Biotechnology Journal
www.biotechnology-journal.com

Biotechnol. J. 2014, 9, 1033–1043

DOI 10.1002/biot.201400115

Research Article

PEGylation of lysine residues improves the proteolytic stability of fibronectin while retaining biological activity

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Excessive proteolysis of fibronectin (FN) impairs tissue repair in chronic wounds. Since FN is essential in wound healing, our goal is to improve its proteolytic stability and at the same time preserve its biological activity. We have previously shown that reduced FN conjugated with polyethylene glycol (PEG) at cysteine residues is more proteolytically stable than native FN. Cysteine-PEGylated FN supported cell adhesion and migration to the same extent as native FN. However, unlike native FN, cysteine-PEGylated FN was not assembled into an extracellular matrix (ECM) when immobilized. Here, we present an alternative approach in which FN is preferentially PEGylated at lysine residues using different molecular weight PEGs. We show that lysine PEGylation does not perturb FN secondary structure. PEG molecular weight, from 2 to 10 kDa, positively correlates with FN–PEG proteolytic stability. Cell adhesion, cell spreading, and gelatin binding decrease with increasing molecular weight of PEG. The 2-kDa FN–PEG conjugate shows comparable cell adhesion to native FN and binds gelatin. Moreover, immobilized FN–PEG is assembled into ECM fibrils. In summary, lysine PEGylation of FN can be used to stabilize FN against proteolytic degradation with minimal perturbation to FN structure and retained biological activity.

Received	24 FEB 2014
Revised	12 APR 2014
Accepted	06 MAY 2014
Accepted article online	07 MAY 2014

Supporting information available online

Keywords: Bioengineering · Biopolymers · Fibronectin · Polyethylene glycol · Proteolytic stability

1 Introduction

Fibronectin (FN) is a key component of the tissue scaffold or extracellular matrix (ECM) and is necessary for tissue repair. This 250–270 kDa dimeric protein attracts and binds different molecules and cells and mediates biological responses at the wound site [1, 2]. In chronic or hard-to-heal wounds, a protease rich environment retards tissue repair due to excessive degradation of FN [3–5]. Furthermore, FN fragments resulting from proteolysis stimulate the production of more proteases [6, 7] leading to a destructive cycle of inflammation and FN degradation that further damages the surrounding tissue [3–5]. Therefore, targeting FN stability in chronic wounds may present a novel therapeutic approach for mitigating poor tissue repair.

Conjugation of polyethylene glycol (PEG) to proteins or protein PEGylation has been used in the pharmaceutical industry to stabilize protein therapeutics [8–10]. PEGylated protein therapeutics are more stable against proteolysis, pose less harmful immunogenic reactions and are often more effective and safer than native proteins [8–10]. However, PEG chains have the potential to block the active sites of proteins and decrease their biological activity. This is particularly important for large proteins such as FN, where all multiple domains must remain bioactive in order for this protein to perform its biological function.

We previously demonstrated that cysteine-PEGylated FN had higher proteolytic stability than native FN [11]. Cysteine-PEGylated FN also elicited comparable biological responses to native FN with regards to cell adhesion, focal adhesion formation, and cell migration. However, unlike native FN, cysteine-PEGylated FN was not assem-

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Abbreviations: BSA, bovine serum albumin; **CD**, circular dichroism; **ECM**, extracellular matrix; **ELISA**, Enzyme-Linked Immunosorbent Assay; **FN**, fibronectin; **FN–PEG**, PEGylated fibronectin; **mPEG-SCM**, methoxypolyethylene glycol succinimidyl carboxyl methyl ester; **PBS**, phosphate buffer solution; **PEG**, polyethylene glycol; **SDS–PAGE**, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

bled into an ECM when immobilized on a surface [11]. Since the activity of PEGylated proteins depends on a number of factors such as PEG molecular weight and PEGylation site, past work suggests that PEGylation parameters can be manipulated to create FN–PEG conjugates with varying stabilities and activities.

The goal of this study was to determine the effect of varying PEG molecular weight on the stability and activity of FN. FN was preferentially PEGylated on lysine residues with PEG precursors of varying molecular weight. In our previous work, conjugation of PEG diacrylate to FN was possible only through reduction of cystines in the amino-terminal of FN [11]. However, this approach can disrupt gelatin-binding interactions, which are localized in a 70-kDa amino-terminal fragment of FN and are essential for interactions with the ECM [12–14]. As an alternative to cysteine PEGylation, lysine PEGylation is advantageous because it does not require FN reduction, and thereby minimizes potential perturbation to FN structure.

Here we address the problem of stabilizing FN against proteolysis through lysine PEGylation. In contrast to previous work on PEGylation through cysteine residues, this approach helps to avoid disruption of the protein structure. Most protein PEGylation studies have been conducted on proteins with molecular weights less than 100 kDa [8–10]. PEGylation of FN presents additional challenges in the sense that this protein must remain capable of interacting with its binding partners after PEGylation and thus past PEGylation findings with other proteins cannot be extrapolated to FN PEGylation.

The justification for using surface lysine residues for PEGylation stems from the fact that approximately 3% of the 2386 amino acids residues in plasma FN are lysines (Universal Protein knowledgebase number P02751). This indicates that only a small fraction of the amino acid residues in FN would be perturbed by lysine PEGylation. The PEGylation strategy herein uses methoxypolyethylene glycol succinimidyl carboxyl methyl ester (mPEG-SCM). mPEG-SCM is advantageous compared to PEG diacrylate used previously because: (i) the ester group in mPEG-SCM reacts irreversibly with primary amines found in lysine residues; (ii) the terminal methoxy group is inert, which eliminates cross-linking between conjugates; (iii) conjugation with mPEG-SCM occurs efficiently in a mild and buffered environment without the need for protein reduction; and (iv) the amide bond formed between PEG and the protein is hydrolytically stable [15].

The objective of the study was to determine the influence of lysine PEGylation and the role of PEG molecular weight on the proteolytic stability and biological activity of FN. The challenge in FN PEGylation is that it is a large protein with multiple functional domains. Therefore, PEGylation may impact a number of biological activities in FN. Here we examine the role of PEGylation on two functionalities in FN that are critical to its activity [1, 2]; cell and gelatin binding. The cell binding domain is crucial for cell attachment and both the cell and gelatin binding domains are important for ECM assembly [1, 2]. The effect of PEG molecular weight on proteolytic stability and cell adhesion, cell spreading, and gelatin binding are reported. The conjugates developed may be useful in addressing the problem of FN degradation and dissecting molecular mechanisms involved in FN degradation while at the same time preserving the cell binding and ECM assembly capability of PEGylated FN.

2 Material and methods

2.1 Purification of FN

FN was isolated from frozen human blood plasma, which was obtained from the blood bank at Loyola University Medical Center. Blood plasma was first thawed and centrifuged to remove residual cells and other precipitates and then passed through a column packed with Sepharose 4B beads (Sigma–Aldrich, St Louis, MO). The Sepharose column flow-through was then passed through a column packed with Gelatin Sepharose beads (GE Healthcare Bioscience, Pittsburgh, PA). The bound FN was eluted with 6 M urea (Fisher Scientific, Pittsburgh, PA) in phosphate buffer saline (PBS, Fisher Scientific). The concentration of eluted FN solution was determined by taking light absorbance readings at 280 nm. The molecular weight of the purified FN was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by silver staining. The yield of FN was approximately 30 mg for 150 mL of human plasma. The stocks of purified FN in 6 M urea in PBS were stored at −80°C. Prior to PEG conjugation, FN was thawed on ice and then dialyzed in phosphate buffered saline PBS. Dialysis was done twice against 1000× PBS volume so that the final urea concentration would be in the μM range. This was further diluted to the working concentration and the estimated concentration of residual urea was in the nM range. At this concentration of urea FN remains folded in its native structure. This was checked through circular dichroism studies.

2.2 FN PEGylation and characterization

FN was PEGylated by mixing it with 2, 5, or 10 kDa mPEG-SCM (Creative PEGWorks, Winston-Salem, NC). mPEG-SCM with different molecular weights was first dissolved in dimethyl sulfoxide (Sigma–Aldrich) to make a 10 mM stock solution. FN was diluted to 2 μM in PBS and mixed with excess mPEG-SCM at a molar ratio of 1:50. The mixture was then placed on a rotator and incubated for an hour at room temperature. The reaction was quenched by adding 1 M Tris pH 8.8 and the resulting solution was dialyzed against PBS overnight. The con-

centration of PEGylated FN after dialysis was determined using Bicinchoninic acid assay (Pierce, Rockford, IL).

The molecular weight of the products of FN PEGylation was determined by SDS–PAGE using Coomassie staining and immunoblotting. Aliquots of the PEGylation reaction were collected 0.5 and 1 h after the onset of PEG-SCM addition. The aliquots were mixed with reducing electrophoresis sample buffer, which contained 6.6% sodium dodecyl sulfate (SDS, Fisher Scientific), 33% glycerol (Fisher Scientific) and 0.33 M dithiothreitol (Sigma–Aldrich). For Coomasie blue, 1 μg of protein was analyzed using standard procedure. For immunoblotting, 100 ng of FN or an equivalent amount of the reaction mixture at different time points was electrophoresed on a 7% polyacrylamide gel, transferred to nitrocellulose membrane and probed with monoclonal antibody 7.1 (Developmental Studies Hybridoma Bank, Iowa City, IA). The primary antibody was used at a dilution of 1:1000 and applied to the nitrocellulose membrane for 1 h at room temperature. The nitrocellulose membrane was then washed and incubated with goat anti-Mouse IgG (H + L) horseradish peroxidase conjugates (Invitrogen, Carlsbad, CA) at a dilution of 1:5000 for 1 h. Antibody labeling was detected with SuperSignal West Pico chemiluminescent reagents (Pierce) and imaged by a ChemiDoc XRS+ system (Bio-Rad, Hercules, CA). The molecular weight of FN positive bands was determined by comparing the distance migrated of FN positive bands against that of Precision Plus Protein Prestained standards (Bio-Rad).

2.3 Circular dichroism (CD) of PEGylated proteins

CD studies were conducted to determine the secondary structure of native and PEGylated FN. CD spectra of native and PEGylated FN at concentration of 0.75 mg/mL were collected between 190 and 300 nm in a J715 spectropolarimeter (Jasco, Easton, MD). We used a 0.1-cm path length cuvette, a temperature of 20°C, a bandwidth of 5 nm, and a scan rate of 100 nm/min to obtain ellipticity. Five scans were averaged for each individual sample. The measured ellipticity, θ, in degrees was converted to molar ellipticity $[\theta]$ in deg cm^2/dmol by the following relationship:

$$[\theta] = \theta \div (c \times l) \quad (1)$$

where c is the molar concentration in dmol/cm^3 of peptide bonds and l is the path length in cm.

2.4 Proteolysis of PEGylated FN

Tosyllysine chloromethyl ketone-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 [11]. In brief, PEGylated and native FN at a concentration of 50 μg/mL were mixed with α-chymotrypsin at a protein to protease mass ratio of 25:1. Aliquot samples were collected at specific time points from 0 to 30 min. Proteolysis in the aliquots was inhibited by the addition of 2 mM phenylmethylsulfonyl fluoride (Fisher Scientific). The samples were then mixed with reducing electrophoresis buffer, resolved in a 7% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with monoclonal antibodies 7.1.

2.5 Monoclonal 7.1 antibody binding to adsorbed PEGylated FN

The amount of monoclonal 7.1 antibodies bound to FN or FN–PEG adsorbed on polystyrene surfaces was determined by enzyme-linked immunosorbent assays (ELISA). Forty eight-well polystyrene dishes were incubated with 50 μg/mL FN or PEGylated FN at 37°C for 1 h. The wells were washed and blocked with 1% bovine serum albumin (BSA, Fisher Scientific) in PBS for 1 h at 37°C, then washed twice with PBS. The wells were incubated with monoclonal 7.1 antibodies followed by secondary antibodies, conjugates and substrates using methods described in previous studies [16, 17]. Treatments were blanked with wells containing PBS.

2.6 Binding of PEGylated FN to gelatin

Binding of native or PEGylated FN to gelatin was carried out as follows. Forty eight-well polystyrene dishes were incubated with 50 μg/mL gelatin (Fischer Scientific) in PBS at 37°C for 1 h. The plates were blocked with 1% BSA in PBS, washed twice with PBS and then incubated with 50 μg/mL FN or PEGylated FN for 1 h at 37°C. ELISAs with 7.1 monoclonal antibodies were carried out using methods previously detailed by our laboratory in order to determine the amount of FN or PEGylated FN bound to gelatin [16, 17]. The spectrometer readings were blanked against wells containing PBS.

2.7 Cell culture

NIH 3T3 fibroblasts (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (Fisher Scientific) supplemented with 10% bovine calf serum (Fisher Scientific) at 37°C with 5% CO_2. NIH 3T3 fibroblast passages 15–21 were used for this study. The cells were grown to 80–90% confluency in 10 cm dishes.

2.8 Static cell adhesion assay

Glass coverslips were cleaned with 70% ethanol (Fisher Scientific) and washed twice in PBS. The coverslips were incubated in 50 μg/mL native or PEGylated FN in PBS at 37°C for 1 h. After incubation the coverslips were rinsed

twice with PBS and blocked with 1% BSA in PBS for 30 min at 37°C. The coverslips were then rinsed twice with PBS. NIH 3T3 fibroblast cells at 80–90% confluency on 10-cm dishes were trypsinized with 1 mg/mL of TPCK trypsin (Fisher Scientific) in 0.01% ethylenediaminetetraacetic acid (Fisher Scientific) in PBS for 5 min at room temperature. Trypsin was inactivated by adding 0.5 mg/mL soybean trypsin inhibitor (Fisher Scientific) in PBS. The cell suspension solution was centrifuged and resuspended in serum free Dulbecco's modified Eagle's medium solution. Cells were then added at a density of 4×10^4 cells per well to the glass coverslips and then incubated for 1 h at 37°C in 5% CO_2. After incubation the samples were washed twice with PBS, fixed with 3.7% paraformaldehyde (Fisher Scientific) in PBS and permeabilized with 0.5% detergent NP-40 (Fisher Scientific) in PBS. The samples were then rinsed twice with PBS and stained with fluorescein conjugated phalloidin (Invitrogen) and Hoechst 33258 (Fisher Scientific). Fluorescein–phalloidin and Hoechst 33258 were used at a dilution of 1:50 and a concentration of 1 μg/mL, respectively. Staining was carried out at 37°C for 30 minutes in 2% ovalbumin (Sigma–Aldrich) in PBS. 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).

2.9 FN matrix assembly on coated surfaces

Fibronectin matrix assembly studies were used to characterize the assembly of coated PEGylated FN into ECM fibrils by cells. The procedure for matrix assembly is described in greater detail in the study by Zhang et al. [11]. 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, once with water and then imaged.

2.10 Fluorescence microscopy and image analysis

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

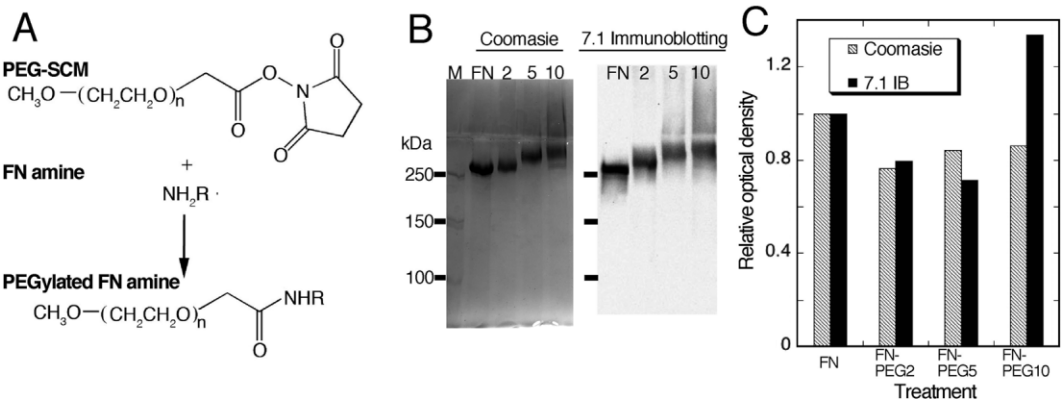
2.11 Statistical analysis and data treatment

The treatments comprised FN PEGylated with 2, 5, and 10 kDa mPEG-SCM. 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 areas were randomly selected from each treatment and analyzed for cell area. A one-factor analysis of variance was used to statistically evaluate cell adhesion and spreading. A two-sided significance level of 5% was used to conduct student's *t*-tests when comparing the means of different treatments. *p*-values less or equal to 0.05 were considered statistically significant.

3 Results

3.1 PEGylation of FN on lysines

The goal of this study was to determine the influence of lysine PEGylation and PEG molecular weight on the stability and biological activity of FN. FN was PEGylated on lysines with mPEG-SCM and the extent of PEGylation was determined by SDS–PAGE. The reaction scheme used is shown in Fig. 1A. Linear mPEG-SCMs with molecular weights of 2, 5, and 10 kDa were conjugated to FN to form PEGylated products, FN–PEG2, FN–PEG5, and FN–PEG10, respectively. Figure 1B shows Coomasie staining and immunoblotting of mPEG-SCM and FN an hour after mixing. There is an increase in the molecular weight of the FN bands an hour after the addition of mPEG-SCM as determined with both analytical methods. The changes in molecular weight after mPEG-SCM addition positively correlate with the molecular weight of mPEG-SCM used. The absence of a band corresponding to the molecular weight of native FN an hour after mPEG-SCM addition indicates that all FN molecules are PEGylated within this time period. FN PEGylated by mPEG-SCM had a narrower molecular weight range than FN–PEGs synthesized from PEG diacrylate [11]. The latter showed the presence of dimers and n-mers that were crosslinked due to the bifunctional nature of PEG diacrylate.



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Treatment	Molecular Weight (kDa)		Average Number of PEG Molecules
	Average	Range	
FN	284	273-293	-
FN-PEG2	304	290-318	10
FN-PEG5	378	355-398	19
FN-PEG10	415	393-441	13

Figure 1. PEGylation of FN by mPEG-SCM and characterization of PEG conjugates. (**A**) Scheme of FN PEGylation. (**B**) Coomassie blue stained gel and 7.1 immunoblot of electrophoresed FN and FN plus 2, 5 and 10 kDa mPEG-SCM 1 h after mixing. The 7.1 antibody is specific for human FN. The mass of protein analyzed for Coomassie staining and immunoblotting is 1 μg and 100 ng, respectively. The lane with FN alone serves as the loading control. The **M** lane represents molecular weight markers. (**C**) Densitometric analysis of the bands from Coomassie staining and 7.1 immunoblotting normalized to FN. (**D**) Molecular weight analysis of native FN and FN PEGylated with 2, 5, and 10 kDa mPEG-SCM or FN–PEG2, FN–PEG5, and FN–PEG10.

Even though equal amounts of the native and PEGylated protein were loaded into the gel and blot in Fig. 1B, there were differences in the intensity of staining with each analytical method and in the intensity of staining between the two methods. Densitometric analyses were carried out to correlate the amount loaded with the amounts in gels and blots. Coomassie staining in Fig. 1C shows that there is 75–85% less FN–PEG2, FN–PEG5, and FN–PEG10 than native FN. As synthetic polymers PEGs are polydisperse which results in polydispersity of the conjugates [9, 18]. Polydispersity may be the reason that bands containing PEGylated FN are more spread and show lower intensity than native FN in Fig. 1C. Differences in the amount of FN and PEGylated FN detected by Coomassie staining could also be due to molecular weight cut-off and the sensitivity of the molecular imager. The trends in the amount of protein in the immunoblot mirror those seen in Coomassie staining except for FN–PEG10. Interestingly, immunoblotting detects 50% more protein than that detected by Coomassie staining in the latter case. This increase may be due to background from non-specific binding between 10 kDa PEG and the antibody. This would result in higher than normal values of antibody detection of FN–PEG10.

Molecular weight analysis of PEGylated products, FN–PEG2, FN–PEG5, and FN–PEG10 was conducted on immunoblots to determine how many PEG molecules bound to native FN after PEGylation. The results of the molecular weight analysis of the products of FN PEGylation with mPEG-SCM are shown in Fig. 1D. PEG conjugation results in covalent bonding of 10–19 PEG molecules per FN molecule. This corresponds to PEGylation of 12–24% of the 78 lysine residues in FN or PEGylation of 0.4–0.8% of the amino acid residues in FN.

3.2 CD studies of secondary structure of PEGylated and native FN

In order to verify that PEGylation through lysine residues did not affect secondary structure CD was used to examine PEGylated FN [19–21]. Although the secondary structure of proteins is usually not affected by PEGylation it is necessary to confirm this is the case, especially with a large multifunctional protein like FN. This analysis is crucial as there are no previous CD studies of PEGylated FN. Figure 2 shows the molar ellipticities of native FN as well as FN–PEG2, FN–PEG5, and FN–PEG10. The spectrum of FN in Fig. 2 is in good agreement with that reported in literature by Lai and Wolff [19] and Sakai and co-workers [20]. The similarities with reported data include: (i) a negative amplitude at approximately 212 nm corresponding to β-sheets; (ii) a positive amplitude at approximately 227 nm corresponding to aromatic residues; and (iii) comparable values of the peak intensities. The molar ellipticities of FN and FN–PEG conjugates were within 10% of each other which is within the allowable measurement

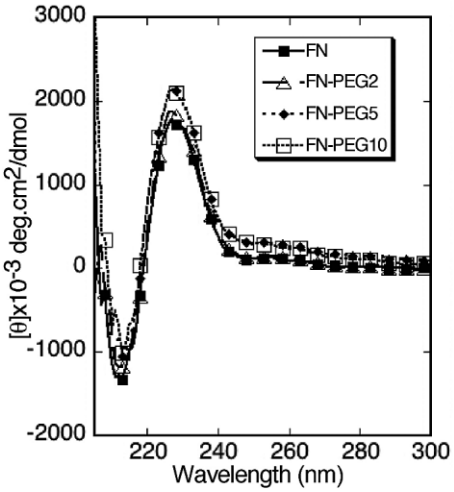

Figure 2. Characterization of native and PEGylated FN secondary structure by CD. Molar ellipticities, [θ], of FN, FN–PEG2, FN–PEG5, and FN–PEG10 are averaged from five scans.

error associated with CD studies [22]. Thus, based on the molecular weight values and CD spectra, lysine PEGylation does not have a significant impact on the secondary structure of FN.

3.3 Proteolytic stability of FN PEGylated with 2–10 kDa mPEG-SCM

We had previously shown that cysteine-PEGylated FN was more proteolytically stable than native FN in the presence of α chymotrypsin [11]. We characterized the proteolytic stability of lysine-PEGylated FN and examined the relationship between PEG molecular weight and proteolytic stability. Dialyzed FN–PEG2, FN–PEG5, and FN–PEG10 were treated with α-chymotrypsin. An immunoblot of FN–PEG conjugates as well as native FN, collected before proteolysis and at 15 and 30 min after proteolysis was initiated, is shown in Fig. 3A. Figure 3A shows higher protease degradation of FN than of FN–PEG2, FN–PEG5, or FN–PEG10. This is characterized by greater fragmentation of FN after 15 and 30 min compared to PEGylated FN. Figure 3B represents densitometric analyses of the immunoblots of intact FN or FN–PEG before protease addition and at 15 and 30 min after protease addition. The data in Fig. 3B was normalized to the amount of intact protein at time zero. After 30 min of proteolysis, 4, 34, 43, and 65% of the starting amount of FN, FN–PEG2, FN–PEG5, and FN–PEG10, respectively, was present.

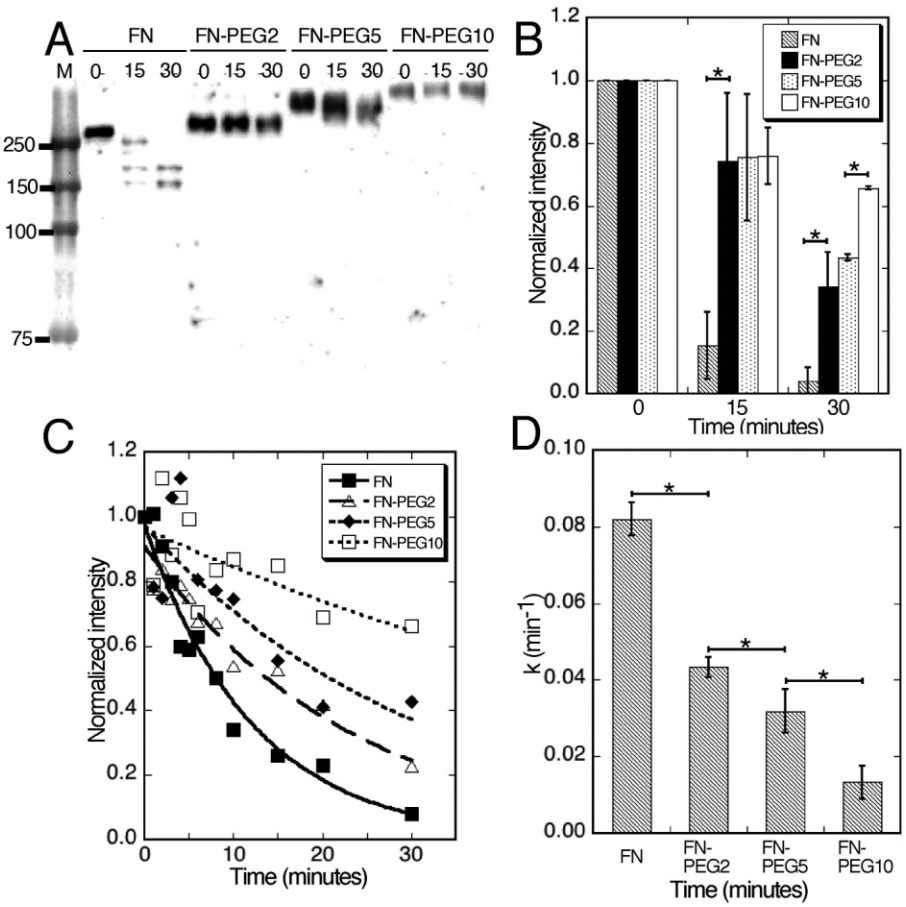

Figure 3. Proteolytic stability of PEGylated FN. (**A**) Immunoblot of FN and FN–PEG conjugates before and after 15 and 30 min treatment with α chymotrypsin. Lane M contains the molecular weight standards. The second lane contains 100 ng of FN and is the loading control. The 7.1 antibody is specific for human FN. (**B**) Densitometric analysis of immunoblot in A. (**C**) Kinetics of degradation of FN and PEGylated FN from densitometric analysis of immunoblots of proteolysis at different time points. (**D**) First order kinetic rate constants of proteolysis, *k*, obtained from the data in C. The data is from two different experiments. Error bars represent the standard error of the mean and "*" represents statistically significant differences.

The FN fragments in PEGylated FN were not recognized by 7.1 monoclonal antibodies which is in line with what we reported for cysteine PEGylation of FN [11]. One reason could be that the amount of fragments is below the detection limit of the molecular imager. Another reason is that the presence of PEG may sterically alter cleavage sites and subsequently the molecular weight distribution of fragments. This may lead to fragments that do not contain an epitope for 7.1 monoclonal antibodies and thus cannot be detected by 7.1 immunoblotting. The data indicates that lysine PEGylation stabilizes FN against proteolysis and that the molecular weight of PEG conjugated to FN positively correlates with proteolytic stability.

A kinetic analysis of proteolysis was carried out to determine the correlation between PEG molecular weight and proteolytic stability. Proteolysis was carried out as before but samples were collected at more time points and then immunoblotted. Figure 3C represents densitometric analyses of the bands in the immunoblots corresponding to intact FN, FN–PEG2, FN–PEG5, or FN–PEG10 at different time points during proteolysis. The densitometric data was fit to a first order kinetic rate law. A logarithm transformation of the data in Fig. 3C resulted in linear fits that had R values of 0.98, 0.98, 0.88, and 0.69 for FN, FN–PEG2, FN–PEG5, and FN–PEG10, respectively. The slope of the linear fit of the logarithm-transformed data corresponds to a first order kinetic rate constant. The first order kinetic rate constant decreased with increasing PEG molecular weight, or the larger the conjugated PEG the slower the rate of proteolysis (Fig. 3D). Thus, the molecular weight of PEG conjugated to FN determines the proteolytic stability of the FN–PEG conjugate.

3.4 Binding interactions with 7.1 monoclonal antibodies and gelatin

ELISAs with 7.1 monoclonal antibodies were used to determine if PEGylation perturbed the antibody-binding site. PEGylated and native FN were adsorbed on polystyrene surfaces in conditions that result in surface saturation [23–25]. Figure 4A shows ELISA absorbance readings that reflect the quantity of antibodies bound to FN, FN–PEG2, FN–PEG5, and FN–PEG10 adsorbed on polystyrene surfaces. These absorbance readings represent

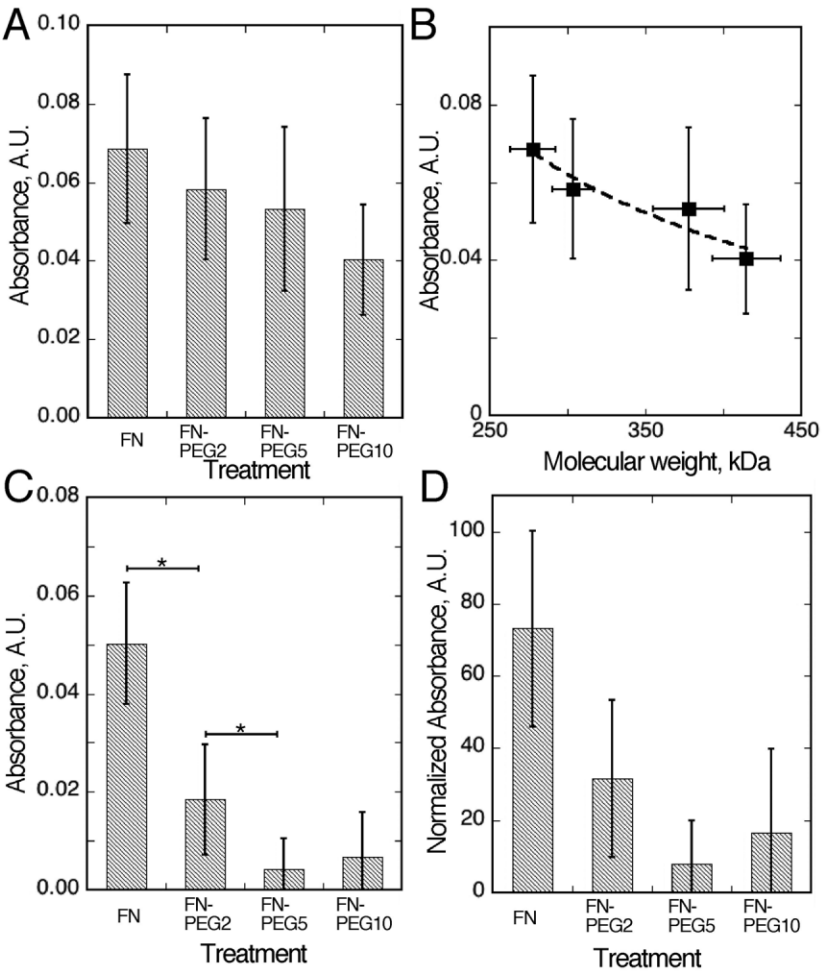

Figure 4. Quantitative analysis of binding interactions between PEGylated FN with monoclonal 7.1 antibodies and gelatin. (**A**) FN and FN–PEG conjugates adsorbed on polystyrene and detected by ELISAs of monoclonal 7.1 antibodies that bind to the cell binding domain of FN. (**B**) ELISA absorbance in (A) versus conjugate molecular weight. (**C**) FN and FN–PEG conjugates incubated on gelatin coated polystyrene wells and detected with ELISAs using monoclonal 7.1 antibodies. (**D**) The results in (C) normalized to the amount of molecules on the surface. Error bars represent the standard error of the mean of duplicates in two different experiments.

the quantity of the aforementioned molecules on the surface. The absorbance readings obtained on surfaces with adsorbed PEGylated FN are less than on native FN and decrease with increasing PEG molecular weight. This trend in the amount of FN or FN–PEG adsorbed on polystyrene could be due to differences in molecular size of FN and FN–PEG or due to differences in antibody binding.

One potential explanation for higher antibody detection of FN compared to FN–PEG could be that differences in the amount of adsorbed protein are due to differences in molecular weight; that is, the larger the molecule, the less the number of adsorbed molecules on the surface. The role of PEG size on the amount of FN detected on the surface can be analyzed by making some assumptions. These are: (i) the adsorption of FN and FN–PEG follows the model proposed by Grinnell [26] that postulates that FN molecules adsorbed on a polystyrene surface from high solution concentrations can be approximated as spheres packed on the surface; (ii) monolayer coverage; and (iii) increases in the physical size of the FN–PEG are proportional to the total molecular weight of the grafted PEG. The assumption of packed spheres can be safely made if one considers the excluded area during the random sequential adsorption of molecules. The linear relationship between increases in size and total weight of grafted PEGs was shown by Fee and Van Alstine [27] where they found a correlation between the viscous radii of PEGylated proteins (determined by size exclusion chromatography) and total molecular weight (determined by matrix-assisted laser desorption/ionization-time of flight mass spectrometry). Based on the assumption of tightly packed spheres, the number of molecules on a surface can be approximated as being inversely proportional to the molecular weight raised to a two-thirds power.

ELISA results for monoclonal antibody binding plotted against molecular weight of the conjugate in Fig. 4B shows that the data fits a power law relationship $y = 36.2\ (\pm\ 5.65)\ x^{-1.11(\pm\ 0.297)}$ where y represents absorbance and x represents molecular weight. The R value of the fit is 0.94. The upper limit of the exponent is comparable to the packed sphere model estimate indicating that FN and FN conjugates on the surface can be approximated as packed spheres. It is also possible that differences between the model and experimental data may arise from altered antibody binding sites, particularly in the largest conjugate. Nonetheless, the assumption that the number of conjugates adsorbed on the surface is inversely proportional to the total molecular weight of the conjugate is supported by the ELISA results.

FN binds to gelatin at the 70 kDa amino terminal [12–14]. We examined whether gelatin binding was significantly perturbed by lysine PEGylation through solid phase binding assays. Gelatin was adsorbed on polystyrene surfaces and then FN or FN–PEG conjugates were incubated on the gelatin coated surfaces. ELISAs with 7.1 antibodies were used determine the amount of FN or FN–PEG bound to gelatin. The results shown in Fig. 4C indicate a significant reduction in the binding of FN–PEG2, FN–PEG5, and FN–PEG10 to gelatin compared to that of native FN. The results in Fig. 4C were normalized for quantity of ligands on the surface. Figure 4D shows that after normalization with the amount on the surface, gelatin binding decreases with increasing PEG molecular weight. Binding of gelatin to FN–PEG5 or FN–PEG10 is significantly lower than that of gelatin to native FN. Therefore, increasing the molecular weight of PEG conjugated to lysines in FN leads to decreased gelatin binding.

3.5 Effect of lysine PEGylation and PEG molecular weight on cell adhesion and spreading

FN mediates cell adhesion through the cell binding domain [28, 29]. We had earlier demonstrated that cysteine PEGylation of FN does not perturb cell adhesion and spreading [11]. We examined what the effect of lysine PEGylation was on cell adhesion and spreading. The influence of PEGylation and PEG molecular weight on cell adhesion and spreading was determined through static adhesion assays. Figure 5A shows fluorescently stained cells after 1 h of culture in serum free media on surfaces immobilized with the different proteins. Qualitatively, there is more robust cell spreading and adhesion on surfaces with adsorbed FN and FN–PEG2 than on surfaces with FN–PEG5 or FN–PEG10.

Quantitative analyses of cell adhesion and spreading on glass surfaces adsorbed with native and PEGylated FN were carried out. The results of this analysis are shown in Fig. 5B and 5C, respectively, and they demonstrate that cell adhesion and spreading decreases with increasing PEG molecular weight. The adhesion and spreading results were normalized to the amount of FN or FN–PEG conjugates on glass coverslips (Supporting information, Fig. S1). On surfaces where cell adhesion was normalized to the amount of conjugate on the surface, the adhesive response still decreased with increasing PEG molecular weight. FN–PEG2 had a comparable adhesion response to native FN (Supporting information, Fig. S1). After normalization for the amount on the surface, cell spreading was significantly lower on all the conjugates than on native FN and decreased with increasing PEG molecular weight. These results suggest that PEGylation does not occur at the cell binding domain but may occur near this domain and thus perturb cell adhesion at large PEG molecular weights.

3.6 Matrix assembly on glass surfaces coated with native and PEGylated FN

The cell and gelatin binding domains are essential for FN matrix assembly [30]. We had previously reported that, unlike native FN, FN PEGylated on cysteine residues was

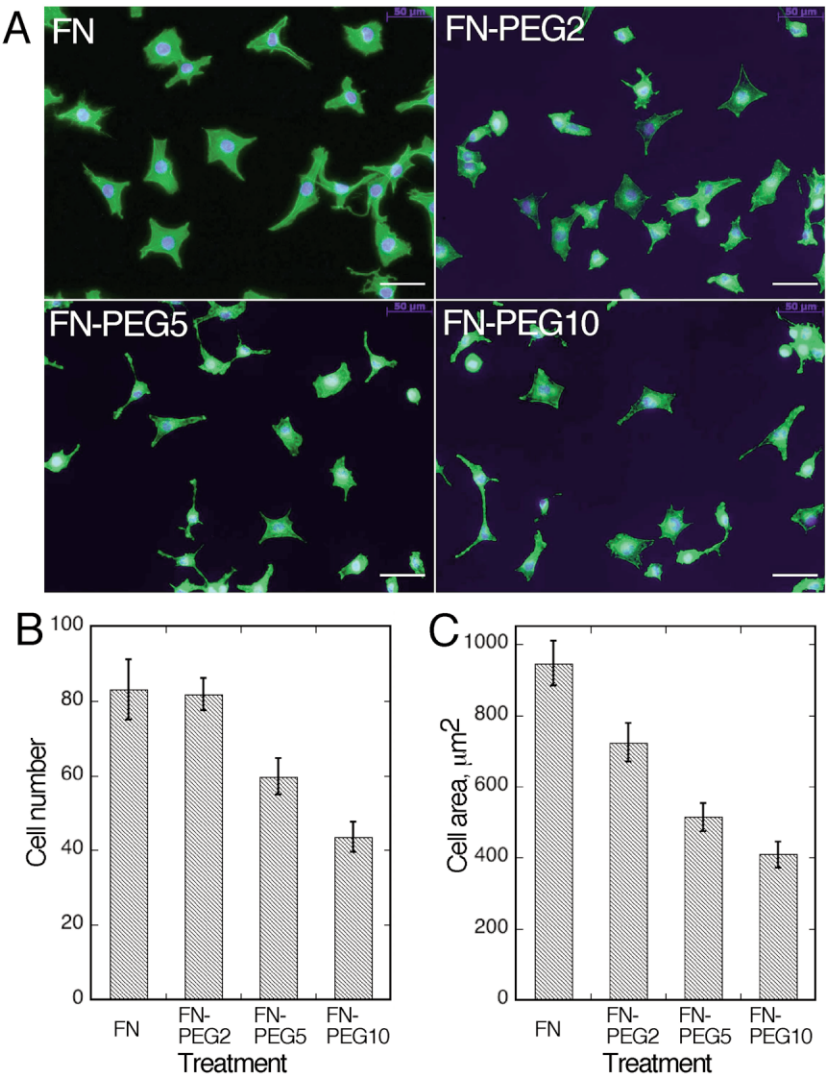

Figure 5. Cell adhesion and spreading on surfaces coated with PEGylated or native FN. (**A**) Fluorescence microscopy images of cell adhesion and spreading on glass cover slips coated with native or PEGylated FN. Nuclei and actin filaments are stained blue and green, respectively. Scale bar in *A* = 50 µm. (**B**) Image analysis of cell number in a 10× field. (**C**) Image analysis of cell area under 20× magnification. Error bars in B represent the standard error of the mean of 16 random areas for two repeats in each treatment in experiments conducted twice. Error bars in C represent the mean of a minimum of 72 cells per treatment for experiments conducted twice.

not assembled into ECM fibrils when coated on surfaces [11]. The gelatin binding domain of FN is cysteine rich [12] and binding interactions in this domain may be perturbed by cysteine PEGylation. We examined whether lysine-PEGylated FN could be assembled into an ECM when coated on surfaces. Figure 6 is an immunofluorescence micrograph of NIH 3T3 mouse fibroblasts cultured on surfaces coated with native FN or lysine-PEGylated FN. Fibrillar structures positive for human FN staining were observed at the periphery of cells cultured on surfaces coated with FN, FN–PEG2, FN–PEG5, and FN–PEG10. For clarity only the 2 and 10 kDa PEG conjugates are shown. Qualitatively, there were more fibrillar structures present on FN coated surfaces than on surfaces coated with the conjugates (Fig. 6). Although the amount of FN fibrils assembled on surfaces coated with FN–PEG conjugates is qualitatively less than that on surfaces with the native FN, lysine PEGylation does not hinder FN fibril assembly.

4 Discussion

In this study, we determined the influence of PEG molecular weight on the proteolytic stability and activity of FN–PEG conjugates. Our results show that PEGylation on accessible lysines resulted in conjugation of approximately 15 PEG molecules per FN molecule. No significant changes in the secondary structure of FN were detected after PEGylation. In all the PEG molecular weights sampled, completely PEGylating FN on accessible lysines results in conjugates that are more proteolytically stable than native FN. The greater the molecular weight of PEG conjugated to FN, the higher the proteolytic stability. In immunoblotting studies, the binding site of monoclonal 7.1 antibodies in the cell binding domain did not seem to be perturbed by lysine PEGylation in FN–PEG2 and FN–PEG5 but appeared to increase in FN–PEG10. Cell adhesion on surfaces with adsorbed FN–PEG2 and native FN is comparable. However, cell adhesion decreases with

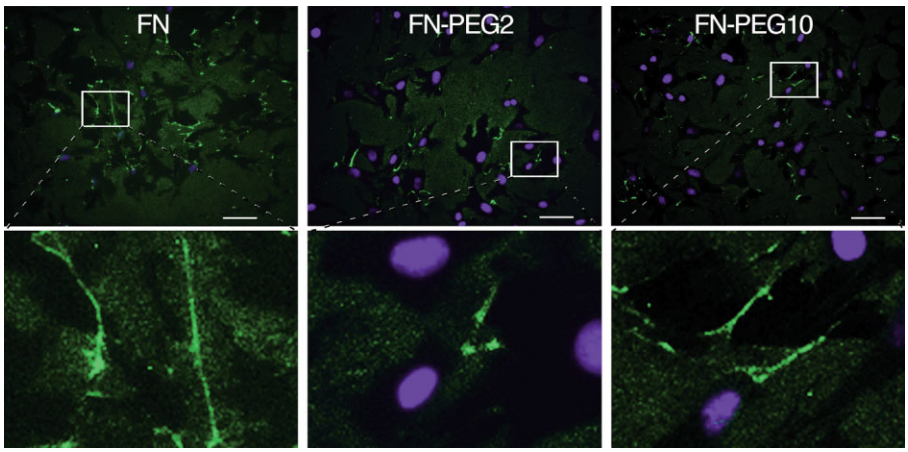

Figure 6. FN matrix assembly on surfaces coated with FN, FN–PEG2, and FN–PEG10. Fluorescence microscopy of FN (green) and nuclei (blue). Scale bar is 50 μm. Lower panel images are enlarged images of boxed areas.

increasing PEG molecular weight. Cell spreading also decreases with PEG molecular weight on surfaces with adsorbed FN–PEG conjugates. Furthermore, the amount of PEGylated FN bound to gelatin is dependent on PEG molecular weight. FN–PEG conjugates with PEG molecular weights of 5 and 10 kDa bind poorly to gelatin while FN conjugated to 2 kDa PEG has about half the gelatin binding capacity of native FN. FN and FN–PEGs are assembled into fibrils by cells when immobilized on surfaces but more fibril formation is observed on FN coated surfaces. Among the different PEG molecular weights sampled, the 2 kDa FN–PEG conjugate appears to show an optimal balance between resistance to proteolysis and bioactivity retention. Thus, PEGylation on lysines enhances proteolytic stability and PEG molecular weight can be used to modulate multiple biological responses in FN.

PEGylation has long been used as a strategy for increasing protein life time, decreasing immunogenicity, and decreasing renal clearance in protein therapeutics [8–10]. Most of these approaches have targeted peptides and small proteins [8–10]. Each protein or peptide has a unique set of PEGylation variables that can be used to optimize its stability and activity. Therefore, past PEGylation findings with smaller proteins and peptides cannot always predict the effect of PEGylation on larger and more complex proteins such as FN. This study presents new evidence of the relationship between the molecular weight of PEG, FN PEGylation approach (through lysine residues in this work) and FN activity and stability. The decrease in FN bioactivity with increasing molecular weight of conjugated PEG may be attributed to steric hindrance by PEG. But this trade off in loss of activity is counteracted with increased lifetime of the protein due to slower degradation as has been observed for other protein therapeutics [31].

This study demonstrates how lysine PEGylation can be used to enhance proteolytic stability of an ECM protein and the effect of FN PEGylation on its ability to bind cells, antibodies, and other ECM components. It is important to note that the PEGylation approach used here is preferential but not specific for lysine residues [15]. Therefore, there is the potential of PEGylating other moieties on the surface of FN [15]. Nonetheless, our studies lay an important foundation in PEGylating ECM proteins such as FN because they demonstrate the feasibility in controlling the stability and activity of two binding sites in FN by manipulating the molecular weight of conjugated PEG. Cell and gelatin binding are just two of many binding partners in FN that can be targeted in terms of activity. In addition to cysteines and lysines, other sites in FN could be PEGylated through choice of different PEG precursors. To the best of our knowledge this is the first work that illustrates how the effects of PEGylation of ECM proteins are more complex than previously considered, which is particularly important for large, multifunctional proteins such as FN. This current study contributes to the understanding of biological activities and stabilities of specific FN–PEG conjugates.

The authors are grateful to Dr. Ahmed Mirza from Loyola University Medical Center for help with acquisition of blood plasma. We are also grateful to Dr. Nick Menhart from the Biology Department at the Illinois Institute of Technology and to Dr. Gerd Prehna at the Center for Structural Biology at the University of Illinois at Chicago for the use of a spectropolarimeter and advice on CD. We are grateful to Anand Ramanathan and Zhongyang Wang for technical assistance with CD and microscopy studies.

This research is supported by start-up funds to Dr. Karuri. The other authors declare no financial or commercial conflict of interest.

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The cover shows a refreshing drink made of limes and is inspired by the summer, the football World Cup in Brazil and the article by Willrodt et al. on limonene formation from glycerol (http://dx.doi.org/10.1002/biot.201400023). © Paulista – Fotolia.com

Biotechnology Journal – list of articles published in the August 2014 issue.