

Insights into Mechanisms Controlling PEGylated Fibrin Degradation

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Statement of Purpose: Cell therapy promises to help heal ischemic and other tissue damaged by disease or trauma. A mechanism for site-specific delivery that will degrade and be remodeled by biological processes is needed. Fibrin gels fulfill these requirements. As part of the normal wound healing process, fibrinogen is cleaved by thrombin resulting in a three-dimensional fibrin gel. Proteolytic enzymes present during wound healing eventually digest the gel as cellular ingrowth occurs.

At a relatively low concentration of 10mg/ml, fibrinogen is conducive to cell proliferation, but degrades more quickly than desired for many cell therapies. Current fibrin sealants containing protease inhibitors such as aprotinin or ϵ -amino caproic acid slow the degradation rate, but have been shown to have deleterious affects on the healing process¹.

Our previous work has demonstrated a tunable method of controlling Fibrin degradation by the addition of various bi-functional, amine reactive PEGs. Understanding the mechanism by which the degradation rate is reduced needs elucidation.

Methods: Fibrinogen (porcine, Sigma) was added to tris buffered saline, pH 7.8 (TBS, Sigma) and mixed thoroughly. PEG derivatives at varying concentrations were added to TBS. (PEG-(SMB)₂ or (PEG-(SMB)₁, Nektar or PEG-(NPC)₂, SunBio). Each solution was sterile filtered and used immediately.

Equal volumes of the PEG and fibrinogen solutions (or TBS as a control) were mixed and allowed to incubate for 60 minutes at 37°C. 500 μ l of each mixture was added to a microcentrifuge tube (2ml graduated, Fisher). An equal volume of thrombin (human, Sigma) diluted to 25U/ml in 40mM CaCl₂ was added to the PEGylated fibrinogen and mixed by pipetting rapidly two times. The gel was allowed to crosslink for 10 minutes at 37°C followed by the addition of reduced serum media (DMEM, Sigma) plus 1% penicillin-streptomycin (Gibco) plus 2% FBS (Invitrogen). All gels were placed in the incubator (37C, 5% CO₂, humidified). Sample media was collected at 2 hours and then every week followed by replacement with freshly made media. The weight of each sample was recorded after several washing and lyophilization steps and normalized to the day 0 mass. The protein content of each sample was determined by BCA analysis (Pierce) following manufacturer's instructions. The size and preliminary connectivity of the PEGylated fibrinogen molecules was determined by SDS-PAGE using a 5-15% Tris-HCL gel under reducing conditions.

Results/Discussion: PEGylated fibrin gels formed using bi-functional amine reactive PEG degraded quite slowly, retaining up to 50% of their mass at 56 days (Fig. 1). Interestingly, mono-functional PEG-SMB₁ derivatives show similar mass retention over this timeframe to PEG-SMB₂ derivatives. Results were further validated by protein content analysis of the removed media each week (data not shown).

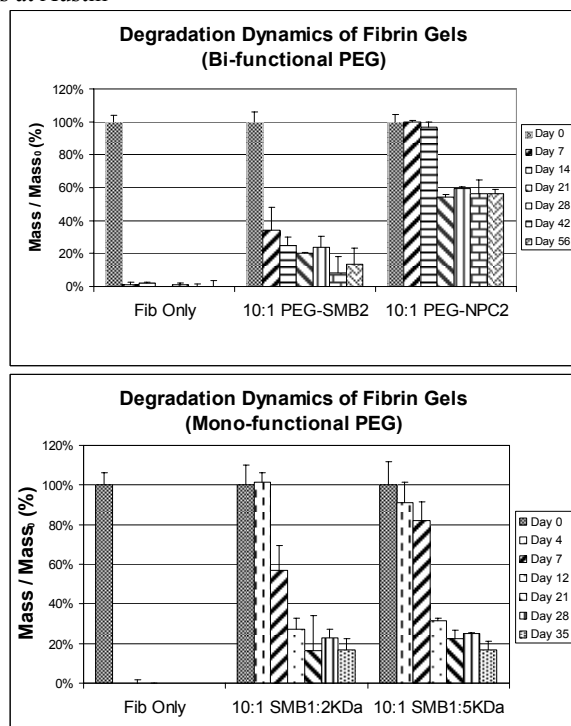


Figure 1. Mass Change in PEGylated Fibrin Gels. Not shown are nonreactive and 1:1 molar ratios of PEG to fibrinogen which degrade by week 1.

In addition to the work above, we demonstrated that the gel will fully degrade via the naturally occurring fibrinolytic protease plasmin. SDS-PAGE data demonstrate similar PEGylated fibrinogen distribution for all combinations shown based on the molecular weight of the PEG derivative used.

Conclusions: Current thinking suggests additional crosslinks formed between fibrinogen subunits is the method by which degradation is reduced. If this were the case, the monofunctional PEG would react similar to a non-reactive PEG control. Since both mono-functional and bi-functional PEG derivatives resulted in similar mass degradation over similar timeframes, the method of degradation must be at least partially related to methods other than cross-linking. Migration of the PEGylated fibrinogen molecules in a polyacrylamide gel was retarded similarly for fibrinogen molecules PEGylated with either the monofunctional or difunctional derivative of the amine reactive polymer. The similarity indicates that fibrinogen molecules are not being crosslinked by PEG under the reducing conditions of SDS-PAGE. Further tests are necessary to elucidate the mechanism(s) by which PEG reduces the degradation rate of fibrin gels.

References: [1] Krishnan L. Fibrinolysis inhibitors adversely affect remodeling of tissues sealed with fibrin glue. *Biomaterials* 24 (2003) 321–327.