the KSV sigma 701 tensiometer by using distilled water as a solvent. The samples were immersed to a depth of 10 mm at a speed of 5 mm/min ignoring the first 2 mm length. The advancing and receding contact angle values were obtained using the software (sg server) provided with the instrument (Finosh et al., 2013).

2.3.1.3. Determination of equilibrium water content (EWC) and swelling ability. The equilibrium water content (EWC) and the swelling efficiency of hydrogels were determined by previously published procedures (Finosh and Jayabalan, 2012). The freeze-dried samples were allowed to attain maximum swelling in distilled water. From the dry weight and the wet weight, EWC and swelling ability were determined.

2.3.2. Determination of surface and pore morphology

The morphology and pore size of the freeze-dried scaffolds were determined by ESEM at low vacuum mode. The pore average length of scaffolds was calculated from the ESEM images using ImageJ software using multi-measure plugin.

2.3.3. Determination of crosslink density and mechanical strength

The crosslink density of the crosslinked hydrogel materials AP-PM and AP-PN was calculated as per the previously published protocols (Jayabalan, 2009).The samples were taken as circular disks and density was calculated using the volume and the dry weight of hydrogels. The hydrogels were allowed to attain maximum swelling in series of organic solvents having different solubility parameters (acetone, methanol, tetrahydrofuran, ethanol, dimethyl acetamide, toluene and dimethyl formamide) and in water. Since water exhibited maximum swelling, it was selected for further studies. The freeze-dried hydrogels were allowed to attain equilibrium swelling in water for 2 days. Both the dry weight and wet weights were accurately measured for the determination of θ using the following equation.

$$\text{Swelling coefficient } (\theta) = \frac{\text{Weight of the solvent in swollen polymer}}{\text{Weight of the swelled polymer}} \times \frac{\text{Density of polymer}}{\text{Density of solvent}}$$

From the swelling coefficient, the volume fraction *Vr* was calculated. The solubility parameter of water was taken the solubility as parameter of solvent and polymer. The crosslink density (γ) was calculated using the modified Flory–Rehner's equation; *Vr* is the volume fraction, *dr* is density of the polymer, χ is polymer–solvent interaction and *Vo* is molar volume of the solvent.

$$\text{Cross link density, } \gamma = -\frac{[Vr + \chi Vr^2 + \ln(1-Vr)}{drVo(\sqrt[3]{Vr} - (Vr/2))}$$

The tensile properties of the crosslinked hydrogel materials AP-PM and AP-PN were determined using water-swollen samples. The samples were cut to dumb bell shape using die and tensile properties were determined as per ISO 527-2 type 5A as reported already (Finosh et al., 2013). A universal automated mechanical test analyzer (Instron, model 3345) connected with long travel extensometer was used.

2.4. In vitro degradation studies

2.4.1. Stability in DMEM

The stability of hydrogels in cell culture medium (DMEM) was determined by swelling them in the medium at physiological temperature and the solubility was noted after 7 days. Three samples were used from each system.

2.4.2. Degradation in PBS

The long-term stability of hydrogels was determined by aging in the PBS for 30 days as reported already (Finosh et al., 2013). The weight loss and changes in pH, conductivity and total dissolved solids were monitored in regular intervals of time using the pH meter (cyber scan pc510). The initial weight was noted. After aging, samples were removed from the medium at an interval of 7 days and freeze-dried. The dry weight after lyophilisation was determined. The weight loss was calculated. The changes in pH, conductivity and total dissolved solids in the medium were monitored.

2.5. Biological evaluation

2.5.1. Effect of AP hydrogels on blood compatibility

The influence of crosslinked hydrogel materials AP-PM and AP-PN on blood compatibility was evaluated using *in vitro* RBC aggregation assay, hemolysis assay, plasma protein adsorption assay and platelet adhesion studies.

2.5.1.1. In vitro RBC aggregation assay. Red blood cells (RBC) from healthy donor was collected with informed consent and diluted to ten times with sterile 0.9% NaCl saline solution after washing thrice with the same. The hydrogels were extracted in sterile 1 × PBS for 48 h. 100 µl of this PBS was mixed with 100 µl dilute RBC suspension and incubated at 37 °C for 30 min. After incubation, cells were microscopically examined for RBC aggregation. Positive (+ve) and negative (–ve) controls were used for comparison.

2.5.1.2. Hemolysis assay. The hemolysis induced by the material extract to the RBC suspension was evaluated by the standard procedures reported elsewhere (Seo et al., 2009).

2.5.1.3. Plasma protein adsorption assay. Plasma was separated from blood by centrifuging at 1500 rpm for 10 min and diluted to 10 ml with 1 × PBS. 1 ml diluted plasma was added

to the PBS-swelled hydrogels and incubated for 2 h at 37 °C on a shaker incubator. Then hydrogels were removed and the protein fraction left out was determined by the Lowry's method using a BSA standard (Hahm, 2011). The percentage of proteins adsorbed onto hydrogels was quantified with respect to the total protein content.

2.5.1.4. *Platelet adhesion studies*. Platelet-rich plasma (PRP) was prepared from anticoagulated-blood by centrifuging at 2500 rpm for 5 min. Then the blood was further centrifuged at 4000 rpm for 15 min to collect PPP (platelet poor plasma). PRP count was adjusted to 2.0–2.5 $\times 10^8$/ml with PPP, carefully added on the center of the PBS-swelled hydrogels, and then incubated at room temperature for 30 min in a shaker incubator. Afterwards, hydrogels were washed 3 times with PBS (pH-7.4) and platelets adhered on hydrogels were fixed with 2.5% glutaraldehyde (in PBS) solution as per published protocols (Dhandayuthapani et al., 2012). These samples were then freeze-dried and examined in an environmental scanning electron microscope.

2.5.2. *In vitro evaluation of cytotoxicity*

The cytotoxicity of hydrogels was evaluated by MTT assay, the direct contact method and Live/Dead Assay.

2.5.2.1. *Cell culture*. The mouse fibroblast cells (L929) were grown in DMEM supplemented with 10% FBS and penicillin streptomycin and amphotericin-b (5000 units) in a humidified incubator at 5% CO_2 at 37±0.2 °C. The cells were regularly monitored. The medium was changed once in three days. The confluent monolayer was split for maintenance and future studies.

2.5.2.2. *Evaluation of the toxicity of hydrogel extracts*. The cytotoxicity of hydrogels extracts was evaluated as per ISO 10993-5 on L-929 mouse fibroblast cell culture. The extract of the hydrogel was prepared with DMEM medium. Cell suspension containing approximately 1×10^5 cells/ml in the above medium were seeded onto a 24 well tissue culture plate and incubated at 37 °C and 5% CO_2 for 72 h. The percentage of the surviving fibroblast cells were quantified by the standard MTT assay (Idris et al., 2010).

2.5.2.3. *Direct contact method*. The cytotoxicity of hydrogels under the direct contact of cell was determined by direct contact assay. L929 fibroblast cells (1×10^4 cells/m) were seeded on to a 12 well plate (BD Falcon) and allowed to proliferate for 24 h to form a sub-confluent layer. Then the hydrogel (1 cm diameter) was placed over the monolayer and allowed to proliferate for 24 h in a CO_2 incubator. After the incubation, cells were evaluated with respect to a control (cells grown without hydrogels) under inverted phase contrast microscope attached with an imaging camera. The images were captured using imaging software.

2.5.2.4. *Live/dead assay*. The L929 cells grown in contact with the hydrogel for 5 days were evaluated with Live/Dead Assay. A mixture of acridine orange (100 μg/ml) and ethidium bromide (100 μg/ml) were added to the L929 cells and immediately viewed under an epifluorescence microscope (Optika SRL) using a blue filter for acridine orange and green filter for ethidium bromide. Two images were taken from the same field without changing settings of microscope using both filters. The images were merged by imaging Photoshop8 CS software (Finosh et al., 2013).

2.5.2.5. *Determination of long-term viability of fibroblast by MTT assay*. The long-term viability of fibroblast adhered on to the surface and migrated to the inner pores of all the AP hydrogel was determined by a modified version of MTT assay (Park et al., 2007). Around 2×10^5 cells were seeded to the DMEM-swelled hydrogels and allowed to proliferate and infiltrate for 3 weeks. Once on 3rd day fresh medium was supplied to cultures and on every 6th day, hydrogels were washed with PBS to remove the free and loosely bound cells. Then 1 ml MTT solution (1 mg/ml) was added and incubated at 37 °C for 3 h. After incubation, hydrogels were extracted with the solution containing 0.01 N HCl in isopropanol. The contents were vortexed for 30 min to detach all cells adhered in hydrogels. It was then centrifuged at 10,000 rpm for 10 min to settle the hydrogel particles and cell debris. Then the OD of the supernatant was measured at 570 nm. A control was run with cells grown without hydrogel and a reagent blank containing scaffold without cells was run in a similar manner. From the OD values the long-term viability of cells grown inside the hydrogel were calculated.

2.6. *Statistical analysis*

All experiments consisted of 5 or 6 samples from each group. The values are presented as means±standard deviations. Statistical analysis was done with one way ANOVA using online calculator, Statistics Calculator version-3 beta and the level of significance was set at $p<0.05$ for all calculations.

3. Results and discussion

3.1. *Preparation and evaluation of chemically cross-linked and mechanically favorable biosynthetic hydrogel scaffolds*

The present studies involve the chemically cross-linked and mechanically favorable biosynthetic hydrogel. The hydrogel was prepared using the biosynthetic copolymer, poly (propylene fumarate)-co-alginate copolymer, PA. The biosynthetic copolymer PA is an oligomer with number average molecular weight 1784 and weight average molecular weight 2010. The addition of PEGDA to AP increases the hydrophilicity due to its abundant hydroxyl groups and mechanical properties due to the cross linking with the double bonds of PPF. Moreover, the present crosslinking with PEGDA allows unreacted excess double bonds for further crosslinking with other vinyl monomers, 2-hydroxy ethyl methacrylate (HEMA), methyl methacrylate (MMA) and N N' methylene bis acrylamide (NMBA) to form the chemically crosslinked and mechanically favorable hydrogels AP-PH, AP-PM and AP-PN, respectively. These vinyl monomers gained importance due to their biocompatibility and their ability to enhance mechanical properties (Mitha and Jayabalan, 2009; Vallbacka and Sefton, 2007;

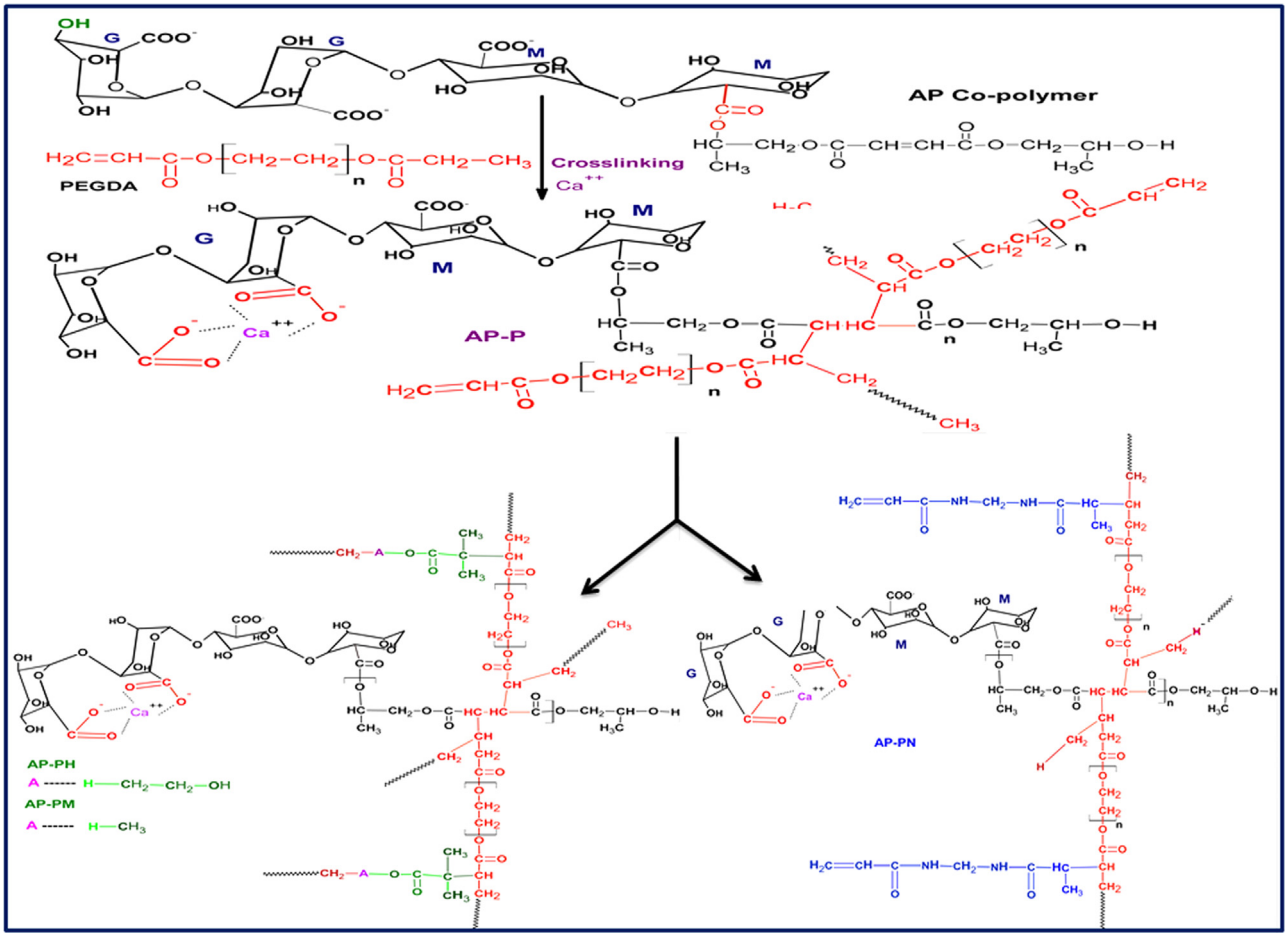

Fig. 1 – Schematic representation of the synthesis of AP-P based hydrogels.

Jagur-Grodzinski, 2010). The details of the synthesis are given in Fig. 1.

The alginate used for this study is composed of 39% guluronic acid (G) and 61% mannuronic acid (M) and possessed medium viscosity (Product no. A2033, Sigma-Aldrich, USA). It was reported that the amount of G residues determines the mechanical properties of the alginate gels formed by crosslinking with divalent ions, since the gelling is the result of chelation of divalent ions with the carboxylate anionic groups of the G residues (Drury et al., 2004). In alginates with high M content, the degree of polymerization is low when compared with alginates with high G content; gels resulting from these alginates will be mechanically weak (Becker et al., 2001). Even though gels made from high G content alginate are mechanically stable, the inherent brittleness results in the increased permeability of bioactive molecules like antibodies (Stewart and Swaisgood, 1993; Klein et al., 1983; Klijck et al., 1997). Moreover, the susceptibility of high G content alginates to fibrosis and immunological responses which is much higher when compared with that of high M content alginate (Klijck et al., 1997; De Vos et al., 1997).

Since the biocompatibility and mechanical properties are essential for the performance of hydrogel as scaffolds for cardiac applications, we improved the mechanical properties of high M content alginate by copolymerizing with a mechanically robust

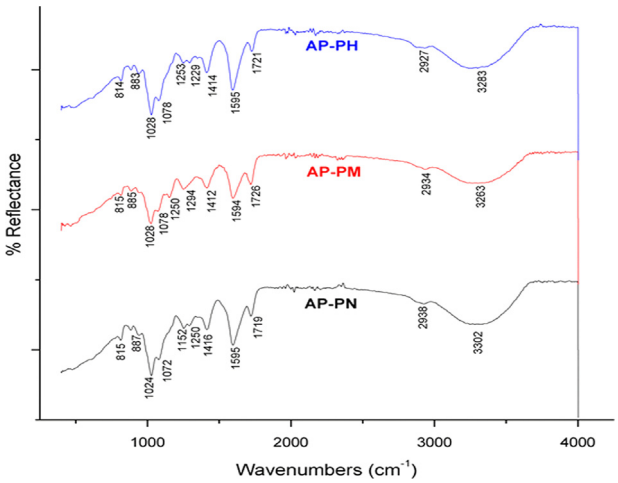

Fig. 2 – IR spectrum of freeze-dried AP-PH, AP-PM and AP-PN hydrogels showing the effective cross linking of the vinyl monomers.

and biocompatible polymer, the PPF to form the AP copolymer. The mechanical properties were further increased by reacting with PEGDA and subsequent crosslinking with the vinyl monomers like HEMA, MMA and NMBA as discussed in the latter section.

The IR spectrum revealed (Fig. 2) a broad peak around 3200–3300 cm^{-1} for AP-PH, AP-PM and AP-PN showing the presence of –OH groups of the alginate on the surface of the hydrogel. With AP-PN, this peak was shifted to 3300 cm^{-1} indicating the stretching of both –OH and –NH groups. With AP-PH, AP-PM and AP-PN, the peaks around 2900 cm^{-1} showed the asymmetric stretching of –CH– groups and peaks around 1700 cm^{-1} revealed the carbonyl stretching, both indicating the ester bond formation. The peaks around 1594–1595 cm^{-1} and 1412–1416 cm^{-1} were due to the asymmetric and symmetric stretching of carboxylate groups indicating their presence in the hydrogel surface. The peaks around 1229–1294 cm^{-1} and 1024–1028 cm^{-1} were due to the asymmetric and symmetric stretching of –C–O–C– of alginate. The ATR analyses confirmed the ester bond formation and effective cross linking of the vinyl monomers.

Contact angle measurements quantify the hydrophilicity of hydrogels. Materials having a contact angle value less than 30° is considered as highly hydrophilic; if it is less than 10°, the hydrogel is super hydrophilic. The values between 30° and 90° can be considered as amphiphilic. The values above 90 are indications of hydrophobicity. The hydrogels having the contact angles ranging from highly hydrophilic to amphiphilic range are desirable for their better blood compatibility and cell attachment for tissue engineering applications (van Wachem et al., 1987). The contact angles of the present hydrogels fall in the amphiphilic range (Table 1). Therefore, the present hydrogels will promote better cell responses and compatibility. This amphiphilicity is due to the ample amount of free polar functional groups like carboxylate and hydroxyl, which is imparted especially by alginate and PEGDA.

The swelling and EWC of a hydrogel are proportional to the amount of water entered and imbibed; this water plays a significant role in the diffusion of solutes from the surrounding medium. The micro architecture resulting from cross-linking will also influence the water holding efficiency of the hydrogel. Swelling properties of hydrogels are also enhanced by the presence of ionic groups as these groups impart additional osmotic pressure due to the presence of counter ions. This osmotic pressure neutralizes with the swelling of polymer chains of the hydrogel to accommodate more water inside (Okay, 2009). The swelling and EWC for the AP-P hydrogels are given in Table 1. The data suggest that the AP-PM hydrogels showed maximum swelling and EWC. The other two hydrogels AP-PH and AP-PN showed lesser but appreciable swelling and water holding capacity. A favorable microenvironment is orchestrated by the diluting effect of the water present in hydrogels for cell penetration and survival.

Crosslink density was determined with respect to water because maximum swelling was observed in water. Therefore, the solubility product of hydrogels was close to that of water. The results are given in Table 1. The crosslink density is slightly higher in the case of AP-PM when compared with AP-PN. This will be due to the increased chain length of NMBA crosslinker than that of MMA. The Flory–Rehner equation will not determine the chemical crosslinks exclusively, but a small fraction of physical crosslinks will also be accounted. So the obtained values were also influenced by the physical and ionic interactions of the alginate segments. Moreover, the chain alignment has no effect on crosslink density by Flory-Rehner equation (Xia et al., 2013). The comparatively less swelling and increased tensile properties of AP-PN can be attributed to the presence of two vinyl groups of NMBA that might have imparted higher degree of cross linking than MMA.

Cardiac tissue consists of a well-defined extra cellular matrix (ECM) with inherent mechanical properties, which is crucial for the proper functioning of cardiovascular system. Therefore, hydrogels mimicking the cardiac ECM should be able to bear appreciable mechanical load until the repair and regeneration is completed. The removal of entire cells from the cardiac tissue will not significantly change the mechanical properties of the cardiac ECM (Berry et al., 1975). The evolution has universally fixed the elastic modulus of elastic arteries including aorta of the heart within a short range of 0.3–1 MPa for most organism. This is achieved by regulating the expression of ECM proteins (Wagenseil and Mecham, 2009). The myocardium of rat was reported to have the stiffness of 70 kPa (Boublik et al., 2005). The stiffness of circumferential and longitudinal right ventricle was 54±8 kPa and 20±4 kPa (Engelmayr et al., 2008), respectively. The stiffness of the adult rat left ventricle was found to be 18±2 kPa (Berry et al., 2006). The adult left ventricle was found to have an ultimate tensile strength of 108 kPa with 63.8% elongation (Kallukalam et al., 2008). The tensile properties of the water swelled AP-P based hydrogels are higher than the above-mentioned values showing their applicability for cardiac tissue engineering (Table 2). Since these hydrogels possess higher values than the reported ones, it can compromise the loss of mechanical properties alginate fraction of the polymer due to the exchange of monovalent ions with that of Ca^{2+} ions. The relative higher tensile properties of AP-PN may be due to its higher crosslinking of NMBA with respect to its

Table 1 – Surface and swelling properties of hydrogels.

Parameters ($n=6$)	AP-PH	AP-PM	AP-PN
Surface properties			
Advancing angle ($P<0.001$)	38.87±2.86	32.67±2.9	33.06±3.68
Receding angle ($P<0.001$)	39.63± 2.81	34.28±2.94	34.01±3.36
Swelling properties			
EWC ($P<0.001$)	72.22±1.33	81.49±1.88	71.95±5.13
% Swelling ($P<0.001$)	260.62±17.24	444.72±54.11	267.16±67.58
Swelling coefficient (θ)	—	0.2949	0.2603
Cross-link density (γ) (g/cc)	—	0.1021	0.0845

Table 2 – Mechanical properties of hydrogels.

Parameters ($n=6$)	AP-PH	AP-PM	AP-PN
Average pore length ($P<0.001$)	64.84±11.54	37.96±9.57	43.93±7.13
Density	—	0.5514	0.7206
Tensile strength (KPa) ($P<0.001$)	1051±140	615±108	1640±173
Elongation at break (%) ($P<0.001$)	105.32±8.65	79.66±10.38	61.54±9.87
Young modulus (KPa)($P<0.001$)	2002±311	1212±200	6032±1441

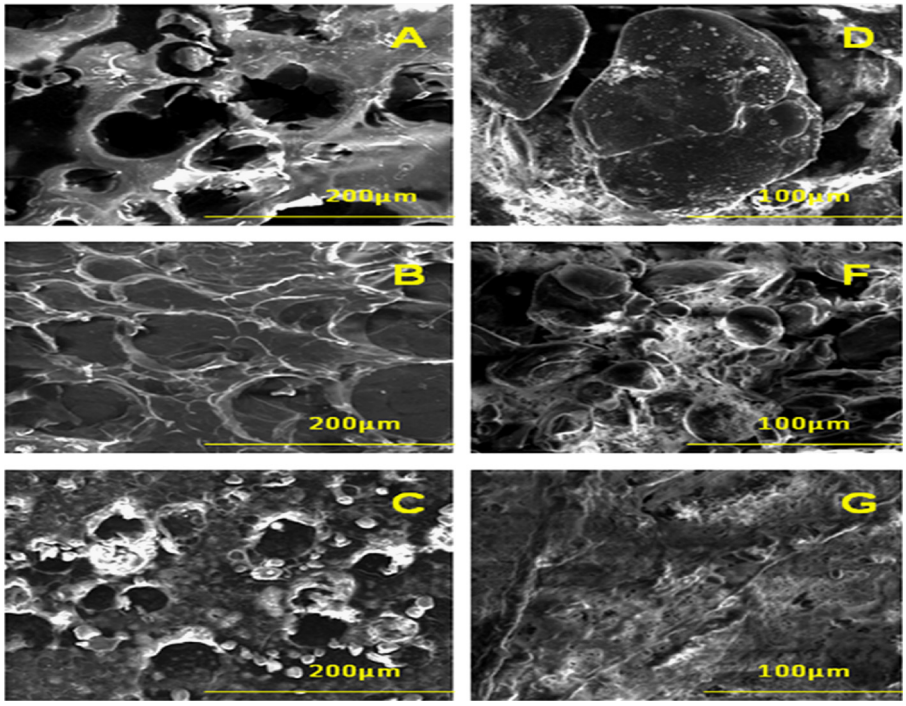

Fig. 3 – ESEM analysis showing the characteristic pore morphology of AP-PH (A), AP-PM (B) and AP-PN (C) and the absence of platelet adhesion after PRP agitation – AP-PH (D), AP-PM (E) and AP-PN (G).

two vinyl functional groups. This is the reason for low tensile properties and water holding ability of AP-PM, even though the vinyl cross linker MMA is hydrophobic than NMBA.

The porosity and their morphology influence the cell response and viability of tissue engineering scaffolds (Bryant et al., 2007). The maximum thickness of the tissue engineering constructs that allows the diffusion of nutrients and oxygen was reported to be 150–200 μm. The porosity also plays a significant role in evoking various biological responses. The reported optimum pore size for neovascularization is 5 μm, 5–15 μm for fibroblast infiltration, 100–350 μm for bone regeneration. The micro-scale architecture can also determine cellular orientation, clustering, penetration, and desired function of the seeded cell (Annabi et al., 2011). If the porosity is too small the cell penetration will be limited and results an over confluence of cell on the seeded surface, similar to 2D culture (Lien et al., 2009). Leor and Cohen claimed that hydrogels for cardiac applications should have an average porosity of 50 μm and appreciable mechanical properties for better coordination of proper signals in a time dependent manner (Leor and Cohen, 2004). The mechanical properties also have implications on porosity and pore distribution; both these properties are influenced by molecular weights of the polymer (Nwe et al., 2009). The ESEM analysis of AP-P based hydrogels showed their characteristic pore morphology (Fig. 3). We preferred ESEM analysis to rule out chances of morphological variations during the analysis due to fixing and coating procedures. Of the three, the AP-PH had comparatively large pores as evaluated by ImageJ software (Table 2). The greater pore length of AP-PH was due to the hydrophilicity imparted by the HEMA crosslinker, which promoted more water adsorption and subsequent formation of comparatively large ice crystals during freezing. The evacuation of these ice crystals resulted in the formation of large pores. In all samples most of the pores remained in a closed state. These pores are opened during the swelling process by absorbing the medium. Due to the large pore size, AP-PH can absorb more medium to swell resulting in rapid degradation than the others can. However, the smaller pore size and higher tensile strength of AP-PN makes it resistant to degradation even though both of them possess very similar swelling and EWC. AP-PM possessed uniform porosity and appreciable tensile properties suited for cardiac tissue engineering.

3.2. Evaluation of degradation of hydrogels

The degradation of the AP hydrogels was evaluated in both DMEM (containing 10% FBS) and in PBS under physiological conditions. All hydrogels except AP-PH were stable for more than 1 week. The DMEM-stable hydrogels, AP-PM and AP-PN were subjected to long-term degradation studies in PBS. AP-PM and AP-PN showed an increase in the dry weight after 1 week and 2 week of aging, respectively. This increase in the dry weight may be due to the entrapment of some water molecules inside the highly crosslinked networks of these hydrogels as the initial degradation phase allows more water particles to penetrate these hydrogels. The medium attained a slight acidic pH. Nevertheless, in the case of AP-PN system the pH was initially slightly acidic and propagates towards neutrality. This slight acidity can easily be buffered by the physiological buffers in vivo after implantation. The ionic contents released to the medium because of degradation will contribute to the conductivity and TDS of the medium, which is an indication of biodegradation. The TDS and conductivity of all hydrogels increase in a time dependent manner that signifies the biodegradation of these hydrogels in the simulated biological fluid, PBS. The data on biodegradation are given in Fig. 4. It was reported that the rate of degradation on the surface is constant which will maintain the bulk structure of the hydrogel. Such degradation provides better opportunities for tissue regeneration and repair (Hahm, 2011).

3.3. Evaluation of hemocompatibility

Hemocompatibility of hydrogels was assessed by RBC aggregation assay, hemolytic assay, platelet adhesion assay and indirectly by the determination of plasma protein adsorption. The initial event occurring when blood encounters a hydrogel is the adsorption of the plasma proteins onto the surface. The compatibility of the material depends on the amount and type of the protein adsorbed on the hydrogel surface (Varon et al., 2000, Sanak et al., 2010). The surface roughness of materials interrupts the normal blood flow leading to the accumulation and aggregation of blood cells and subsequent clot formation. Therefore, the surface smoothness can impart hemocompatibility. For instance the inner lining of the blood vessels, the endothelium, is smooth composed of cells with less than 1 μm thickness (Finosh and Jayabalan, 2013a). RBC membranes are susceptible to damage and rupture upon encountering the rough surface. This leads to RBC aggregation and hemolysis. Moreover, the major hemostatic component, the platelets, is activated upon contact with the implanted hydrogels and lead to thrombosis. WBCs and RBCs can also accumulate and adhere to the fibrin clot and aggravate the immune responses (Finosh and Jayabalan, 2013b). All these events can be prevented by the adsorption of albumin protein from the blood plasma. So the adsorbed albumin layer on the hydrogel surface is crucial for enhancing the hemocompatibility of materials (Liu et al., 2005). All the three AP-P hydrogels are non-hemolytic (Table 3) and no aggregation of RBCs were evident (Fig. 5). These results show that hydrogels will not alter the membrane integrity and function of RBCs upon contact with them. The complete absence of platelets in the PRP treated hydrogels reveal the potential of AP-P hydrogels to prevent the thrombus formation (Fig. 3). The plasma protein adsorption of the AP-PN hydrogels is found to be greater when compared with that of other two (Table 3). Since albumin is the most abundant protein of the blood plasma, it will be the most adsorbed one on the hydrogel surface. The SDS-PAGE analysis showed that the band corresponding to the molecular weight of albumin was prominent on all three hydrogels (Fig. 6). All these results confirmed that all three AP-P hydrogels are hemocompatible and will not evoke any adverse effects in blood flow and compatibility.

Table 3 – Hemolysis and Protein adsorption of hydrogels.

Parameters ($n=6$)	AP-PH	AP-PM	AP-PN
% Hemolysis	0.71%	0.71%	0.71%
% Protein adsorption	4.45%	3.92%	26.7%

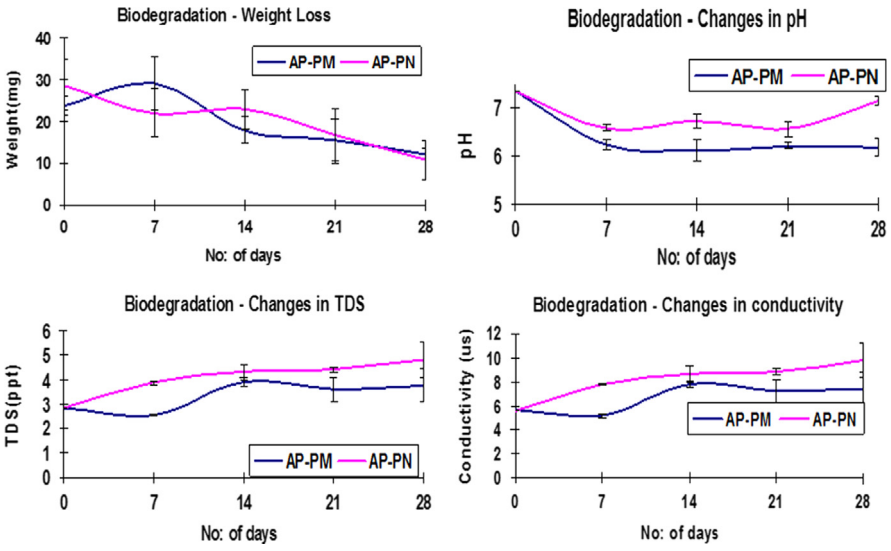

Fig. 4 – Biodegradation profile of AP-PM and AP-PN hydrogel scaffolds.

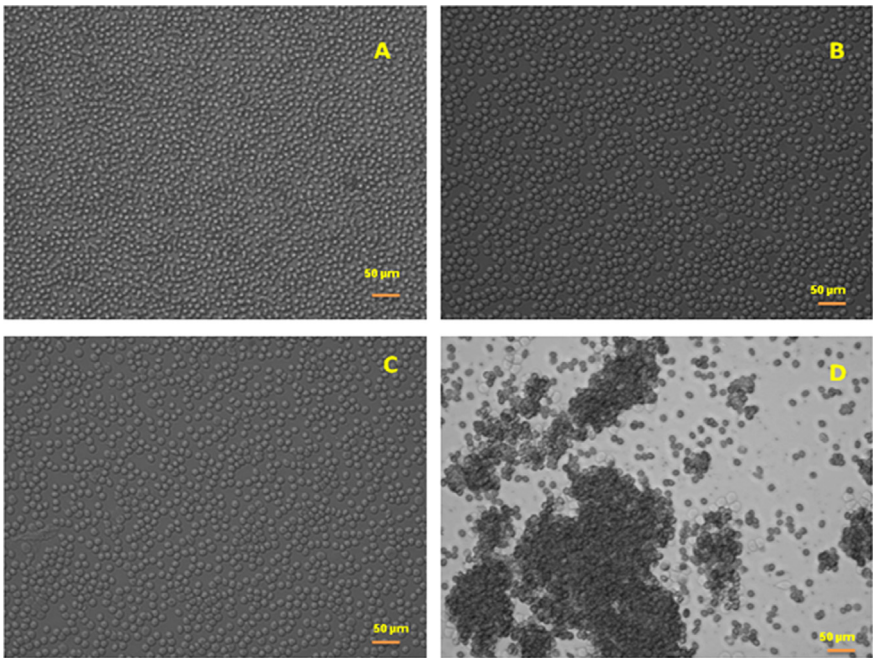

Fig. 5 – RBC aggregation studies. AP-PH (A), AP-PM (B) and AP-PN (C) showing no aggregation when compared to that of PEI treated +ve control (D).

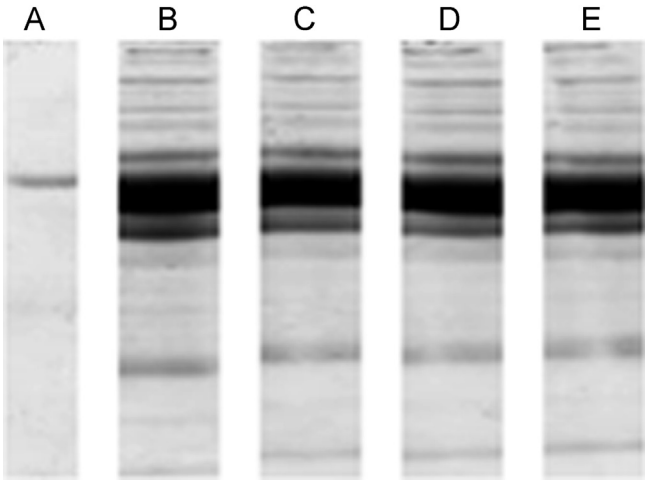

Fig. 6 – SDS-PAGE analysis of AP-PH (C), AP-PM (D) and AP-PN (E) hydrogels after incubating with plasma showing the prominent band corresponding to the adsorption of albumin when compared to that of control plasma (B) and bovine serum albumin (A).

3.4. *Cytocompatibility of hydrogels*

The cytotoxicity assays revealed the non-toxic nature of the AP-P hydrogels. All hydrogels exhibited viability greater than 85% (Fig. 7). The AP-PH hydrogels were found to have less stability in the culture medium. Still it promotes the viability to 90%. These are clear indications of non-toxic nature of degradation products or byproducts. The direct contact of hydrogels with the sub-confluent monolayer of L929 cells did not evoke any changes in the cell morphology and proliferation as an evident from direct contact assay (Fig. 8).

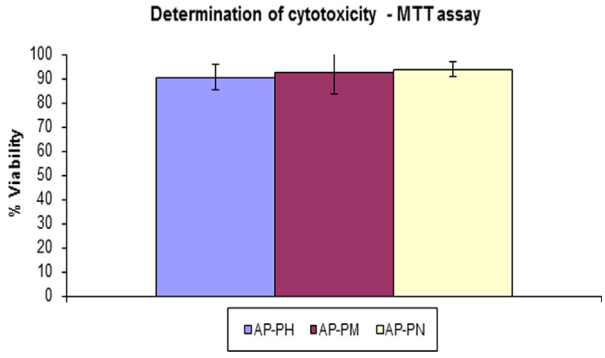

Fig. 7 – Determination of cytotoxicity by MTT assay on hydrogel extracts.

Live/dead assay revealed that all cells seeded on to the AP-PN and AP-PM hydrogels were able to maintain their proper health status as evident by the green fluorescence (Fig. 8). The control cells used for live/dead assay were grown on the tissue culture plate without hydrogels. Such cells (in 2d environment) form a monolayer after attaining confluence and display a uniform distribution of cells (Fig. 8F). Nevertheless, hydrogels offer a three dimensional environment to cells due to their porous structure. Therefore, cells grown on hydrogels seemed to be lesser in number, which can be attributed to the increased surface area of hydrogels. It was obvious from the ESEM analysis that the average pore length of AP-PN was greater than that of AP-PM. This will be due to the increase in chain length of the NMBA segments that form the cross links with comparatively larger distance than done by MMA in AP-PM. The cells prefer to adhere and proliferate on the edges these pores where the nutrient availability will be greater. This will result in the uneven distribution of cells in hydrogels (Figs. 8D and 8E). From this, it is clear that these

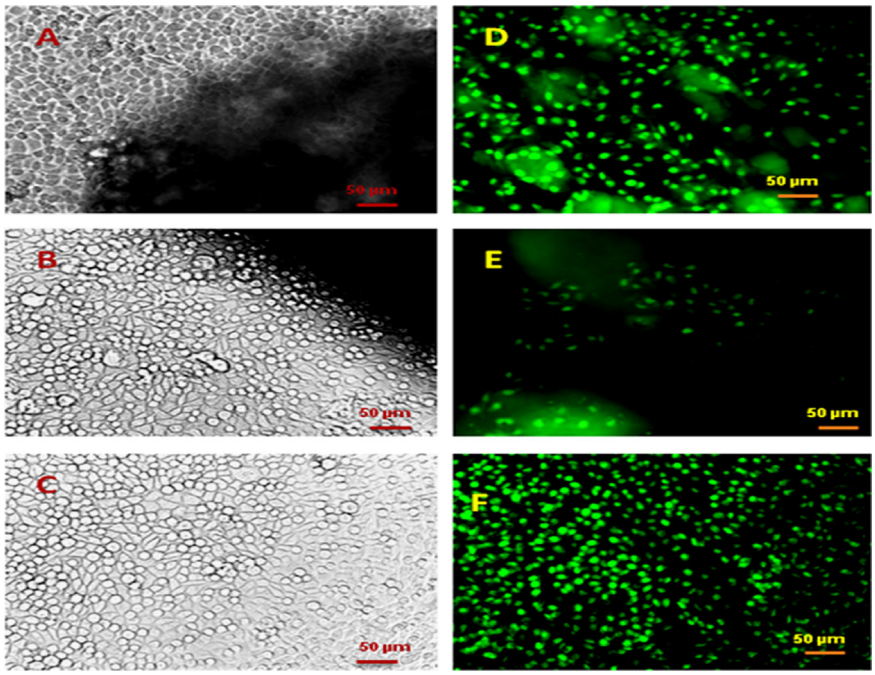

Fig. 8 – Direct contact assay of hydrogels showing the non-toxic nature of AP-PM (A) and AP-PN (B) with respect to the control (C). The live/dead assay also revealed healthy L929 cells with AP-PM (D) and AP-PN (E) compared with that of the control (F).

hydrogels were able to provide a favorable microenvironment for the growth of cells.

3.5. *Mechanical strength vs. long-term cell viability in hydrogels*

The mechanical properties of hydrogels can affect cell behaviors such as proliferation, migration, and differentiation (Engler et al., 2006, Khatiwala et al., 2006, Pelham, Wang, 1997). The stiffness of the extracellular matrix (ECM) provides vital instructional cues to migrating smooth muscle cells (Engler et al., 2004, Peyton and Putnam, 2005, Wong et al., 2003) and fibroblasts (Pelham, Wang , 1997, Lo et al., 2000) and induce cells to migrate in a directional fashion from softer substrates to stiffer substrate. The long-term viability of L929 cells on AP-PM and AP-PN hydrogels were quantified for 18 days (Fig. 9) and both hydrogels presented appreciable viability even after 18 days. There was no considerable difference between the viability. Even though the AP-PH hydrogel was compatible, its lower mechanical strength in the culture medium forms a hurdle for its application in the field of cardiac tissue engineering. During the course of time, hydrogels will get degraded and pave way to the enlargement of existing and opening of newer pores towards the inner side. This will enhance the nutrient circulation, which will attract more cells towards the interior of the scaffolds.

The mechanical properties of hydrogels should be matched with the anatomical site of implantation. This offers challenge for developing organ specific tissue engineering scaffolds especially in the case of heart. Moreover, the implanted scaffolds have to get mechanical integrity with the host until the regeneration of the target site is completed. Still there should be a balance between the mechanical properties and porosity of hydrogels for the better infiltration

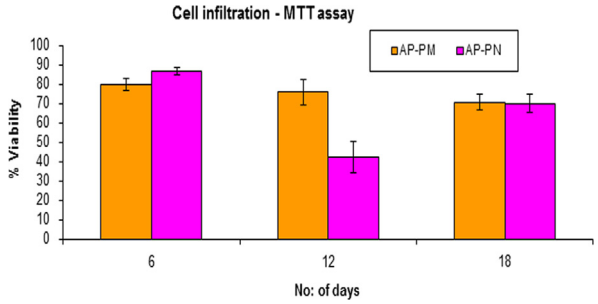

Fig. 9 – Cell infiltration assay of L929 cells with AP-PM and AP-PN hydrogels.

of cells (O'Brien, 2011). The AP-PH hydrogel was compatible but its lower mechanical strength in the culture medium forms a hurdle for its application in the field of cardiac tissue engineering as a scaffold. The physiochemical, mechanical and biological properties of the other two hydrogels AP-PM and AP-PN were found to be appropriate for cardiac applications.

4. Conclusion

Mechanically robust and chemically crosslinked biosynthetic hydrogels comprising alginate, polyproplyelene fumarate and crosslinkers polyethylene glycol diacrylate (PEGDA) and vinyl monomer were prepared. The effect of PEGDA and vinyl monomers, 2-hydroxy ethyl methacrylate (HEMA), methyl methacrylate (MMA) and N N' methylene bis acrylamide (NMBA) on the degradation and mechanical stability and cell viability was assessed. All three hydrogels were amphiphilic, hemocompatible and non-cytotoxic and exhibited appreciable water holding capacity. Comparatively, AP-PN hydrogel

displayed larger pore size, increased crosslinking and higher tensile strength, which make it resistant to degradation even though both AP-PN and AP-PH based hydrogels possess very similar swelling and EWC. AP-PH degraded in a faster rate compared with that of others. Hydrogel AP-PN was found to be mechanically strong and showed better biological responses with long-term viability of fibroblasts viz 18 days. AP-PN hydrogel is more promising for cardiac tissue engineering.

REFERENCES

Andersen, T., Strand, B.L., Formo, K., Alsberg, E., Christensen, E.B. E., 2012. Alginates as biomaterials in tissue engineering. Carbohydr. Chem. 37, 227–258.

Annabi, N., Nichol, J.W., Zhong, X., Ji, C., Koshy, S., Khademhosseini, A., Dehghani, F., 2011. Controlling the porosity and microarchitecture of hydrogels for tissue engineering. Tissue Eng. Part B 16, 371–383.

Becker, T.A., Kipke, D.R., Brandon, T., 2001. Calcium alginate gel: a biocompatible and mechanically stable polymer for endovascular embolization. J. Biomed. Mater. Res. Part-A 54, 76–86.

Berry, C.L., Greenwald, S.E., Rivett, J.F., 1975. Static mechanical properties of the developing and mature rat aorta. Cardiovasc. Res. 9, 669–678.

Berry, M.F., Engler, A.J., Woo, Y.J., Pirolli, T.J., Bish, L.T., Jayasankar, V., Morine, Gardner, T.J., Discher, D.E., Sweeney, H.L., 2006. Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance. Am. J. Physiol. Heart Circ. Physiol. 290, 2196–2203.

Boublik, J., Park, H., Radisic, M., Tognana, E., Chen, F., Pei, M., Vunjak-Novakovic, G., Freed, L.E., 2005. Mechanical properties and remodeling of hybrid cardiac constructs made from heart cells, fibrin, and biodegradable, elastomeric knitted fabric. Tissue Eng. 11, 1122–1132.

Bryant, S.J., Cuy, J.L., Hauch, K.D., Ratner, B.D., 2007. Photo-patterning of porous hydrogels for tissue engineering. Biomaterials 28, 2978–2986.

De Vos, P., De Haan, B., Van Schilfgaarde, R., 1997. Effect of the alginate composition on the biocompatibility polylysine microcapsules. Biomaterials 8, 273–278.

Dhandayuthapani, B., Varghese, S.H., Aswathy, R.G., Yoshida, Y., Maekawa, T., Sakthikumar, D., 2012. Evaluation of anti thrombogenicity and hydrophilicity on Zein-SWCNT electrospun fibrous nanocomposite scaffolds. Int. J. Biomater. Article ID 345029, 10 pp. ⟨http://dx.doi.org/10.1155/2012/345029⟩.

Drury, J.L., Dennis, R.G., Mooney, D.J., 2004. The tensile properties of alginate hydrogels. Biomaterials 25, 3187–3199.

Engelmayr Jr., G.C., Cheng, M., Bettinger, C.J., Borenstein, J.T., Langer, R., Freed, L.E., 2008. Accordion-like honeycombs for tissue engineering of cardiac anisotropy. Nat. Mater. 7, 1003–1010.

Engler, A., Bacakova, L., Newman, C., Hategan, A., Griffin, M., Discher, D., 2004. Substrate compliance versus ligand density in cell on gel responses. Biophys. J. 86, 617–628.

Engler, A.J., Sen, S., Sweeney, H.L., Discher, D.E., 2006. Matrix elasticity directs stem cell lineage specification. Cell 126, 677–689.

Finosh, G.T., Jayabalan, M., Vandana, S., Raghu, K.G., 2013. Growth and survival of cells in biosynthetic poly vinyl alcohol–alginate IPN hydrogels for cardiac applications. Colloids Surf. B: Biointerfaces 107, 137–145.

Finosh, G.T., Jayabalan, M., 2012. Regenerative therapy and tissue engineering for the treatment of end-stage cardiac failure: new developments and challenges. Biomatter 2, 1–14.

Finosh, G.T., Jayabalan, M., 2013a. Biosynthetic hydrogels—studies on chemical and physical characteristics on long-term cellular response for tissue engineering. J. Biomed. Mater. Res. Part-A (Epub ahead of print, ⟨http://dx.doi.org/10.1002/jbm.a.34895⟩).

Finosh, G.T., Jayabalan, M., 2013b. Influence of plasma protein–hydrogel interaction moderated by absorption of water on long-term cell viability in amphiphilic biosynthetic hydrogels. RSC Adv. 3, 24509–24520. ⟨http://dx.doi.org/10.1039/C3RA43710H⟩.

Hahm, J., 2011. Functional polymers in protein detection platforms: optical, electrochemical, electrical, mass-sensitive, and magnetic biosensors. Sensors 11, 3327–3355.

Idris, S.B., Dånmark, S., Finne-Wistrand, A., Arvidson, K., Albertsson, A., Bolstad, A.I., Mustafa, K., 2010. Biocompatibility of polyester scaffolds with fibroblasts and osteoblast-like cells for bone tissue engineering. J. Bioact. Compat. Polym.. 25, 567–583.

Jagur-Grodzinski, J., 2010. Polymeric gels and hydrogels for biomedical and pharmaceutical applications. Polym. Adv. Technol. 21, 27–47.

Jayabalan, M., 2009. Studies on poly (propylenefumarate-co-caprolactone diol) thermoset composites towards the development of biodegradable bone fixation devices. Int. J. Biomater. Article ID 486710, 10 pp. ⟨http://dx.doi.org/10.1155/2009/486710⟩.

Jayabalan, M., Shalumon, K.T., Mitha, M.K., 2009. Injectable biomaterials for minimally invasive orthopedic treatments. J. Mater. Sci.: Mater. Med. 20, 1379–1387.

Kallukalam, B.C., Jayabalan, M., Sankar, V., 2008. Injectable polyethylene glycol terminated poly(propylene fumarate)/acrylamide biodegradable materials for cardiac applications. Hacet. J. Biol Chem. 36 (4), 283–290.

Kasper, F.K., Tanahashi, K., Fisher, J.P., Mikos, A.G., 2009. Synthesis of poly(propylene fumarate). Nat. Protoc. 4 (4), 518–525.

Khatiwala, C.B., Peyton, S.R., Putnam, A.J., 2006. Intrinsic mechanical properties of the extracellular matrix affect the behavior of pre-osteoblastic MC3T3-E1 cells. Am. J. Physiol.: Cell Physiol. 290, 1640–1650.

Klein, J., Stock, J., Vorlop, K.D., 1983. Pore size and properties of spherical Ca alginate biocatalysts. Eur. J. Microbiol. Biotechnol. 18, 86–91.

Klijck, G., Pfefferrnann, A., Ryser, C., Grijhn, P., Kuttler, B., Zimmermann, H.H.U., 1997. Biocompatibility of mannuronic acid-rich alginates. Biomaterials 18, 707–713.

Leor, J., Cohen, S., 2004. Myocardial tissue engineering: creating a muscle patch for wounded heart. Ann. N.Y. Acad. Sci. 1015, 312–319.

Li, Z., Guan, J., 2011. Hydrogels for cardiac tissue engineering. Polymers 3, 740–761.

Lien, S.M., Ko, L.Y., Huang, T.J., 2009. Effect of pore size on ECM secretion and cell growth in gelatin scaffold for articular cartilage tissue engineering. Acta Biomater. 5, 670.

Liu, T., Lin, W., Huang, L., Chen, S., Yang, M., 2005. Hemocompatibility and anaphylatoxin formation of protein-immobilizing polyacrylonitrile hemodialysis membrane. Biomaterials 26, 1437–1444.

Lo, C.M., Wang, H.B., Dembo, M., Wang, Y.L., 2000. Cell movement is guided by the rigidity of the substrate. Biophys. J. 79, 144–152.

Mitha, K.M., Jayabalan, M., 2009. Studies on biodegradable and crosslinkable poly(castor oil fumarate)/poly(propylene fumarate) composite adhesive as a potential injectable biomaterial. J. Mater. Sci.: Mater. Med. 20, 203–211.

Novakovic, G.V., Tandon, N., Godier, A., Maidhof, R., Marsano, A., Martens, T.P., Radisic, M., 2010. Challenges in cardiac tissue engineering. Tissue Eng. Part B. Rev. 16 (2), 169–187.

Novikova, L.N., Mosahebi, A., Wiberg, M., Terenghi, G., Kellerth, J., Novikov, L.N., 2006. Alginate hydrogel and matrigel as potential cell carriers for neurotransplantation. J. Biomed. Mater. Res. Part-A 77, 242–252.

Nwe, N., Furuike, T., Tamura, H., 2009. The mechanical and biological properties of chitosan scaffolds for tissue regeneration templates are significantly enhanced by chitosan from Gongronella butleri. Materials 2, 374–398.

O'Brien, F., 2011. Biomaterials and scaffolds for tissue engineering. Mater. Today 14 (3), 88–95.

Okay, O., 2009. General properties of hydrogels. In: Gerlach, G., Arndt, K.-F. (Eds.), Hydrogel Sensors and Actuators, Springer Series on Chemical Sensors and Biosensors, 6; 2009 http://dx.doi.org/10.1007/978-3-540-75645-3_1.

Park, J.S., Woo, D.G., Sun, B.K., Chung, H., Im, S.J., Choi, Y.M., Park, K., Huh, K.M., Park, K., 2007. In vitro and in vivo test of PEG/PCL-based hydrogel scaffold for cell delivery application. J. Control Release 124, 51–59.

Pelham Jr, R.J., Wang, Y., 1997. Cell locomotion and focal adhesions are regulated by substrate flexibility. Proc. Natl Acad. Sci. U. S. A. 94, 13661–13665.

Peppas, N.A., Hilt, J.Z., Khademhosseini, A., Langer, R., 2006. Hydrogels in biology and medicine: from molecular principles to bionanotechnology. Adv. Mater. 18, 1345–1360.

Peyton, S.R., Putnam, A.J., 2005. Extracellular matrix rigidity governs smooth muscle cell motility in a biphasic fashion. J. Cell Physiol. 204, 198–209.

Rosellini, E., Cristallini, C., Barbani, C., Vozzi, N., Giusti, P., G., 2009. Preparation and characterization of alginate/gelatin blend films for cardiac tissue engineering. J. Biomed. Mater. Res. Part-A 91, 447–453.

Sanak, M., Jakieła, B., Węgrzyn, W., 2010. Assessment of hemocompatibility of materials with arterial blood flow by platelet functional tests. Bull. Polish Acad. Sci.: Tech. Sci. 58, 317–322.

Seo, K.H., You, S.J., Chun, H.J., Kim, C., Lee, W.K., Lim, Y.M., Nho, Y. C., Jang, J.W., 2009. In vitro and in vivo biocompatibility of γ-ray crosslinked gelatin-poly(vinyl alcohol) hydrogels. J. Tissue Eng. Regen. Med. 6, 414–418.

Stewart, W.W., Swaisgood, H.E., 1993. Characterization of calcium alginate pore diameter by size-exclusion chromatography using protein standards. Enzymes Microb. Technol. 15, 922–927.

Vallbacka, J.J., Sefton, M.V., 2007. Vascularization and improved in vivo survival of VEGF-secreting cells microencapsulated in HEMA-MMA. Tissue Eng. 13, 2259–2269.

van Wachem, P.B., Hogt, A.H., Beugeling, T., Feyen, J., Bantjies, A., Detmers, J.P., van, A.W.G., 1987. Adhesion of cultured human endothelial cells onto methacrylate polymers with varying surface wettability and charge. Biomaterials 8, 323–328.

Varon, D., Dardik, R., Shenkman, B., Kotev-Emeth, S., Farzame, N., Tamarin, I., et al., 2000. A new method for quantitative analysis of whole blood platelet interaction with extracellular matrix under flow conditions. Thromb. Res. 99, 353–361.

Wagenseil, J.E., Mecham, R.P., 2009. Vascular extracellular matrix and arterial mechanics. Physiol. Rev. 89 (3), 957–989.

Wong, J.Y., Velasco, A., Rajagopalan, P., Pham, Q., 2003. Directed movement of vascular smooth muscle cells on gradient-compliant hydrogels. Langmuir 19, 1908–1913.

Xia, Z., Patchan, M., Maranchi, J., Elisseeff, J., Trexler, M., 2013. Determination of crosslinking density of hydrogels prepared from microcrystalline cellulose. J. Appl. Polym. Sci. 127 (6), 4537–4541. ⟨http://dx.doi.org/10.1002/app.38052⟩.