NEW FIBRIN-POLYMER INTERPENETRATING NETWORKS: A POTENTIAL SUPPORT FOR HUMAN SKIN CONSTRUCT

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Interpenetrating polymer network (IPN) architectures have been conceived to improve the mechanical properties of a fibrin gel. Self-supported biomaterials are synthesized rapidly (one pot – one shot process) and combine the properties of both a protein gel and a synthetic polymer. IPN architectures have been characterized with biochemical (ELISA), chemical (solvent extraction) and physicochemical (rheology, DMA) methods. Mechanical properties of a fibrin gel were improved (viscoelasticity x 100) by associating it with a polymer network (PEO, PVA) inside IPN architecture. The network composition insures the material biodegradability through enzyme hydrolysis. These co-network IPNs are the first ones to be potentially biodegradable through tunable fragmentation, then elimination. They also exhibit the unique feature for a protein-based biomaterial of being non-retractable when used as support for fibroblast culture. The material is biocompatible as demonstrated with human dermal fibroblasts. Adhesion, viability and proliferation of human dermal fibroblasts have been measured for various IPN compositions with Live/dead test and by confocal microscopy. This innovative biomaterials present good potentiality as supports for skin construct.

Keywords: IPN, tissue engineering, biodegradability

INTRODUCTION

Among the large field of soft biomaterials, hydrogels (Slaughter *et al*., 2009) occupy a major position as they are usually biocompatible and occasionally biodegradable. Hydrogels may be synthesized from natural or synthetic compounds or from a mixture of them. Hydrogels made from biomolecules have the advantage to mimic the physiological microenvironment of tissues and to be enzyme-responsive (Khetani and Bhatia, 2006). Fibrin hydrogels show promising biological properties for clinical applications in tissue engineering and damaged tissue regeneration (Anitua *et al.*, 2006; Shevchenko *et al.*, 2010); however, they are very soft and not easily handled when they are synthesized at the physiological concentration (Ahmed *et al.,* 2008).

To confer it good mechanical properties a fibrin network was entrapped inside interpenetrating polymer networks (IPN) architecture. IPN is defined as a combination of two polymer networks that are cross-linked in the presence of the other. This co-network was synthesized by copolymerization of serum albumin (SA) - a protein conferring the network biodegradability - and PVA (polyvinyl alcohol) – a synthetic polymer giving the network rigidity - both modified with methacrylate functions. PVA-SA co-network were then associated with fibrin gel inside IPNs through a one pot – one shot process. Their mechanical properties were evaluated by rheological measurements. Their biodegradability through enzyme hydrolysis was followed and the hydrolysis rate related to the protein content. Finally, fibroblast viability and growth on the different IPN surfaces were examined to demonstrate their biocompatibility.

EXPERIMENTAL PROCEDURES

IPNs were synthesized as follows: PVA and/or SA (total concentration 100 mg), both bearing methacrylate groups were mixed with 5 mg fibrinogen and 0.042% (w/v) Irgacure 2959 all dissolved in 1 mL Tris buffer 50mM at pH 7.4 containing $CaCl_2$ and NaCl, then 0.20 unit thrombin were added. Gelation study was performed at 37°C in a rheometer equipped with U.V source (4.46 mW/cm²) using a cone-plate geometry. Mechanical properties were analyzed with 1% imposed deformation at 1 Hz.

Cell behavior was followed using Human fibroblast from foreskin (FB-BJ, ATCC CRL 2522). Viability tests were performed by staining cells with 0.2µM calcein AM and 0.2 µM ethidium bromide dimer for 30 min at 37°C. The staining of nuclei with DAPI was carried out for cell density evaluation.

RESULTS

Synthesis and Properties of the Different IPNs

While the co-network was carried out by free radical photopolymerization, the fibrin gel was synthesized by the enzymatic hydrolysis of fibrinogen by thrombin at 37°C. Different IPNs were synthesized varying the PVA/SA ratio. The *in situ* synthesis of PVA_{100} /Fb_5, $PVA_{50}SA_{50}/Fb_5$ and SA_{100}/Fb_5 IPNs has been developed. In each case, a solid material was obtained (Figure 1).

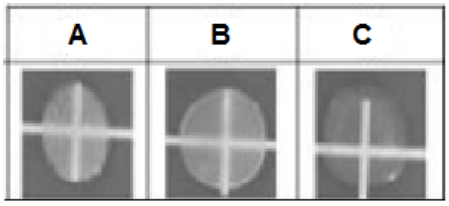

Figure 1. (A) PVA_{100}/Fb_5; (B) PVA_{50}-SA_{50}/Fb_5, and (C) SA_{100}/Fb_5 IPNs just after synthesis

The SA_{100}/Fb_5 IPN (Figure 1, C) is more transparent than IPNs containing PVA (Figure 1, A and B). All the IPNs keep their round shape after synthesis which is indicative of good mechanical properties. In addition, they can be stable for months when immersed in sterile buffer.

The synthesis was thus followed by rheology measurements and gel times determined (Table 1). With every composition, a material was obtained after 15 min.

Table 1. Gel time and storage modulus (G') for various IPNs

Scaffold composition	Fb based IPN	
	Gel time (min)	G' (Pa)
PVA_{100}/Fb_5	3	3240 ± 420
PVA_{50}-SA_{50}/Fb_5	3	640 ± 20
SA_{100}/Fb_5	15	870 ± 420

After 60 min irradiation, the storage moduli were measured and compared to that of the fibrin hydrogel (G' = 80 Pa) which is not easy to handle. All IPNs containing either SA or/and PVA are self-supported (G' > 100 Pa). The choice of IPN architecture has been then validated by a large improvement of the mechanical properties of the fibrin gel at physiological concentration. Indeed, the addition of any synthetic co-network to a fibrin gel leads to a large increase in storage modulus (G') from 8 to 40 fold (Table 1). The partner co-networks well fulfill their role of transforming the fibrin gel into an easily handled gel.

To check the effective formation of the networks in IPN architecture, extractions of soluble fractions of both the synthetic partner and the fibrin of the materials were performed. The part of methacrylate polymers which is not included in the synthetic network is high (from 12 to 16 %), but correct for polymer networks synthesized from a diluted precursor solution. In addition, the presence of PVA-SA co-network does not inhibit the enzymatic formation of fibrin network as all the fibrinogen has been turned to fibrin and included in the material.

Biodegradability

The degradation of IPNs was then tested. In order to quickly prove that SAm confers biodegradability to the materials, they were incubated in a concentrated thermolysin (a zinc endopeptidase)solution for 24 h and macroscopically observed (Figure 2).

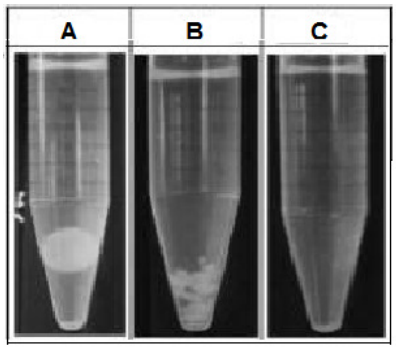

Figure 2. (A) PVA_{100}/Fb_5; (B) PVA_{50}-SA_{50}/Fb_5, and (C) SA_{100}/Fb_5 IPNs after 24 h in a protease solution

As suspected, the PVA_{100}/Fb_5 IPN (Figure 2, A) was not degraded by the protease under those conditions. The protease may degrade the fibrin network inside the IPN, but this protein representing only 5 wt% of the solid fraction of the materials, its proteolysis does not lead to the dislocation of the material; for example PVA_{100}/Fb_5 IPN global integrity is maintained after fibrin hydrolysis When SA was included in PVA network, the IPN can be totally or partly degraded upon enzyme action. Thus, many fragments were obtained after immersion of PVA_{50}-SA_{50}/Fb_5 IPN in thermolysin solution (Figure 2 B), indicating that SA can be degraded inside the IPN and that it was also well distributed in the co-network. Moreover, the SA_{100}/Fb_5 IPN was totally degraded by the protease. This material is thus well biodegradable. The degradation rate depends on the SA weight proportion, ranging from no degradation (no SA) to total one (no PVA).

The degradation kinetics was also measured (Figure 3). The proteolysis was measured for several IPNs all containing 0.5 (w/v) % fibrin and 10 (w/v) % polymers, including from 0 to 10 (w/v) % SAm. The hydrolysis rate depends on the concentration of proteins (fibrin + serum albumin) in the material. A fast hydrolysis was observed for the IPNs containing more than 50% serum albumin in their crosslinked network (PVA_{50}-SA_{50}/Fb_5 and SA_{100}/Fb_5 IPNs) while the hydrolysis was slow when only fibrin may be hydrolyzed by the protease (PVA_{100}/Fb_5 IPN).The absorbance at the equilibrium directly depends on the protein concentration and is well fitted by the relation Abs_{280} = 0.034 total protein concentration (%) + 0.042 (R^2= 0.9775). These experiments show that SA in the material is accessible to the protease and may be hydrolyzed even in the IPN. The complete degradation of the IPNs is possible only if the SA is evenly distributed in the material.

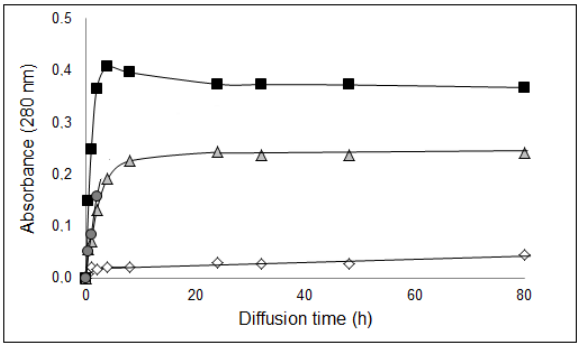

Figure 3. Protein absorbance at 280 nm as a function of immersion time in water for various IPNs. ◇: PVA_{100}/Fb_5 IPN, ▲PVA_{50}-SA_{50}/Fb_5 IPN, ■ SA_{100} /Fb_5 IPN

The degradation rate of the IPNs is depending on the total protein concentration (fibrin + serum albumin). Moreover, the hydrolysis process follows a Michaelis – Menten kinetics, indicating that the diffusion of the protease is not limited inside the solid material. These results show that controlling the protein total concentration allows tuning the IPN degradability.

Cell Viability

Viability of cells in contact with the materials has been measured with BJ fibroblasts. A cell suspension (50 000 cells/ cm^2; confluent density) was spread on the surface of PVA_{100} /Fb_5, PVA_{50}-SA_{50}/Fb_5 and SA_{100}/Fb_5 IPNs. After incubation between 24 h and 3 weeks, a metabolic assay showed the ability of cells to survive on the surface of these IPNs (Figure 4).

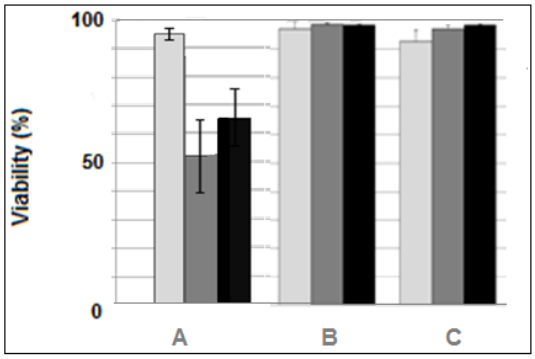

Figure 4. Cell viability on the surface of various materials A: PVA_{100} /Fb_5 IPN; B: PVA_{50}-SA_{50}/Fb_5 IPN; C: SA_{100}/Fb_5 IPN after 24h (■),1 week (■) and 3 weeks (■) of culture

While cells rapidly die in contact to PVA single network surface [Bidault *et al*., 2013]; the presence of fibrin in the PVA_{100}/Fb_5 IPN slowed down their death. However, this material is not suitable as a cell culture support. When SA was present in the IPNs, cell viability stayed close to 100% after 3 weeks (Figure 4), indicating that the protein environment favored cell viability. In polymer biomaterials, cell growth has been linked to biodegradability, our results are in concordance with this assessment as the biodegradability increased with the protein concentration (Figure 3).

Then the proliferation capability of cells on the surface of these different materials was explored (Figure 5).

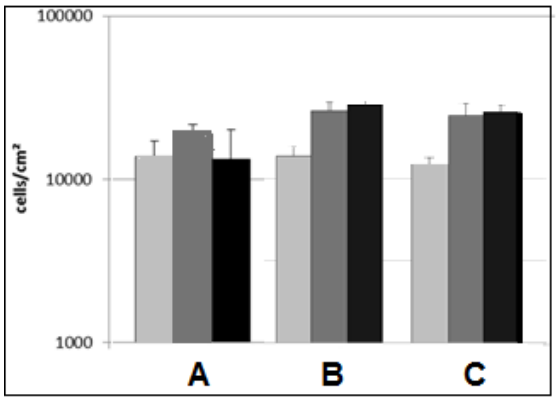

Figure 5. Cell density on the surface of various materials A: PVA_{100} /Fb_5 IPN; B: PVA_{50}-SA_{50}/Fb_5 IPN; C: SA_{100}/Fb_5 IPN after 24h (■),1 week (■) and 3 weeks (■) of culture

Cells survived but did not proliferate on PVA_{100} /Fb_5 IPN. Contrarily, the introduction of SA into the network allowed fibroblasts to proliferate up to total recovery of the material surface and their number largely increased (Figure 5) when

they were cultivated on either SA_{100}/Fb_5 IPN or PVA_{50}-SA_{50}/Fb_5 IPNs. Comparison with single networks without fibrin showed that the effects of fibrin and SA were synergistic on cell development. This feature is one of the main interests of IPN architectures which allow benefiting of the advantages of each network.

CONCLUSION

Fibrin gel has been associated with different PVA/SA co-networks inside interpenetrating polymer networks architecture. The materials are synthesized rapidly and they are all self-supported because the polymer network allowed improving the mechanical properties of the fibrin gel. These biomaterials are biodegradable by proteases due to SA introduction. This degradability due to the incorporation of SA in the PVA scaffold helped to increase very significantly the bioactivity of materials. When SA was included in PVA-SA/Fb IPNs, the surface of materials can be completely covered by fibroblasts within two weeks. All these properties indicate that the IPNs here synthesized are good supports for cell growth.

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