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European Journal of Pharmaceutics and Biopharmaceutics 66 (2007) 21–27
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Maintenance of vascular endothelial growth factor and potentially other therapeutic proteins bioactivity during a photo-initiated free radical cross-linking reaction forming biodegradable elastomers

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Received 1 June 2006; accepted in revised form 10 August 2006
Available online 22 August 2006

Abstract

Previously, we prepared a biodegradable elastomeric device that can release different therapeutic proteins at a nearly constant rate in nanomolar concentrations with high bioactivity. The elastomer device was fabricated using a photo-initiated free radical cross-linking reaction of acrylated *star*(ε-caprolactone-*co*-D,L-lactide) in organic solvent in the presence of solid protein particles. The objective of this study was to examine the effect of various parameters used for fabricating the photo-cross-linked elastomeric device on the stability of a therapeutic protein, vascular endothelial growth factor (VEGF), to determine which factor plays the dominant role in protecting VEGF. VEGF was lyophilized with or without bovine serum albumin (BSA) and then suspended in solid state in a macromer (acrylated *star*-poly(ε-caprolactone-*co*-D,L-lactide)) solution containing different concentrations of a free radical initiator, 2,2-dimethoxy-2-phenylacetophenone (DMPA). The protein suspension was then UV-irradiated at different intensities. UV irradiation with the generation of free radicals was detrimental to VEGF stability. BSA preserved the VEGF bioactivity during UV irradiation but provided little protection in the presence of the photo-initiator DMPA. The acrylated macromer acted as a free radical scavenger and effectively preserved VEGF and BSA stability during UV-initiated photo-polymerization. The detrimental effect of UV radiation with free radical generation on VEGF stability during device manufacture can be eliminated by choosing the proper bulking agents coupled with an efficient photo-polymerization reaction.

Keywords: Photo-cross-linking; Vascular endothelial growth factor; Protein stabilization; Protein bioactivity

1. Introduction

Protein drugs are the most rapidly growing segment of the biopharmaceutical drug market in this decade [1]. For therapeutic proteins that are secreted locally and have a short in vivo half-life, it would be highly desirable to develop a delivery device that can provide a high dosage at a controlled rate in the vicinity of the target area, without distributing the drugs in systemic circulation. In recent years, there has been increasing interest in developing photo-polymerized materials for protein drug delivery applications [2–11]. The advantages of using photo-polymerization in the development of a protein drug delivery device are that the reaction initiation and termination can be well controlled, and the reaction proceeds rapidly under ambient conditions [2–11].

The articles published to date have focused principally on the preparation of photo-cross-linkable hydrogels, wherein the protein is in solution during cross-linking. Of the articles published, few have examined the stability of the protein encapsulated during the photo-cross-linking procedure, and for those that do, the reports are conflicting. For example, West and Hubbell [2] entrapped tissue plasminogen activator in a PEG-*block*-lactide-diacrylate hydrogel during photo-cross-linking with UV light and found that the bioactivity of the protein was unaffected by the

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doi:10.1016/j.ejpb.2006.08.006

cross-linking procedure, whereas Baroli et al. [6] using PEG diacrylate found that the bioactivity of horseradish peroxidase and α-glucosidase decreased upon photo-cross-linking with a blue light system. The maintenance or loss of protein bioactivity in these situations is likely protein-specific and loss of protein activity is probably due to the fact that both protein and the free radicals generated during cross-linking are in the aqueous phase. This situation makes it more likely for the protein molecule to come into contact with the highly reactive free radical and become denatured.

In a different approach, we have demonstrated the possibility of entrapping solid protein particles, specifically interferon-γ, interleukin-2, and vascular endothelial growth factor (VEGF), in an elastomer network. The free radical initiator and the cross-linkable macromononer in this approach are in an organic phase, separate from the protein phase. In our previous work, 7.8 kDa *star*-poly(ε-caprolactone-*co*-D,L-lactide) was prepared by ring-opening polymerization of D,L-lactide and ε-caprolactone initiated by glycerol [12]. The polymer was end-capped with an acrylate functional group through reaction with acryloyl chloride to generate an acrylated macromonomer. To incorporate the therapeutic protein into the elastomer, the protein was co-lyophilized with bovine serum albumin (BSA) and trehalose, and the lyophilized protein formulation was ground and suspended in the acrylated macromer solution. The elastomer device was fabricated through a photo-initiated polymerization of the macromer using 2,2-dimethoxy-2-phenylacetophenone (DMPA) as free radical initiator using 100 mW/cm^2 long-wave ultraviolet light (320–480 nm) at room temperature for 5 min. More than 80% of the bioactivity of the therapeutic proteins was retained after the free radical polymerization reaction.

Although the proteins retained significant bioactivity, the mechanism by which this occurred was not clear. There were a number of possible protective mechanisms. The loading of therapeutic protein in the lyophilized powders was less than 0.1 w/w (%). Thus, one possible source of protection could have been the overwhelming presence of the co-lyophilized excipients BSA and trehalose. In a mock-cross-linking reaction wherein the co-lyophilized powders were exposed to UV irradiation in the presence of the photo-initiator, unacrylated star copolymer, and solvent, it was demonstrated that BSA provided a much greater protective effect than the trehalose [11]. BSA may act as a bulking agent and thus become a readily available target for radiation and free radical damage. Previous studies have shown that BSA exposed to gamma-irradiation undergoes oxidative degradation, which can lead to either irreversible aggregation or/and fragmentation into oligomers [13,14] and this denaturation may also be occurring in our situation. Such degradation is undesirable as the denatured aggregates (and/or fragments) can increase immunogenicity, and compromise the biocompatibility of the elastomer device. Another possibility is that the polymer itself absorbs sufficient UV energy to protect the proteins. Finally, the acrylate groups may be reacting with the primary radicals generated by the decomposition of the photo-initiator before the radicals can react with the protein present. It was the objective of this work to determine which mechanism was responsible for the protection afforded to the entrapped therapeutic protein. VEGF was used as a representative therapeutic protein for this purpose.

2. Materials and methods

Human recombinant VEGF and its ELISA kit were purchased from Peprotech (Ottawa, Ont., Canada). D,L-Lactide and ε-caprolactone were purchased from Purac (Gorinchem, the Netherlands) and Alfa Aesar (Pelham, NH, USA), respectively. The human VEGF receptor-2/Fc chimera antibody and all other reagents were purchased from Sigma Aldrich (Oakville, Ont., Canada) unless otherwise specified.

2.1. Polymer synthesis

Linear copolymers of D,L-lactide and ε-caprolactone were prepared by melt polymerization at 140 °C of D,L-lactide and ε-caprolactone at a molar ratio of 50:50 with 1-octanol as the initiator and stannous 2-ethylhexanoate as a catalyst (Fig. 1). Different molar ratios of the monomers and 1-octanol were used to prepare copolymers of 1200, 2700, 4800, and 7800 g/mol. Polymers were end-capped at the hydroxyl terminus with acrylate by reaction with acryloyl chloride using the same procedures as previously described [12]. The evidence of acrylation was confirmed by ^{1}H NMR in d$_6$-DMSO at 400 MHz (Bruker, Canada). The acrylated linear macromers possess peaks at δ 6.02,

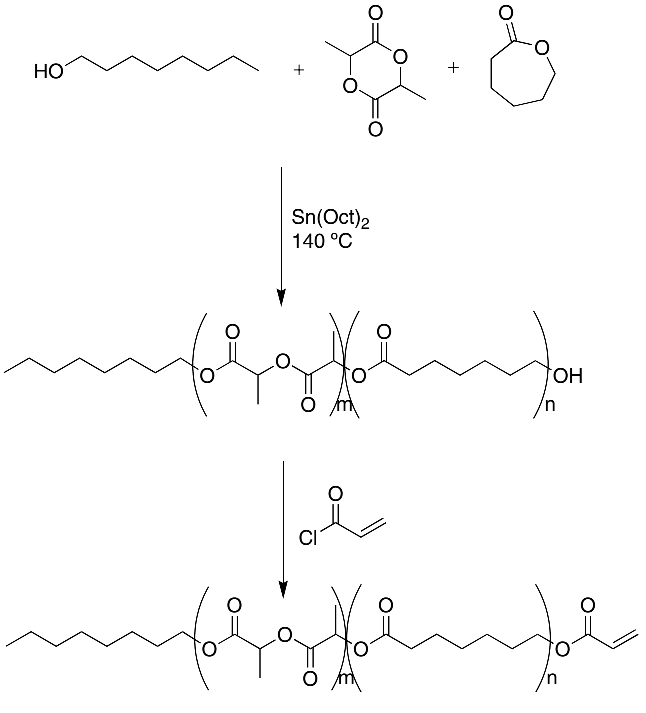

Fig. 1. Synthesis of linear 1-octanol initiated and terminally acrylated poly(ε-caprolactone-*co*-D,L-lactide).

6.25, 6.39, and 6.41 ppm which correspond to the vinyl protons on the acrylate functional group, and the absence of hydroxyl peaks at 5.5 and 4.3 ppm corresponding to lactide and caprolactone end groups, respectively.

2.2. VEGF concentration and bioactivity measurement

VEGF concentration was measured by ELISA as described previously [10]. The VEGF bioactivity was measured by using a VEGF receptor KDR ELISA. KDR is the dominant VEGF receptor used for transducing angiogenic signals during blood vessel formation [15]. The receptor domain of KDR binds to five amino acid residues (Phe17, Ile46, Glu64, Gln79, and Ile83) on VEGF [16]. Thus, the concentration of VEGF detected by the KDR ELISA represents the fraction of VEGF that can induce biological signaling pathways via the KDR receptor. In this study, the mass concentration and VEGF bioactivity were measured immediately before and after the photo-cross-linking reaction.

2.3. Effect of lyophilization and solvent on protein stability

Approximately 1 μg of VEGF was dissolved in a sterile 0.5 mM succinate buffer containing 0.1 w/v (%) BSA. The protein formulation solution was frozen in liquid nitrogen, then lyophilized on a Modulyo D freeze dryer (Thermosavant, USA) at 80 μbar and −50 °C for 48 h. The lyophilized powders were ground using a mortar and pestle, and sieved through a Tyler 100 sieve to yield particles of less than 145 μm diameter. These particles were suspended in tetrahydrofuran (THF), which is the organic solvent used to dissolve the macromonomer. Protein particles in the solvents were then collected by vacuum filtration, and dried in vacuum at room temperature for 24 h to ensure complete solvent evaporation. The dried protein powders were then suspended in 0.1 M pH 7.4 PBS. The stability profiles of BSA and VEGF were assayed according to the procedure described below.

2.4. Effect of UV light and free radical initiators on protein stability

The lyophilized and ground protein particles were suspended in THF containing various concentrations of a free radical initiator, 2,2-dimethoxy-2-phenylacetophenone (DMPA). The protein suspension was stirred at low speed on a magnetic stirrer, and then exposed to long-wave UV radiation (320–480 nm) at a distance of 1.5 cm from the solvent surface at room temperature using an EXFO E3000 high-intensity lamp at a range of intensities of 50–1000 mW/cm^2 for 300 s. The protein particles were then dried and assayed using the procedures described below.

2.5. Mass fraction of soluble protein

To recover BSA and VEGF from the particular radiation treatment steps, protein suspensions were dried in vacuum overnight to remove THF from the formulation. The recovered protein particles were then reconstituted in deionized water, and filtered using a 0.1 μm syringe filter. The protein mass in the filtrate was determined by measuring the concentration spectrophotometrically via UV absorbance at 280 nm. The mass recovery yield was calculated as the mass fraction of soluble protein recovered after the UV and free radical treatments over the initial mass used.

2.6. Effect of polymer solution and acrylate functional group on protein stability

The lyophilized and ground protein particles were suspended in THF containing 1 mg/mL of linear copolymer with various molecular weights. To the protein–polymer suspension was then added 0.3 w/v (%) DMPA, and the suspension exposed to UV light at an intensity of 100 mW/cm^2. The protein powders were then dried and assayed.

2.7. BSA stability

BSA molecular-weight distributions before and after free radical UV radiation were measured using gel permeation chromatography (GPC). The GPC detection system consisted of a Precision PD2100 light scattering detector possessing scattering detectors at angles of 90° and 15° from the incident light, and a Waters 2487 UV–visible light absorbance detector. The separation was carried out using a series of three Ultrahydrogel™ columns (120, 250, and 2000). The mobile phase was 50 mM PBS at pH 7.4. The protein samples were filtered and injected at 100 μL with a Waters 717plus autosampler into the columns at 0.8 mL/min and 30 °C. A BSA calibration curve made of freshly reconstituted BSA was prepared for each experiment. All data were obtained and processed in the Precision Detectors Precision Aquire32 and Discovery32 software programs.

3. Results and discussion

Before assessing which factor provided the greatest protective effect to the VEGF during photo-cross-linking, it was necessary to have an established baseline for VEGF bioactivity during the preformulation steps. Fig. 2 shows the effect of BSA on VEGF stability after freeze-drying and grinding. The presence of BSA greatly improved VEGF bioactivity during freeze-drying. Over 85% of the VEGF co-lyophilized with BSA was bioactive in comparison to only 28% of the VEGF lyophilized without BSA. BSA has previously been demonstrated to be an effective lyoprotectant for cytokines [17]. This effect has been attributed in part to a pH drop that occurs during lyophilization and possible preferential BSA adsorption to surfaces of the lyophilizing container. Fig. 2 also demonstrates that grinding had no deleterious effect on the VEGF in the presence of

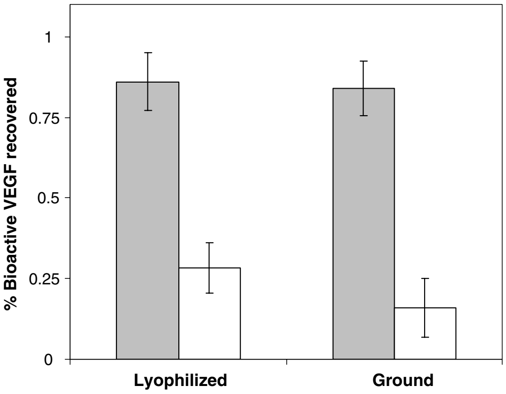

Fig. 2. Effect of particle preparation on VEGF bioactivity. The lyophilized VEGF with and without BSA particles were ground and sieved, and then re-suspended in PBS solution. The black and white bars correspond to VEGF lyophilized with BSA and without BSA, respectively.

BSA. There was a small decrease in bioactivity of the lyophilized VEGF after grinding in the absence of BSA, but no change in VEGF bioactivity, which remained at approximately 85%, when it was ground in the presence of BSA.

To characterize the effect of UV radiation on VEGF stability, lyophilized VEGF particles were resuspended in tetrahydrofuran (THF), and then exposed to UV light at different intensities. The bioactivity of the recovered VEGF was normalized with respect to the bioactivity of the lyophilized VEGF without UV irradiation. BSA greatly improved VEGF bioactivity during UV radiation, as shown in Fig. 3. When VEGF particles without BSA were irradiated with UV light at 50 mW/cm^2 for 5 min, over 60% of the VEGF lost bioactivity. Virtually no bioactive VEGF was recovered when the UV intensity was greater than 500 mW/cm^2. In the presence of BSA, over 80% of the VEGF was bioactive when irradiated at 50–200 mW/cm^2. As the radiation intensity increased to over 200 mW/cm^2, VEGF bioactivity decreased in a near linear fashion. The loss of VEGF bioactivity after UV radiation was likely due to photo-oxidation of the VEGF methionine residues. Duenas et al. [18] exposed an aqueous VEGF solution to fluorescent light at an intensity of 160 mW/cm^2 in a light box at 27 °C for 4 weeks. They found that all methionine residues on VEGF were oxidized within 2 weeks and the VEGF bioactivity decreased by 73% in week 4. In comparison to the low intensity and visible light source, used by Duenas et al., the use of high-intensity and long-wave UV radiation could have greatly accelerated VEGF oxidation in our case. In the presence of BSA, VEGF bioactivity was greatly improved under the same UV radiation intensity. This observation supports the proposed mechanism that BSA acts as a bulking agent and thus becomes a readily available target for radiation and free radical damage. When VEGF was co-lyophilized with BSA, the two proteins were suspended in a succinate buffer at pH 5.0. Given that the concentration of BSA was much higher than that of VEGF and that the p*I* values of VEGF and BSA are 8.5 [19] and 4.7 [20], respectively, VEGF can form a complex with BSA molecules by electrostatic interaction. It is possible that VEGF is buried within or surrounded by many BSA molecules in the lyophilized powder. When exposed to high-intensity UV radiation, the presence of BSA could have reduced the possibility of VEGF being directly exposed to radiation thereby preserving VEGF bioactivity.

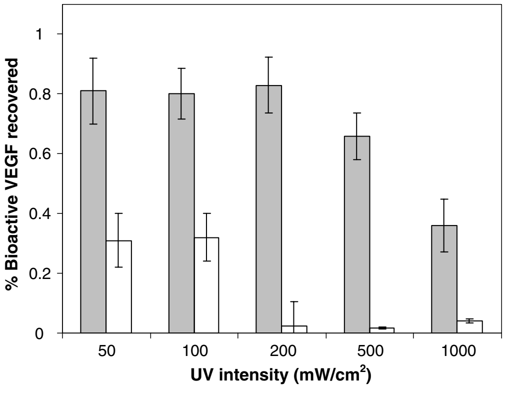

Fig. 3. Effect of UV radiation on VEGF bioactivity. The black and white bars correspond to VEGF lyophilized with BSA and without BSA, respectively.

The effect of UV radiation on VEGF stability in the presence of DMPA is shown in Fig. 4. The lyophilized protein particles were suspended in THF containing different concentrations of DMPA and were then irradiated under UV light at 100 mW/cm^2. After exposure to UV light, a portion of the protein particles was no longer soluble in PBS and the fraction of insoluble protein after irradiation increased as the free radical initiator concentration increased. When exposed to 100 mW/cm^2 of UV light in the presence of 0.003 mg/mL DMPA, the VEGF mass recovery yield was 60% in the absence of BSA. Under the

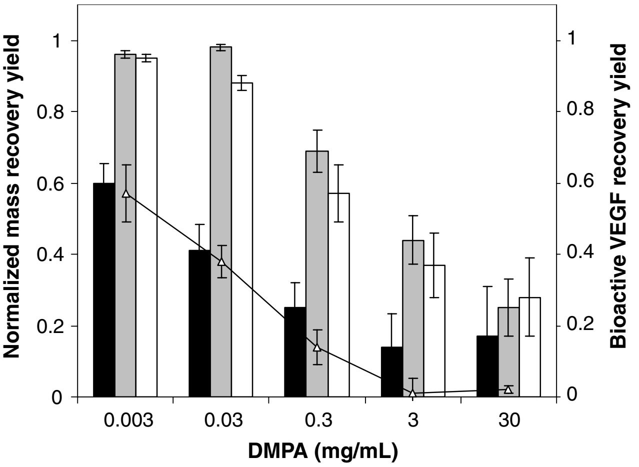

Fig. 4. Effect of DMPA concentration on VEGF and BSA soluble fraction and VEGF bioactivity. The black and gray bars represent the soluble mass fraction of VEGF co-lyophilized with and without BSA after UV radiation. The white bars represent the soluble mass fraction of BSA retained after radiation. The scattered symbols represent the fraction of bioactive VEGF retained after UV radiation. The UV intensity was 100 mW/cm^2.

same irradiation condition, the fraction of soluble VEGF in the presence of BSA was close to 96%. As the DMPA concentration increased to 3 mg/mL, over 80% of the VEGF in the particles without BSA was insoluble after radiation in comparison to 23% when BSA was present in the particles. When the DMPA concentration increased to 30 mg/mL, both BSA and VEGF underwent extensive cross-linking, and the mass recovery yields of both proteins were less than 30%. In comparison to the fraction of soluble VEGF, the VEGF bioactivities from these samples were much lower. For the case of 0.003 mg/mL DMPA in the irradiated suspension, although the fraction of soluble VEGF was over 90%, less than 60% of the recovered VEGF was bioactive. As the DMPA concentration increased, the bioactive VEGF recovery yield decreased linearly. When the DMPA concentration was over 0.3 mg/mL, there was virtually no bioactive VEGF present in the recovered sample. These results show that, although BSA could protect the VEGF during irradiation with UV alone, in the presence of the photoinitiator, BSA could not afford the same level of protection. Thus, protection by the bulking effect does not play a dominant role in the maintenance of VEGF stability during device preparation.

Previously, it has been demonstrated that using 0.03 mg/mL of DMPA at a UV intensity of 100 mW/cm^2 was sufficient to cross-link acrylated macromer within 5 min [12]. Using the radiation data as illustrated in Fig. 4, the photo-cross-linking condition could potentially denature over 60% of VEGF during device fabrication. To examine the possible influence of the presence of the polymer alone on protecting bioactive protein, VEGF particles were mixed with 1-octanol initiated D,L-lactide and ε-caprolactone equimolar copolymers at a molecular weight ranging from 1 to 12 kDa, then exposed to 100 mW/cm^2 UV light with 0.3 mg/mL of DMPA in THF. The effect of polymer molecular weight on BSA and VEGF soluble fraction and on VEGF bioactivity is shown in Fig. 5. The presence of polymer during UV irradiation increased the soluble fraction of both BSA and VEGF BSA on average by 10%. Polymer molecular weight had only a slight influence, as the fraction of soluble BSA and VEGF recovered increased slightly on average as the polymer molecular weight increased. For example, in the absence of polymer, 60 ± 5% of the VEGF was soluble, in the presence of the 4 kDa polymer, 73 ± 8% was soluble, and in the presence of 12 kDa polymer, the recoverable fraction increased to 90 ± 12%. This effect of molecular weight is attributed to the increased viscosity of the solution, which slows the transport of the reactive radicals, as polymer molecular weight increases. The presence of 1 kDa polymer also helped preserve VEGF bioactivity. Over 50% of VEGF in the presence of 1 kDa polymer was bioactive after irradiation in comparison to 14% bioactivity without polymer. However, increasing the polymer molecular weight had no effect on VEGF bioactivity during radiation. As the VEGF released from the elastomer device prepared previously had a relative bioactivity of greater than 80%, it does not appear that the presence of the unacrylated polymer plays the dominant role in protecting VEGF during photo-cross-linking.

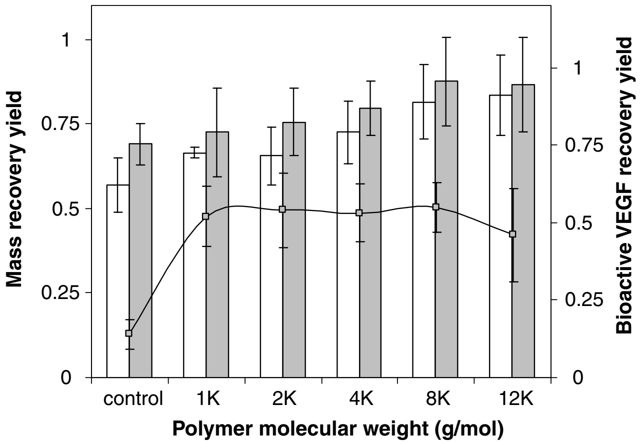

Fig. 5. Effect of polymer molecular weight on VEGF bioactivity during UV radiation in the presence of free radical initiator, DMPA. The concentration of DMPA used in each condition was 0.3 mg/mL. The gray and white bars represent the mass fraction of VEGF and BSA retained after UV radiation. The scattered symbols represent the fraction of bioactive VEGF retained after UV radiation. The controls showed the effect of DMPA and UV radiation on protein mass and bioactivity recovery yields in the absence of polymer.

To characterize the influence of the acrylate functional group on BSA stability, linear polymers of different molecular weight were end-capped with acrylate functional group to generate macromers. Unifunctional linear polymers were used to prevent the formation of a polymer network. As the network would be insoluble, it would not be possible to extract the VEGF and determine its relative bioactivity. The lyophilized protein particles were thus resuspended into a THF solution containing different molecular weight linear unifunctional macromers. DMPA concentrations added to the macromer suspension were normalized to the molar concentration of macromer acrylate functional group. As shown in Fig. 6, the presence of acrylate functional group preserved the VEGF bioactivity during DMPA initiated UV radiation in a dose-dependent manner. When DMPA outnumbered acrylate by a factor of 10,000 ($\text{moles}_{\text{acrylate}}:\text{moles}_{\text{DMPA}} = 1/10{,}000$), the presence of different molecular weight acrylated macromers showed no protective effect on VEGF, as less than 5% of the VEGF retained bioactivity following UV radiation. By reducing the ratio of DMPA:acrylate concentration, VEGF particles mixed with high molecular-weight acrylate macromer had a higher relative bioactivity after reaction than those with lower molecular-weight acrylate macromer. For the 1 kDa acrylated macromer, bioactive VEGF recovery yield was under 30% when the molar ratios of acrylate to DMPA were between 0.001 and 0.01. Under the same ratios, the yield of bioactive VEGF from the 12 kDa acrylate macromer solutions increased linearly to over 70%. By increasing the molar ratios of acrylate and DMPA to 0.1, the VEGF bioactivity yield was increased to 85%, which was similar to the bioactiv-

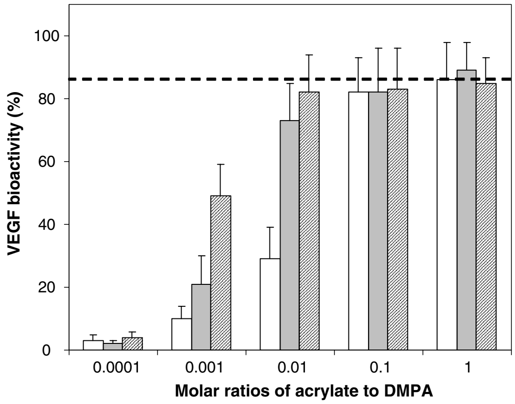

Fig. 6. Effect of acrylated macromer on VEGF stability. The molecular weights of the acrylated macromers were 1 kDa (white bars), 4 kDa (gray bars), and 12 kDa (shaded bars). The dotted line represents the control which is the percentage of bioactive VEGF after lyophilization but without UV radiation. The UV intensity was 100 mW/cm^2.

ity of the lyophilized VEGF that had not undergone radiation.

The likely mechanism by which the acrylate functional group protects VEGF is that it reacts rapidly with the free radicals formed during the photo-cross-linking reaction. Previous studies have shown that the free radicals generated by DMPA react readily with the acrylate functional group, typically in the range of milliseconds [21,22]. Fig. 6 shows that at a low molar ratio of acrylate and DMPA (moles$_{\text{acrylate}}$:moles$_{\text{DMPA}}$ < 0.01), increasing the molecular weight of the acrylated macromer effectively increased VEGF stability. This may be explained by the synergistic effect of acrylate functional group and the polymer viscosity. The polymer viscosity during cross-linking increases as a result of macromer polymerization via acrylated functional termini. In a diluted macromer solution, given the same molar concentration of acrylate functional groups, the polymerization of 12 kDa macromer will yield a higher solution viscosity than that of 1 or 4 kDa macromer. The increase of polymer viscosity in turn creates a cage effect on the free radicals by restricting their mobility in the reaction solution and thereby reducing the attacks on the protein particles [23]. Although Fig. 5 shows the polymer molecular weight did not have a major influence on preserving VEGF bioactivity, the combination of macromer polymerization and free radical scavenging ability of acrylate functional groups increased VEGF bioactivity.

It is well known that γ-radiation can cause protein fragmentation and irreversible aggregation. Exposure to γ-radiation in the presence of free radicals such as hydroxyl, oxygen, or peroxy radicals can alter the monodisperse nature of proteins, due to aggregation and/or fragmentation [14,24–27]. To validate our assertion that the primary factor influencing protein stability in the co-lyophilized particle was the rate of reaction of the acrylate groups with the generated free radicals, we examined the molecular-weight distribution of the BSA. BSA was chosen because of the concentrations required for effective analysis using gel permeation chromatography. We found that the molecular-weight distribution of BSA was significantly altered after UV irradiation in the presence of DMPA (Fig. 7). In comparison to the control (freshly reconstituted BSA) which has a very narrow size distribution, BSA after the UV radiation initiated free radical generation exhibited large aggregates as well as small fragments. Thus, UV irradiation in

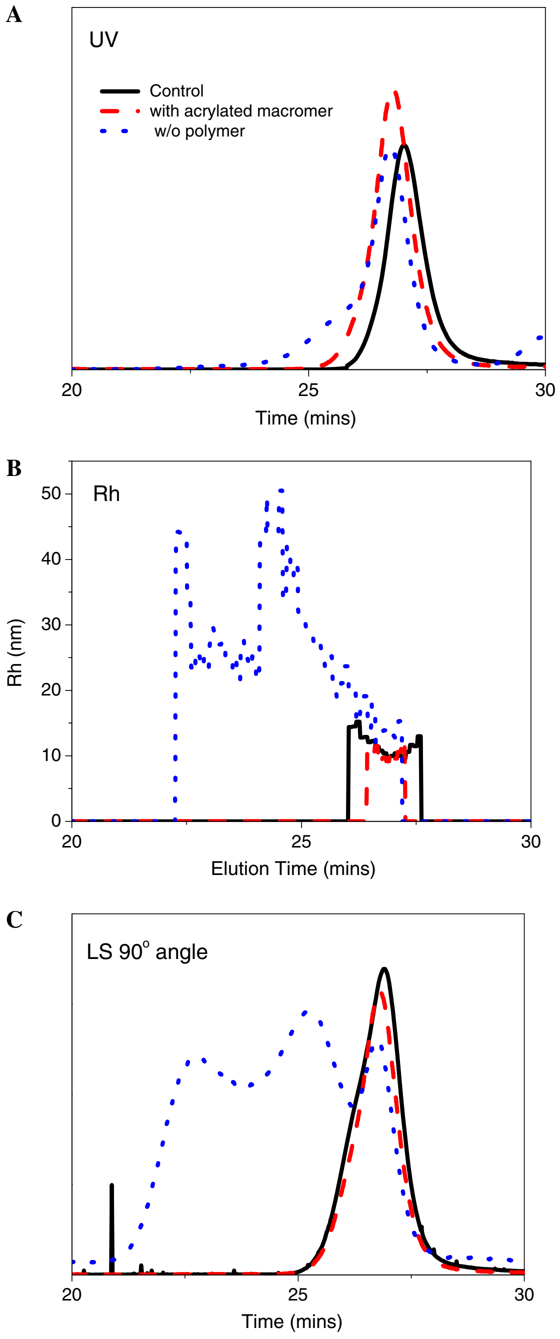

Fig. 7. Effect of acrylated 4 kDa prepolymer during photopolymerization on BSA stability. The solid line represents the elution profile of freshly reconstituted BSA. Effects of free radical UV radiation on BSA in the presence (dashed lines) or absence (dotted lines) of polymer are characterized using GPC and the signals are recorded from a UV detector (A), a hydrodynamic radius detector (B), and a light scattering detector at an angle of 90° to the incident beam (C).

the presence of DMPA has similar effects on proteins compared with literature data on gamma radiation with conventional radicals. However, BSA was monodisperse following photo-cross-linking reaction in the presence of acrylated 4 kDa macromer, and its molecular-weight distribution was very similar to that of freshly reconstituted BSA. These results indicated that BSA particles suspended in the acrylated macromer solution were stable and did not undergo fragmentation and aggregation during photo-cross-linking. If this is the case for BSA, which is present in higher concentration in the lyophilized particles, it is likely the case for VEGF as well.

Although BSA can preserve VEGF stability during lyophilization and the photo-cross-linking reaction, BSA would not be used in practice. A logical replacement is human serum albumin. However, the use of human serum albumin in practice has a significant challenge. Serum albumin is a blood-derived product and as such would necessarily need to be highly purified to eliminate the risk of blood-borne pathogens. Future studies on finding an alternative lyo- and reaction protectant are necessary for this protein delivery approach to be used clinically.

4. Conclusion

The results from this work demonstrate that exposure of lyophilized therapeutic protein particles to UV irradiation can denature the protein. This radiation stress was minimized by co-lyophilizing the therapeutic protein with BSA. The use of BSA, however, did not protect the therapeutic protein in the presence of free radicals such that VEGF and BSA were no longer soluble in PBS after UV radiation. The free radical attacks on proteins can be greatly reduced in the presence of acrylated macromer. Thus, photo-cross-linking a macromonomer in organic solvent in the presence of solid protein particles not only allows polymerization to proceed rapidly at ambient temperature, but also preserves protein stability during the cross-linking reaction.

Acknowledgements

This work was funded through grants awarded by the Canadian Institutes for Health Research and the Natural Sciences and Engineering Research Council of Canada. The assistance of Darryl Knight in performing the gel permeation chromatography of the proteins is gratefully acknowledged.

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