Proteolytically Stabilizing Fibronectin Without Compromising Cell and Gelatin Binding Activity

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Excessive proteolytic degradation of fibronectin (FN) has been implicated in impaired tissue repair in chronic wounds. We previously reported two strategies for stabilizing FN against proteolytic degradation; the first conjugated polyethylene glycol (PEG) through cysteine residues and the second conjugated PEG chains of varying molecular weight on lysine residues. PEGylation of FN via lysine residues resulted in increased resistance to proteolysis with increasing PEG size, but an overall decrease in biological activity, as characterized by cell and gelatin binding. Our latest method to stabilize FN against proteolysis masks functional regions in the protein during lysine PEGylation. FN is PEGylated while it is bound to gelatin Sepharose beads with 2, 5, and 10 kDa PEG precursors. This results in partially PEGylated FN that is more stable than native FN and whose proteolytic stability increases with PEG molecular weight. Unlike completely PEGylated FN, partially PEGylated FN has cell adhesion, gelatin binding, and matrix assembly responses that are comparable to native FN. This is new evidence of how PEGylation variables can be used to stabilize FN while retaining its activity. The conjugates developed herein can be used to dissect molecular mechanisms mediated by FN stability and functionality, and address the problem of FN degradation in chronic wounds. © 2014 American Institute of Chemical Engineers Biotechnol. Prog., 31:277–288, 2015
Keywords: fibronectin, polyethylene glycol, proteolysis, cell adhesion, spreading, extracellular matrix assembly, gelatin binding

Introduction

Chronic wounds are a significant health and economic burden; they cost the tax payer billions of dollars annually and are the major cause of nontraumatic lower leg amputation.[1,2] Pathologically, these wounds differ from normal wounds in a number of ways. One difference is the abnormally high protease load in chronic wounds which results in excessive degradation of fibronectin (FN).[3–5] FN is a key component of the tissue scaffold or extracellular matrix in the wound bed.[6,7] FN degradation may impair tissue repair because FN attracts and binds different molecules and cells, and supports cell adhesion, migration, proliferation and extracellular matrix assembly at the wound site.[6,8] The erosion of the wound bed by proteases is further amplified because FN fragments from proteolysis stimulate the production of more proteases in cells.[9,10] Approaches that inhibit FN degradation may present new strategies for solving the problem of chronic wound healing.

Human FN is a 250–270 kDa dimeric protein and has two physical forms: a compact and soluble dimer found in blood plasma, and an extended and fibrillar multimer assembled by cells in the extracellular matrix of tissues. The range in molecular weight of FN comes from alternative splicing which leads to the inclusion or exclusion of segments of amino acids. The amino acids in FN are folded into units having similar structures or repeats (Figure 1A). These repeats are classified as types I, II, and III based on structural homology. Contiguous repeats in FN harbor functional domains that form intermolecular binding interactions with cells, extracellular matrix components and growth factors[6,11,12] (Figure 1A). Functional domains also facilitate the transformation of FN from a soluble molecule to a fibrillar extracellular matrix by cells. FN has three essential sites for extracellular matrix assembly: the 70 kDa amino-terminal domain which binds gelatin or denatured collagen, the cell-binding domain and the carboxy-terminal dimerization domain [13–16] (Figure 1A). FN in the blood is incorporated into a provisional extracellular matrix in the wound bed after injury and is an integral part of the scaffold needed for tissue repair.[17] The concentration of FN in the blood is 0.3 mg/mL and it is easily isolated from blood plasma by its binding affinity for gelatin.[18] A strategy to stabilize FN against proteolysis must take its structure and the preservation of binding interactions into account.

Our laboratory has demonstrated stabilization of FN against proteolysis by conjugating it to polyethylene glycol (PEG) or PEGylation.[19,20] PEGylation has been used in the pharmaceutical industry to stabilize protein or polypeptide therapeutics.[21–24] However, it has been applied mainly to small proteins and peptides and not large multifunctional proteins such as FN. PEGylation works because it masks the peptide or protein surface and increases the molecular size

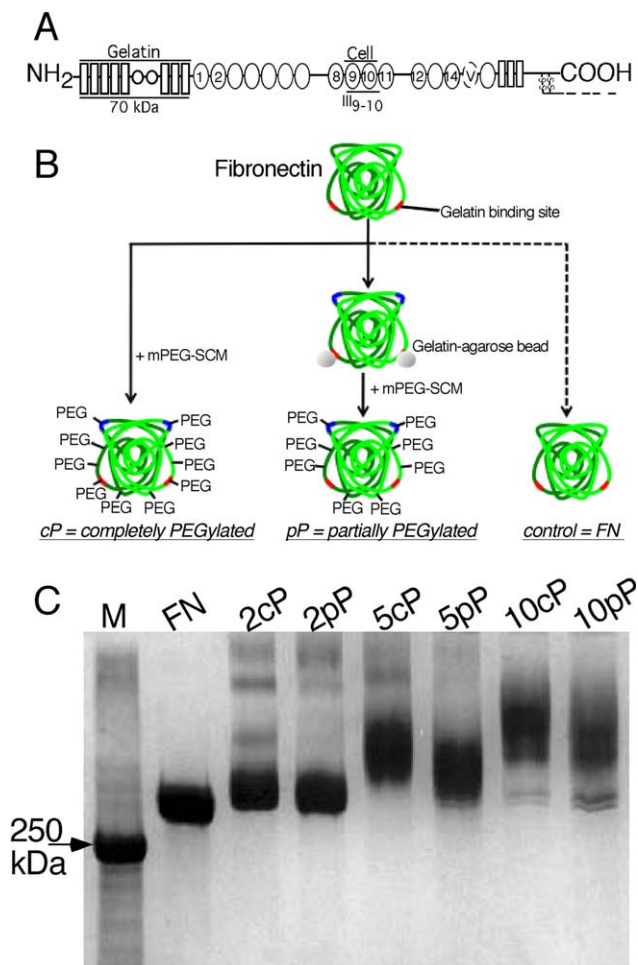Correspondence concerning this article should be addressed to N. Karuri at nkaruri1@iit.edu.

Figure 1. Synthesis and characterization of FN completely and partially PEGylated on lysine residues.

(A) Domain structure of one of the dimer arms of human plasma FN. FN is composed of homologous structural repeats classified as type I (rectangles), II (circles), or III (ovals). The gelatin binding domain at the 70 kDa amino-terminal and the cell binding domain at III_{9-10} are labeled. (B) Scheme for the synthesis of completely PEGylated (cP) and partially PEGylated (pP) FN. In solution FN is a compact molecule with crossed dimer arms. Completely PEGylated FN was synthesized by mixing FN in solution with mPEG-SCM. Partially PEGylated FN was generated by PEGylating FN while it was bound to gelatin sepharose beads. (C) 7.1 immunoblot of the products of PEGylation using the approach in B. The average molecular weights of mPEG-SCM used was 2, 5 and 10 kDa. M represents protein molecular weight standards.

that PEGylation of FN in its native state on lysine residues also results in conjugates that have improved proteolytic stability and does not perturb its secondary structure.[20] Proteolytic stability, in lysine PEGylated FN, positively correlates with PEG molecular weight and negatively correlates with cell and gelatin binding.[20] These relationships between PEG molecular weight and activity, suggest the presence of PEG conjugation sites near the cell binding domain and within the gelatin binding domain.[20] These studies by our laboratory also demonstrate that the stability of FN can be enhanced and its activity retained by manipulating multiple PEGylation variables.

In this study, we describe FN–PEG conjugates designed to test the idea that stability in FN can be enhanced by lysine PEGylation without perturbing intermolecular interactions with cells and gelatin as well as FN matrix assembly. Since previous studies suggest the existence of PEGylation sites within the gelatin binding domain,[20] we show that an active-site protective strategy[22] during PEGylation masks the gelatin binding domain, varies the extent of PEGylation and results in proteolytically stable and biologically active conjugates. The effect of PEG molecular weight and PEGylation extent on the proteolytic stabilities and activities of FN–PEG conjugates is reported. FN–PEG conjugates with comparable cell and gelatin binding and significantly higher proteolytic stability than FN are identified. For FN, the cost in terms of biological activity that arises from enhanced proteolytic stability after lysine PEGylation[20] is minimized by masking the gelatin binding domain during PEGylation.

Materials and Methods

Purification of FN

FN was isolated from human plasma, which was obtained from the blood bank at Loyola University Medical Center. We used gelatin affinity chromatography to purify FN[19,27–29] Plasma FN was centrifuged to remove residual cells and precipitates, and then passed through an agarose Sepharose 4B column (Sigma-Aldrich, St. Louis, MO). The eluate from the Sepharose column was passed through a gelatin agarose column (GE Healthcare Life Sciences, Pittsburgh, PA). Bound FN was eluted with $6M$ urea (Fisher Scientific, Pittsburgh, PA). The concentration of FN was determined by measuring its absorbance at 280 nm. Aliquots of the purified FN were mixed with a reducing buffer containing 6.6% sodium dodecyl sulfate (SDS, Fischer Scientific), 33% glycerol (Fisher Scientific), and $0.33M$ dithiothreitol (DTT, Sigma-Aldrich) at a volume ratio of 1:3. Reduced FN was boiled and analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 7% polyacrylamide gels and then silver stained. A prominent band was observed at 262 kDa in the polyacrylamide gels and no extraneous bands were detected. The yield of FN was approximately 30 mg of protein for 150 mL of human plasma. The purified FN in $6M$ urea was stored at $-80°C$. It was thawed and dialyzed in phosphate buffer saline (PBS, Fisher Scientific) before use.

FN complete and partial PEGylation and characterization

FN was PEGylated at lysine residues to generate two types of conjugate; a completely PEGylated FN where all the available lysines were conjugated to PEG and a partially PEGylated FN where a fraction of the available lysines were

of the protein or polypeptide, thereby reducing renal filtration.[22,24] PEGylation also sterically prevents the approach of binding partners, thus reducing the degradation of the PEGylated protein by proteolytic enzymes.[22,24] Protein–PEG coupling strategies usually bond activated PEG to thiols in cysteine residues, or primary amines which are found in lysine residues or in the amino terminal of the protein.[25] Cysteine residues in FN are concentrated in the 70 kDa amino terminal.[26] PEGylation of cysteines results in a conjugate that has higher proteolytic stability than FN, that binds cells in a comparable manner to native FN and that is assembled into extracellular matrix fibrils by cells when present in solution.[19] However, unlike native FN, cysteine PEGylated FN is not assembled into extracellular matrix fibrils when coated on surfaces.[19] More recent findings show

conjugated to PEG. The scheme for the synthesis of these molecules is shown in Figure 1B. Completely PEGylated FN was synthesized by mixing it with 2, 5, or 10 kDa methoxy-polyethylene glycol succinimidyl carboxyl methyl ester (mPEG-SCM, Creative PEGWorks, Winston-Salem, NC). Ten milimollar mPEG-SCM in dimethyl sulfoxide (DMSO, Sigma) was mixed with 1–1.5 mg/mL FN in PBS at a molar ratio of 1:50. PEGylation was carried out in PBS adjusted to pH 9. The mixture was incubated for an hour at room temperature. The reaction was quenched with $1M$ Tris–HCl pH 8.8 and the resulting solution was again dialyzed against PBS overnight.

Partially PEGylated FN was synthesized by PEGylating FN while it was bound to gelatin Sepharose beads. Gelatin Sepharose beads were washed as per manufacturer's instruction and incubated with FN. The amount of FN incubated with the beads was above the manufacturer specified binding capacity of one gram of FN per liter of beads. The beads were incubated with FN for 1 h at room temperature, and under mild agitation. The beads were isolated by centrifugation and then washed twice with PBS. The beads were incubated with 2, 5, or 10 kDa 10 mM mPEG-SCM. The molar amount of mPEG-SCM used was 50 times greater than the estimated molar amount of FN bound to the beads. PEGylation conditions were similar to those of completely PEGylated FN described above. After quenching the conjugation reaction, the beads were washed twice in PBS and then incubated with $6M$ urea in PBS to release the bound PEGylated FN in solution. The solution was isolated by centrifugation and the urea was eliminated by dialysis. Samples were collected at each step during the synthesis of partially PEGylated FN, mixed with a reducing buffer containing 6.6% SDS, 33% glycerol and $0.33M$ DTT at a volume ratio of 1:3, analyzed by SDS-PAGE in 7% polyacrylamide gels and silver stained.

FN was not lost during washing of the gelatin beads or retained on the beads after treatment with $6M$ urea but over 50% of the FN bound to gelatin Sepharose beads was lost during partial PEGylation. mPEG-SCM may competitively inhibit gelatin binding by PEGylating sites at or near the gelatin binding domain. The optimum binding of gelatin to FN occurs at pH 7–9[30] and we observed that the yield of partially PEGylated FN was maximized when PEGylation was carried out at pH 9 compared to pH 7. The yield of partially PEGylated FN was 20% of the starting material. The concentration of PEGylated FN after dialysis in PBS was determined through absorbance readings and through the Bicinchoninic acid assay (BCA, Pierce, Rockford, IL). The concentrations of the FN–PEG conjugates reported here are based on the FN group. Therefore the mass concentration of FN in 1 mg/mL of FN–PEG conjugate is equivalent to a concentration of 1 mg/mL of native FN. These readings were matched to SDS-PAGE analysis. When the concentrations of FN–PEG conjugates were above 0.3 mg/mL all three methods gave consistent quantitative measurements for concentration. The stock solutions were kept above this concentration. The conjugates were also characterized by: (i) mixing them with a reducing buffer containing 6.6% SDS, 33% glycerol, and $0.33M$ DTT at a volume ratio of 1:3, (ii) SDS-PAGE of reduced samples with 7% polyacrylamide gels followed, and (iii) immunoblotting transferred gels with 7.1 monoclonal antibodies (Developmental Studies Hybridoma Bank, Iowa City, Iowa).[19,28,29] The nomenclature for the completely PEGylated FN molecules used was 2cP, 5Cp,

and 10cP (Figure 1B) corresponding to 2, 5, and 10 kDa mPEG-SCM precursors. Likewise, 2pP, 5pP, and 10pP were the names corresponding to FN partially PEGylated with 2, 5, and 10 kDa mPEG-SCM (Figure 1B).

Proteolysis assays

Tosyllysine Chloromethyl Ketone (TLCK) treated α-chymotrypsin from bovine pancreas (Sigma-Aldrich) was used to characterize the proteolytic stability of PEGylated FN by a procedure similar to one previously described.[19] In brief, PEGylated and native FN at concentrations of 50 μg/mL, based on the FN moiety, were mixed with α-chymotrypsin at a protease to protein mass ratio of 1:25. Aliquots of samples were collected at specific time points from 0 to 30 minutes. Proteolysis in the aliquots was inhibited by the addition of 2 mM phenylmethylsulfonyl fluoride (PMSF, Fisher Scientific). The samples were then mixed with reducing electrophoresis buffer, resolved in a 10% polyacrylamide gel, transferred to nitrocellulose (GE Healthcare Life Sciences) and immuno-blotted with 7.1 monoclonal antibodies. The blots were imaged in a Chemidoc XRS imaging system (Biorad, Hercules, CA). Quantity One software (Biorad) was used for densitometric analysis. The densitometric volume of each band corresponding to intact protein at each time point was obtained.

Cell culture

NIH 3T3 mouse fibroblasts (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Fisher Scientific) supplemented with 10% bovine calf serum (Fisher Scientific) at 37°C with 5% CO_2. NIH 3T3 mouse fibroblast passages 15–30 were used for these studies. The cells were grown to 80–90% confluency in 10 cm dishes before use in cell based assays.

Static cell adhesion assays

Adhesion assays were carried out using established methods.[19,28,29] Glass coverslips in a 24 well plate were incubated in 50 μg/mL native or PEGylated FN in PBS. The incubation temperature and time was 37°C and 1 h, respectively. The coverslips were then washed with PBS and blocked with bovine serum albumin (BSA, Fisher Scientific). Each coverslip was then cultured with 4×10^4 NIH 3T3 mouse fibroblasts for a period of 1 h in serum free media. Nonadherent cells were removed by washing twice with PBS. Adherent cells were fixed with 3.7% paraformaldehyde (Fisher Scientific) in PBS and permeabilized with 0.5% NP-40 (Fisher Scientific) in PBS. The samples were then rinsed twice with PBS and stained with fluorescein conjugated phalloidin (Invitrogen, Grand Island, NY) and Hoechst 33258 (Fisher Scientific). The samples were washed three times with PBS followed by a last wash with deionized water and then mounted for microscopy using prolong antifade (Invitrogen).

FN matrix assembly on coated surfaces

FN matrix assembly studies were used to characterize the assembly of coated PEGylated FN into extracellular matrix fibrils by cells. The procedure for matrix assembly is described in greater detail in the study by Zhang et al.[19] Glass coverslips were coated with 50 μg/mL completely PEGylated, partially PEGylated or native FN. NIH 3T3

mouse fibroblasts at a density of 2×10^5 were cultured on the treated glass coverslips for 24 h. The coverslips were washed and the adherent cells were fixed in 3.7% paraformaldehyde in PBS then washed in PBS. Cell nuclei and FN were stained with Hoechst 33258 (Invitrogen) and 7.1 mouse monoclonal antibodies. A fluorescein conjugated goat anti-mouse IgG (H1L) (Invitrogen) was used to label the primary antibody. The samples were washed thrice with PBS and once with water and then imaged.

Fluorescence microscopy and image analysis

Images of fluorescein and Hoechst stains were collected using a Carl Zeiss Axiovert 40CL microscope coupled to an Axiocam ICM (Zeiss, Thornwood, NY) and analyzed by ImageJ software (National Institute of Health). Both stains were used for cell adhesion and FN matrix assembly studies. In adhesion assays, Hoechst staining in a $10\times$ field was used to determine the number of adherent cells. The area of the fluorescein stained cytoskeleton at $20\times$ magnification was used to quantify cell spreading. Cell area was converted from pixelated units to μm^2 in Axiovision scaling software (Carl Zeiss). In matrix assembly studies, FN fibrils, and cell nuclei were visualized under $20\times$ magnification. Exposure times were kept constant in all treatments.

Gelatin binding assay

The binding of completely and partially PEGylated FN to gelatin Sepharose beads was quantified as follows. A working solution of 0.1 mg/mL, based on the FN moiety, was made from the stock solutions of native and PEGylated FN. Gelatin sepharose beads were incubated with a protein solution above the binding capacity of the beads; 200 μL of 0.1 mg/ml FN, or an equivalent amount of PEGylated FN, was added to 50 μL of beads. The beads were then incubated with the protein solution for an hour and then isolated by centrifugation. The supernatant containing the unbound protein was collected. The beads were washed with PBS and then resuspended in 200 μL of an Electrophoresis Sample Buffer containing 2% SDS, 10% glycerol, 80 mM Tris–HCl pH 6.8, 4 mM EDTA, 0.01% bromophenol blue, and 0.1M DTT. The beads were then boiled to release bound protein in the electrophoresis buffer. The starting solution (30 μL) and the gelatin binding supernatant (30 μL) were mixed with an electrophoresis buffer (10 μL) containing 6.6% SDS, 33% glycerol, and 0.33M DTT and then boiled. The starting solution, gelatin binding supernatant, and protein bound to the beads were electrophoresed in a 10% polyacrylamide gel and immunoblotted with 7.1 antibodies. A 1.7 μL and 7.5 μL volume of the starting solution and supernatant, respectively, and a volume of 1.7 μL of the material bound to the beads was electrophoresed.

Statistical analysis and data treatment

The treatments comprised six FN–PEG conjugates: 2pP, 5pP, 10pP, 2cP, 5cP, and 10cP. Native FN was used as the control. All the experiments were conducted twice with at least two replicates per treatment. The concentration versus time data for α-chymotrypsin degradation was fit to pseudo first order kinetics by taking a logarithm transformation of the data. The LINEST function in Excel was used to perform linear regression on the transformed data and to obtain a rate constant and its associated standard error. For the analysis of

cell adhesion, 16 randomly selected regions per treatment were imaged and used. A minimum of 72 cells were randomly selected from each treatment and analyzed for cell area. A 95% confidence interval for the mean values was obtained by using a Students' t-distribution. A two-sided significance level of 5% was used to conduct a Student's t tests when comparing the means of different treatments. P values less or equal to 0.05 were considered to be statistically significant. These analyses only present pairwise comparisons and cannot be extrapolated to multiple comparisons without adjusting for multiplicity.

Results

The molecular weight of FN completely or partially PEGylated with different sized PEGs

The goal of our study was to PEGylate FN and thus stabilize it while retaining its cell and gelatin binding activities. To do this we used the scheme in Figure 1B to create a collection of FN–PEG conjugates with two different variables; PEG molecular weight and PEGylation extent. Three different PEG molecular weights and two PEGylation extents were sampled. PEGylation extent was varied by masking the gelatin binding domain during PEGylation. Figure 1C shows an immunoblot of FN and the different PEG conjugates of FN. There is an increase in molecular weight of FN when it is completely PEGylated with 2, 5, and 10 kDa mPEG-SCM (FN, 2cP, 5cP, and 10cP in Figure 1C). This is in line with previous findings of FN completely PEGylated on lysines.[20] The partially PEGylated FN conjugates, 2pP, 5pP, and 10pP, have an increase in molecular weight on PEGylation, but this change is lower than that of completely PEGylated FN (Figure 1C). Masking the gelatin binding domain during PEGylation is effective at reducing the number of PEG groups conjugated to FN.

In the immunoblot, the lanes corresponding to the FN–PEG conjugates had bands that were thicker than that of the control native FN. This is in line with the reported polydispersity of commercially available PEG precursors.[22] The lanes with 10cP and 10pP in Figure 1C have some unconjugated FN which is marked by the presence of the thin band at 262 kDa. The largest PEG precursors may have lower diffusion and reaction rates than the smaller PEG precursors thereby resulting in the presence of unconjugated FN. Densitometric analysis showed that the 262 kDa band in the 10cP and 10pP lane was less than 8% of the total lane volume indicating that most of the protein was PEGylated. The prominent bands in all the lanes had more than 90% of the material. We used the protein standards to estimate the molecular weight of the prominent FN positive bands.

SDS-PAGE is a facile and visual method for characterizing molecular weight of PEG conjugates. The results of regression analyses of logarithm of molecular weight of the conjugates versus migration distance in polyacrylamide gels are shown in Figure 2A. Qualitatively they confirm an increase in the molecular weight of FN after PEGylation. The trends indicate that the higher the molecular weight of the PEG precursor, the greater the increase in molecular weight of the conjugate in both completely and partially PEGylated FN. On average each completely PEGylated FN–PEG conjugate had a higher molecular weight than a partially PEGylated conjugate for all PEG molecular weight sampled. Figure 2B represents the results of statistical tests
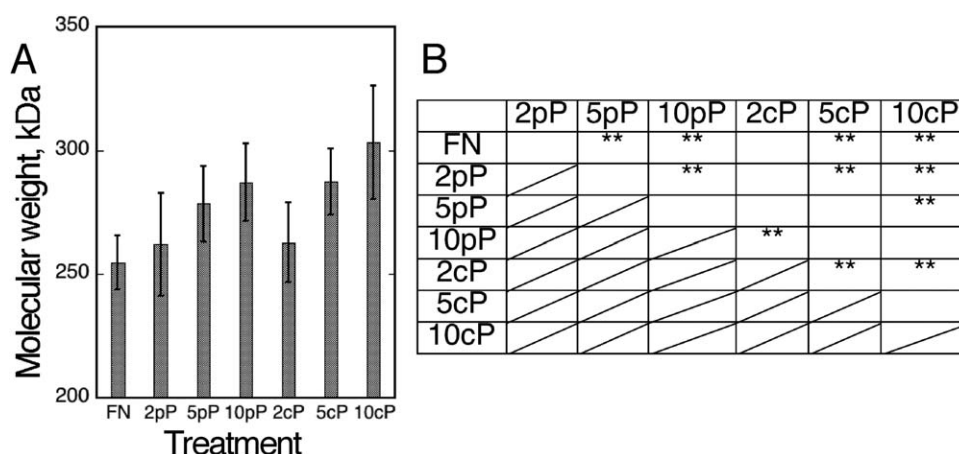
Figure 2. Molecular weight analysis of FN–PEG conjugates.

(A) SDS-PAGE analysis of molecular weight of FN and completely or partially PEGylated FN based on four experiments conducted at different times. (B) Significance testing on molecular weight. "**" represents means that are different at a significance level of 0.05 using two-tailed t test with the assumption of unequal variance in molecular weight among the treatments.

for pairs of significantly different means in Figure 2A. They show significantly different means between FN and FN conjugated pairs with PEG that is greater than 2 kDa. Among the partially PEGylated conjugates the molecular weight of 10pP differs significantly from that of 2pP. In the completely PEGylated conjugates, the molecular weight of 5cP and that of 10cP are significantly different from that of 2cP. While we observed differences in the average distance migrated between the partially and completely PEGylated molecules in polyacrylamide gels, for example between 5pP and 5cP in Figure 1C, the average of measured values from SDS-PAGE gels were not statistically significantly different.

One source of the variability of the data in Figure 2A is the fact that the analyses is based on protein standards that are not PEGylated. Another source of this variability is the decreased resolution of differences in molecular weight of large molecules in SDS-PAGE. FN is a 250 kDa molecule that migrates near the top of the resolving gel. At the top of the resolving gel differences in molecular weight are marked by even smaller differences in the distance migrated compared to molecules at the bottom of the gel. This was observed with 5, 7, and 4–12% gradient gels. A third source of the error bars in Figure 2A is from the distribution of PEG molecular weight which is marked by the wide bands in Figure 1C. Analyses of SDS-PAGE gels demonstrates that there are differences in molecular weight, between the partially and completely PEGylated FN conjugates, and differences in the molecular weight of conjugates within each group that are dependent on PEG molecular weight.

The effect of PEG molecular weight and PEGylation extent on FN stability

We examined the role of PEGylation extent and PEG molecular weight on the proteolytic stability of PEGylated FN. Figure 3A shows immunoblots of FN, 10cP, and 10pP at different proteolysis times. There was more intact 10cP and 10pP than native FN demonstrating that FN completely or partially PEGylated with mPEG-SCM is more proteolytically stable than native FN. The cleavage patterns of FN and its conjugates were different. Figure 3A shows that FN proteolysis results in fragments of discrete molecular weight

whereas proteolysis of PEGylated FN results in smearing indicating a distribution of molecular weights. The range in molecular weights of the products of proteolysis of the conjugates may be due to the polydispersity of conjugated PEG. PEGylation may also reduce protease cleavage sites resulting in different fragmentation patterns of FN.

We quantified the amount of native or PEGylated FN through densitometric volume analysis of the intact bands. Figure 3B shows the amount of intact native and PEGylated FN at different time points after protease addition. The values in Figure 3B have been normalized to the amount of protein at time zero. For clarity, the 5 kDa PEG conjugates have been omitted. As PEG molecular weight increases the amount of intact protein at each time point increases. Decreasing the extent of PEGylation results in a decrease in the amount of intact conjugate during proteolysis. The smallest conjugated, 2pP, has a similar proteolysis pattern to that of native FN (Figure 3B). The intensity versus time data for α-chymotrypsin degradation was fit to pseudo first order kinetics by taking a logarithm transformation of the data in Figure 3B. Figure 3C shows the kinetic rate constant of degradation, k_{deg}, of completely and partially PEGylated FN. The kinetic rate constant of degradation decreases with increasing PEG molecular weight demonstrating that the rate of FN proteolysis is dependent on PEG molecular weight. In all the PEG molecular weights sampled, the kinetic rate constant of degradation of completely PEGylated FN is significantly lower than that of native FN. The partially PEGylated conjugates of FN, 5pP, and 10pP, have significantly lower kinetic rate constants of degradation than native FN. The relationships between the kinetic rate constant of degradation and PEG molecular weight are different for completely and partially PEGylated FN. Partially PEGylated FN has a steeper decline in the kinetic rate constant of degradation with increasing PEG molecular weight than completely PEGylated FN. The 2pP conjugate has the same degradation rate as native FN indicating that there is a lower threshold for PEG molecular weight and PEGylation extent at which there is no effect on FN stability. Similarly, the upper limit of PEG molecular weight at which there is no difference in the proteolytic stability between the completely and partially PEGylated FN is 10 kDa.
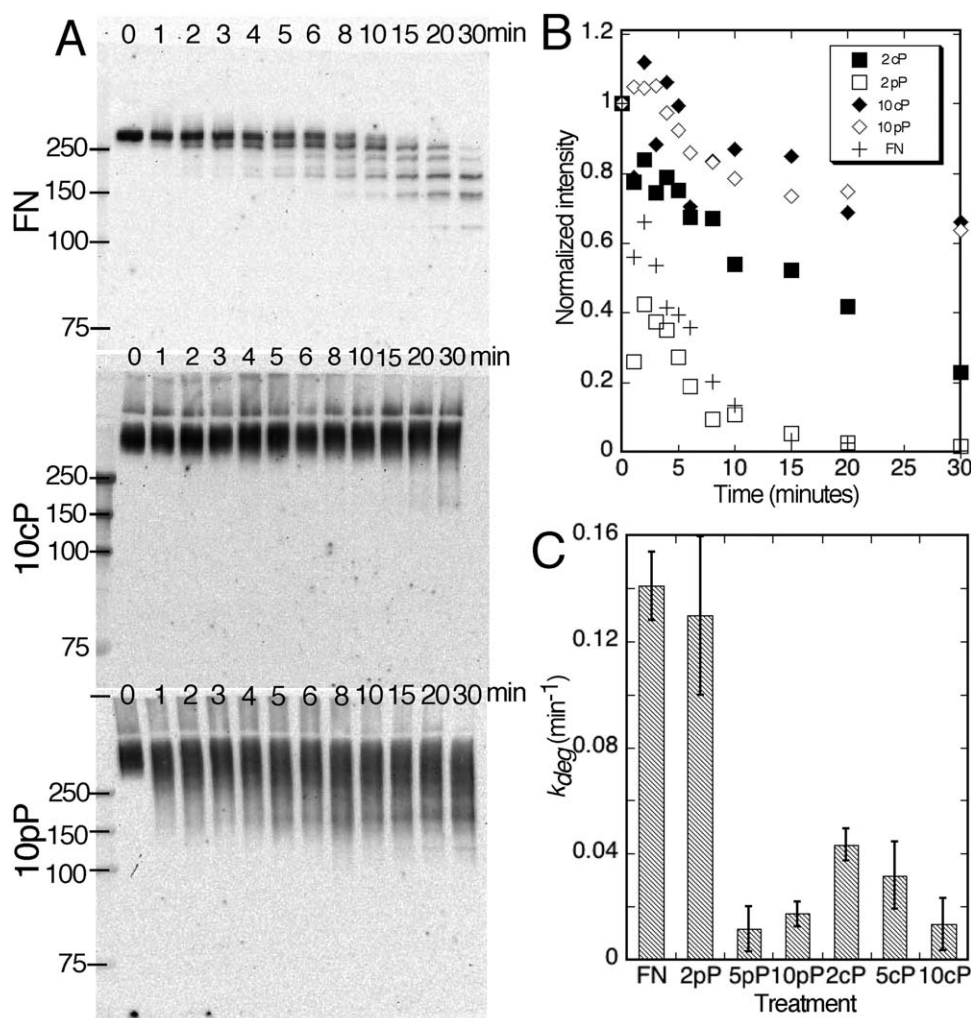
Figure 3. Proteolytic stability of completely and partially PEGylated FN.

α-Chymotrypsin was added to completely and partially PEGylated FN at a mass ratio of 1:25. PEGylation was stopped at different time points and immunoblotted with 7.1 antibodies. (A) Immunoblot of FN, 10Pp, and 10cP. Protein molecular weight standards are shown on the left. (B) Densitometric analysis of intact FN, 2pP, 2cP, 10pP, and 10cP at different time points after α-chymotrypsin addition. The amount has been normalized to the intensity of the intact band at time zero. (C) The first-order kinetic rate constant for α-chymotrypsin degradation, k_{deg}, for native FN and its conjugates. The error bars represent a 95% confidence interval of the mean.

Modulation of cell adhesion by PEGylated FN

We characterized the influence of completely and partially PEGylated FN on adhesion and spreading in NIH 3T3 mouse fibroblasts. Figure 4A shows cells adherent on glass surfaces coated with the different conjugates. Qualitatively, there are more cells and there is more robust cell spreading on the surfaces coated with partially PEGylated FN than on surfaces with completely PEGylated FN. Cells on the control surface coated with native FN have robust attachment and spreading (Figure 4A). Quantitative analyses of cell adhesion and spreading on surfaces coated with the conjugates are shown in Figures 3B and C. For completely PEGylated FN, cell adhesion decreases with increasing PEG molecular weight (Figure 4B). The smallest and completely PEGylated FN conjugate has a comparable adhesion response to native FN. These findings with completely PEGylated FN are in line with previous findings[20] and suggest that there is a PEGylation site near the cell binding domain. Interestingly, the number of adherent cells on glass surfaces coated with partially PEGylated FN is comparable to that on surfaces coated with native FN and is independent of PEG molecular weight (Figure 4B). These studies strongly suggest that masking the gelatin binding domain during PEGylation also masks a PEGylation site near the cell binding domain.

Cell spreading negatively correlated with PEG molecular weight in both completely and partially PEGylated FN. Figure 4C shows that all the PEGylated variants elicit significantly lower cell spreading than native FN but cell spreading is significantly higher on surfaces coated with partially PEGylated FN than on surfaces with completely PEGylated FN. In addition to being dependent on adhesion interactions, cell spreading is also dependent on surface chemistry.[31] We postulate that the ability of PEG groups to resist protein adsorption[32,33] may impact cell spreading on surfaces coated with the FN–PEG conjugates.

Binding of completely and partially PEGylated FN to gelatin

We characterized the binding of FN and PEGylated FN to gelatin Sepharose beads. We had previously reported that binding of completely PEGylated FN to gelatin coated glass
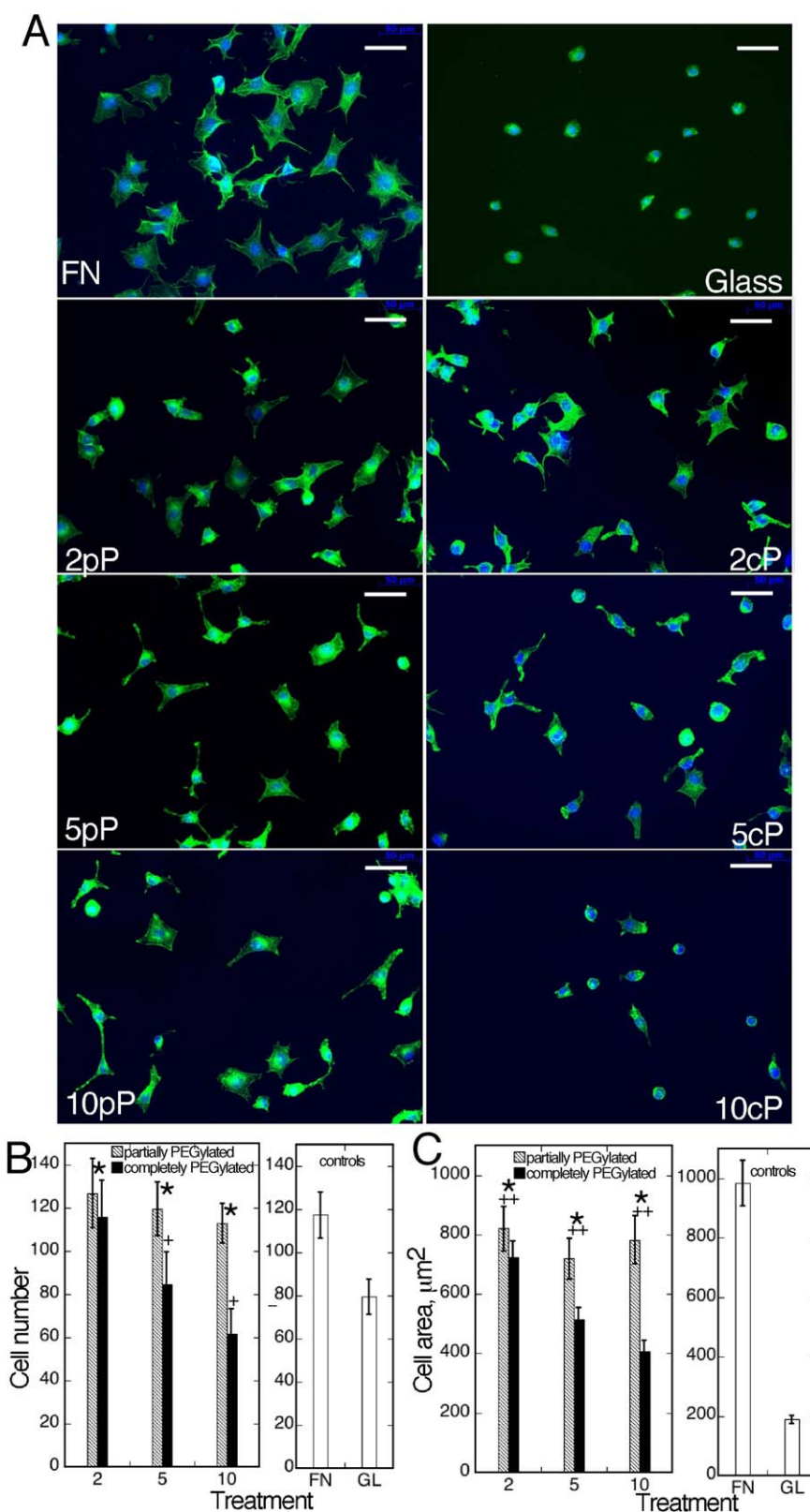
Figure 4. Adhesive response of NIH 3T3 mouse fibroblasts cultured on surfaces coated with native FN and PEGylated FN.

FN was completely (2cP, 5cP, and 10cP) and partially (2pP, 5pP, and 10pP) PEGylated with 2, 5, and 10 kDa mPEG-SCM. (A) Cells were cultured in serum free media for an hour on glass surfaces coated with native and PEGylated FN, then fixed and stained for nuclei (blue) and actin (green). Scale bar is 50 μm. (B, C) Quantification of cell adhesion and spreading on surfaces coated with PEGylated proteins (left panel) and the controls (right panel). The positive and negative control consists of FN coated and uncoated surfaces glass surfaces (GL). The data in both B and C represents the mean of two experiments conducted at different times and two repeats for each treatment. The areas of 30–40 cells for each treatment in C were analyzed. The error bars represent a 95% confidence interval of the mean. "*" represents statistically significant differences between the mean responses of partially and completely PEGylated FN conjugates at a *P* value of 0.05. "+" represents statistically significant differences between the mean responses of the conjugates and native FN.
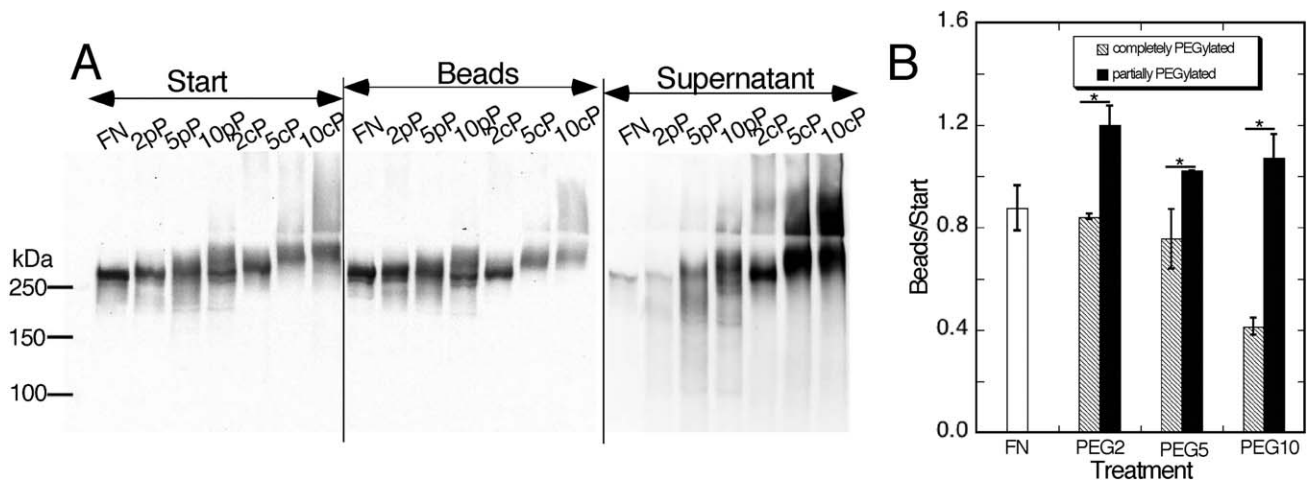
Figure 5. Gelatin binding of completely and partially PEGylated FN.

(A) 7.1 immunoblot of the solutions of native and PEGylated FN before addition to gelatin beads (*Start*) and after incubation with gelatin beads (*Supernatant*) as well as the protein bound to beads (*Beads*). The volumetric amount of *Start* and *Beads* solution loaded is one-fifth the amount of the *Supernatant* solution loaded. Protein molecular weight standards are marked on the left. (B) Densitometric analysis of gelatin binding as a ratio of the amount bound to gelatin beads to the amount at the start (Beads/Start). The error bars represent a 95% confidence interval of the mean. "*" represents a statistically significant difference in the means at a *P* value of 0.05.

surfaces decreased with increasing PEG molecular weight.[20] We used commercially available gelatin beads because their high binding capacity produced more robust binding signal in immunoblots than ELISAs with gelatin coated polystyrene surfaces. We anticipated that binding of partially PEGylated FN to gelatin Sepharose beads would not be perturbed as the gelatin binding domain was masked during PEGylation. Figure 5A is an immunoblot showing the binding of native FN, as well completely and partially PEGylated FN to gelatin Sepharose beads. Binding was from a starting solution with equivalent molar amounts of FN and FN–PEG (*Start* in Figure 5A). FN or FN–PEG bound to the isolated gelatin beads was released into electrophoresis buffer (*Beads* in Figure 5A) with the same volume as the starting solution and the unbound molecules were collected (*Supernatant* in Figure 5A). To better visualize a wider range of concentrations the volumetric amount of *Beads* and *Start* solutions loaded into the gel was one-fifth the *Supernatant* solution.

The figure demonstrates that at the start of the binding study, the amount of native, partially, and completely PEGylated FN is comparable (*Start* in Figure 5A). However, there is less 5cP and 10cP than native FN or the other FN–PEG conjugates bound to the beads (Beads in Figure 5A) and 5cP and 10cP are abundant in the supernatant (*Supernatant* in Figure 5A), which represents unbound molecules. Figure 5B is a quantitative analysis of the amount of protein bound relative to the starting amount. For efficient binding the ratio should be approximately equal to one. The amount of completely PEGylated FN bound to gelatin decreases with increasing PEG molecular weight. This is in line with findings on gelatin coated glass surfaces.[20] Interestingly, masking the gelatin binding domain not only retains gelatin binding activity after PEGylation but also results in significantly higher gelatin binding in 10pP and 5pP than in native FN (Figure 5B). For partially PEGylated FN, the trend in gelatin binding mirrors the trend in cell spreading; it has a minimum at 5pP. The enhanced gelatin binding activity observed in partially PEGylated FN is inline with reports showing that in some cases PEGylated proteins have higher activity than native proteins.[24,34] Thus, masking the gelatin

binding domain during PEGylation of FN retains, and may also increase, its activity in the conjugate.

Matrix assembly on glass surfaces coated with native and PEGylated FN

The cell and gelatin binding domains are essential for FN matrix assembly.[35] We had previously reported that, unlike native FN, FN PEGylated on cysteine residues was not assembled into extracellular matrix fibrils when coated on surfaces.[19] Since proteolytic stability could be enhanced without decreasing the cell and gelatin binding activity by partial PEGylation, we examined whether partially PEGylated FN could be assembled into an extracellular matrix when coated on surfaces. The role of PEG molecular weight and PEGylation extent on FN matrix assembly was also examined. Figure 6 is an immunofluorescence micrograph of NIH 3T3 mouse fibroblasts cultured on surfaces coated with native FN or PEGylated FN. For clarity only the 2 and 10 kDa PEG conjugates are shown. Fibrillar structures positive for FN staining were observed at the periphery of cells cultured on all coated surfaces. These fibrillar structures were not present on uncoated surfaces (Figure 6A). Qualitatively, more fibrillar structures were present on partially PEGylated surfaces (Figures 6C and E) than on the surfaces coated with completely PEGylated FN (Figures 6D and F) or on surfaces coated with native FN (Figure 6B). Masking the gelatin binding domain during lysine PEGylation results in conjugates that are better assembled into an extracellular matrix than completely PEGylated FN.

Discussion

Excessive FN degradation has been implicated in the delayed healing response observed in chronic wounds.[3–5] In this study, we have examined the influence of varying the molecular weight of conjugated PEG and the extent of PEGylation on the proteolytic stability and biological activity of FN. The results from this study allow for comparison with our previous work on cysteine PEGylation[19] or
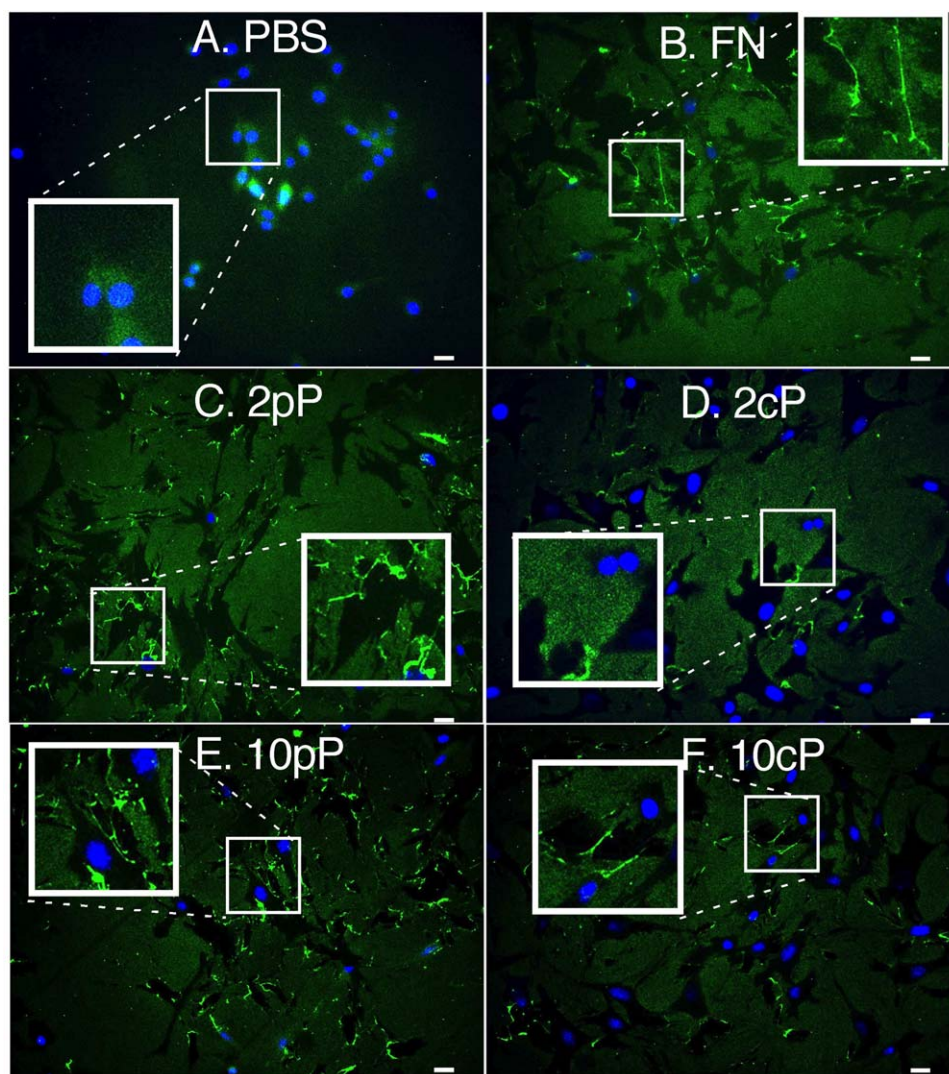
Figure 6.   Assembly of native and PEGylated FN into fibrils by cells.

> NIH 3T3 mouse fibroblasts cultured in complete media for 24 h on glass surfaces coated with native or PEGylated human FN and then fixed and stained with Hoechst for nuclei (blue) and 7.1 antibodies for exogenous human FN. Scale bar is 25 μm.

complete PEGylation of lysine residues[20] in FN. Here we present new evidence that shows that masking the gelatin binding domain during lysine PEGylation results in conjugates that are stable and that have no loss of function with respect to, cell and gelatin binding, as well as FN matrix assembly. Masking the gelatin binding domain during PEGylation, results in lowering the molecular weight of the FN–PEG conjugates. In terms of stability, proteolytic stability positively correlates with PEG molecular weight within partially and completely PEGylated FN. Similar strategies that protect the active site of proteins during PEGylation have resulted in increased proteolytic stability without significant changes in the protein activity.[36,37] All the PEG conjugates, except FN partially PEGylated with 2 kDa PEG, have higher proteolytic stability than native FN. For each PEG molecular weight sampled, completely PEGylated FN is more proteolytically stable than partially PEGylated FN.

Masking the gelatin binding domain in FN during PEGylation decreases changes in its activity caused by PEG conjugation. For instance, cell adhesion and spreading decrease with increasing PEG molecular weight on surfaces coated with completely PEGylated FN, but cell adhesion is independent of PEG molecular weight and is comparable to that of native FN on surfaces coated with partially PEGylated FN. There is greater cell spreading on surfaces coated with partially PEGylated FN than on surfaces coated with completely PEGylated FN. Complete PEGylation results in the loss of gelatin binding but gelatin binding is retained in partially PEGylated FN. There are qualitatively more FN matrix fibrils on surfaces coated with partially PEGylated FN than on surfaces coated with completely PEGylated FN. Partially PEGylated FN conjugates have comparable cell adhesion, gelatin binding and FN matrix assembly to native FN. Only in cell spreading do we see a significantly lower response on surfaces coated with partially PEGylated FN conjugates than surfaces coated with native FN. We attribute this to the ability of PEG within the FN–PEG conjugates to resist protein adsorption and thus decrease cell spreading.[32,33]

The tradeoff for higher activity in partially PEGylated FN is a decrease in proteolytic stability compared to completely PEGylated FN, which can be offset by increasing PEG length. Therefore, the challenge of identifying a conjugate that is more stable (has a lower k_{deg}) than native FN but with similar or greater activity in the library of FN–PEG conjugates is that of optimization of multiple variables. For this small discrete data set, Figure 7 can be used to identify
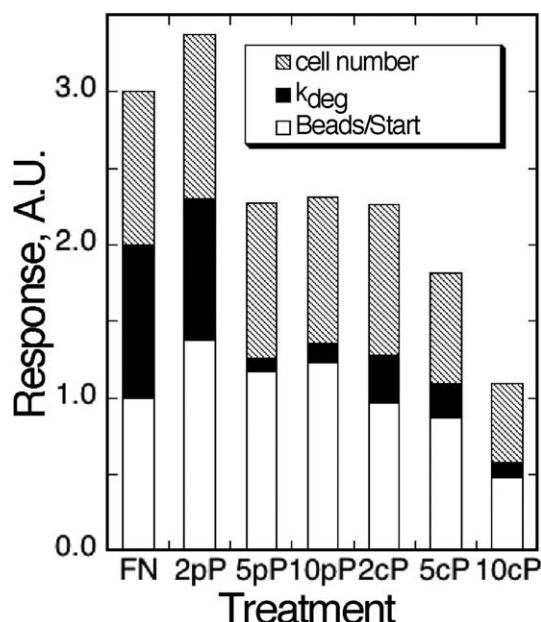
Figure 7.   Summary of cell adhesion, gelatin binding and the degradation rate constants of the conjugates normalized to the values for FN.

Responses for cell adhesion (cell number), proteolytic stability (k_{deg}) and gelatin beading (beads/start) from Figures 2C, 3B, and 4B, respectively have been normalized to FN response for these behaviors.

conjugates with higher proteolytic stability than native FN but with comparable cell and gelatin binding. From the library of conjugates developed, we have identified that the partially PEGylated 5 and 10 kDa FN–PEG conjugates (5pP and 10pP in Figure 7), as well as the completely PEGylated 2 kDa FN–PEG conjugate (2cP in Figure 7) as having higher proteolytic stability (lower k_{deg}) than native FN and comparable cell and gelatin binding. Out of these three conjugates 5pP is the most proteolytically stable and biologically active.

How do these results compare with our previous findings of FN PEGylation[19,20]? Earlier studies from our laboratory had shown that: (i) Cysteine PEGylation stabilized FN against proteolysis, did not perturb cell binding but inhibited matrix assembly of PEGylated FN coated on glass surfaces,[19] and (ii) lysine PEGylation stabilized FN but cell and gelatin binding negatively correlated with the molecular weight of PEG.[20] These past studies with PEGylated FN had resulted in improved stability but with some loss of FN activity. There was also a correlation between PEG molecular weight and FN activity and stability. We had postulated that there was a lysine PEGylation site near the cell binding domain and that PEGylation was occurring in the gelatin binding domain. We proposed that a library of FN–PEG conjugates with varied stabilities and activities could be developed by manipulating PEGylation variables and be used to identify a conjugate with maximal stability and activity. In this study we demonstrate that in addition to PEG molecular weight, PEGylation extent can be used to vary FN proteolytic stability and activity. We take advantage of the binding interaction between gelatin and FN which has been well characterized[38,39] to mask this domain during PEGylation. This strategy of using active-site protecting agent[22] has been used to retain enzyme activity during PEGylation[22] and we are the first to demonstrate that it results in retention of the gelatin and cell binding activity after PEGylation. In addition

to gelatin, FN has other well characterized binding sites for growth factors[12,40,41] and heparin.[38,42–44] We envision a strategy that harnesses these biological activities in active site-protection strategies to create FN–PEG conjugates with varying proteolytic stabilities and biological activities.

An interesting find of our study is the demonstration that masking the gelatin binding domain of FN during PEGylation results in a conjugate that has comparable cell adhesion to that of native FN for all the PEG molecular weights sampled; that is, screening the gelatin binding domain abrogates the effect of PEGylation on the cell binding domain. This would occur if the cell and gelatin binding domains of FN are in close proximity. Like many large proteins, the detailed solution structure of FN is not known but insights of some of the key structural aspects have been garnered from small-angle X-ray and neutron scattering,[45,46] binding studies using recombinant fragments,[35,47–50] fluorescence resonance energy transfer (FRET) studies of soluble FN fragments[51] and FRET studies of FN as it is adsorbed on glass surfaces.[52,53] These studies suggested that FN is an oblate ellipsoid with crossed dimmer arms at $III_{2–3}$ and $III_{12–14}$. The gelatin and cell binding domains are close in proximity to $III_{2–3}$ and $III_{12–14}$, respectively (Figure 1A) and therefore, one would expect that they are in close proximity with one another. The cell and gelatin binding domains are separated from the crossover site by the length of two type III repeats or approximately 100 Å.[54,55] We show that masking the gelatin binding domain of FN during PEGylation results in a conjugate with a better ability to support cell adhesion than completely PEGylated FN. This would suggest that the two domains are in close proximity when FN is bound to gelatin. Thus, the findings here support the dimmer arm crossover model of FN. It is important to point out that FN may undergo conformational changes when bound to gelatin,[56] which may impact access to PEGylation sites. Our PEGylation studies build on the body of evidence of what is known about FN structure by providing useful information about the distance between functional components in the molecule.

In the short term, this study provides a number of FN conjugates with varying stability and biological activity that can be used to dissect molecular mechanisms mediated by FN and its stability. It also demonstrates approaches that can be used to expand the variety of FN–PEG conjugates and that have retained bioactivity. The analyses of the data are based on the assumption that PEGylation occurs at the same sites in FN when using different PEG molecular weights and when screening the gelatin binding domain during PEGylation. The trends in the degradation kinetic constant and biological responses, particularly with the completely PEGylated conjugates suggests this assumption is valid. However, it is important to note, that PEGylation sites may change due to conformation changes FN binds to gelatin beads[56] and changes in the site of PEGylation may influence binding interactions that are involved in proteolysis and biological activity. Future work will focus on characterizing the sites of PEGylation. It is anticipated that the data obtained through the use of these conjugates will provide insight into engineering proteolytic stability in FN.

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