Article

Journal of
Nanoscience and Nanotechnology
Vol. 18, 5292–5298, 2018
www.aspbs.com/jnn

Fabrication of Calixarene Based Protein Scaffold by Electrospin Coating for Tissue Engineering

Esra Maltas Cagil[1, 2, *], Fatih Ozcan[3, 4], and Seref Ertul[3]

[1] *Faculty of Pharmacy, Selcuk University, 42075, Konya, Turkey*
[2] *International Centre of Excellence in Pharmacy, Selcuk University, 42075, Konya, Turkey*
[3] *Department of Chemistry, Selcuk University, 42075, Konya, Turkey*
[4] *Advanced Technology Research and Application Center, 42075, Konya, Turkey*

In this study, calixarene was synthesized by using different functional groups as *p-tert*-butyl-Calix[4]arene ester and amides. Calixarene nanofibers were produced by electrospin coating. Protein immobilization onto the calixarene nanofibers was carried out with human serum albumin (HSA). The maximum amount of binding on produced three different calixarene nanofibers (DE, 2-AMP and 3-AMP) was compared by using a fluorescence technique for protein analysis. Result showed that maximum binding amount was found to be as 177.85 mg cm^{-2} for 3-AMP surface. The protein binding was also characterized by using SEM, TEM, AFM and FT-IR. From obtained results, calixarene-albumin nanofiber was also fabricated by spin coating using 3-AMP which has ability max binding of protein.

Keywords: Nanofiber, Albumin, Protein, Calixarene, Electrospin Coating, Fluorescence, Nanotechnology, Biomedical, Scaffold, Tissue Engineering, Pharmaceuticals.

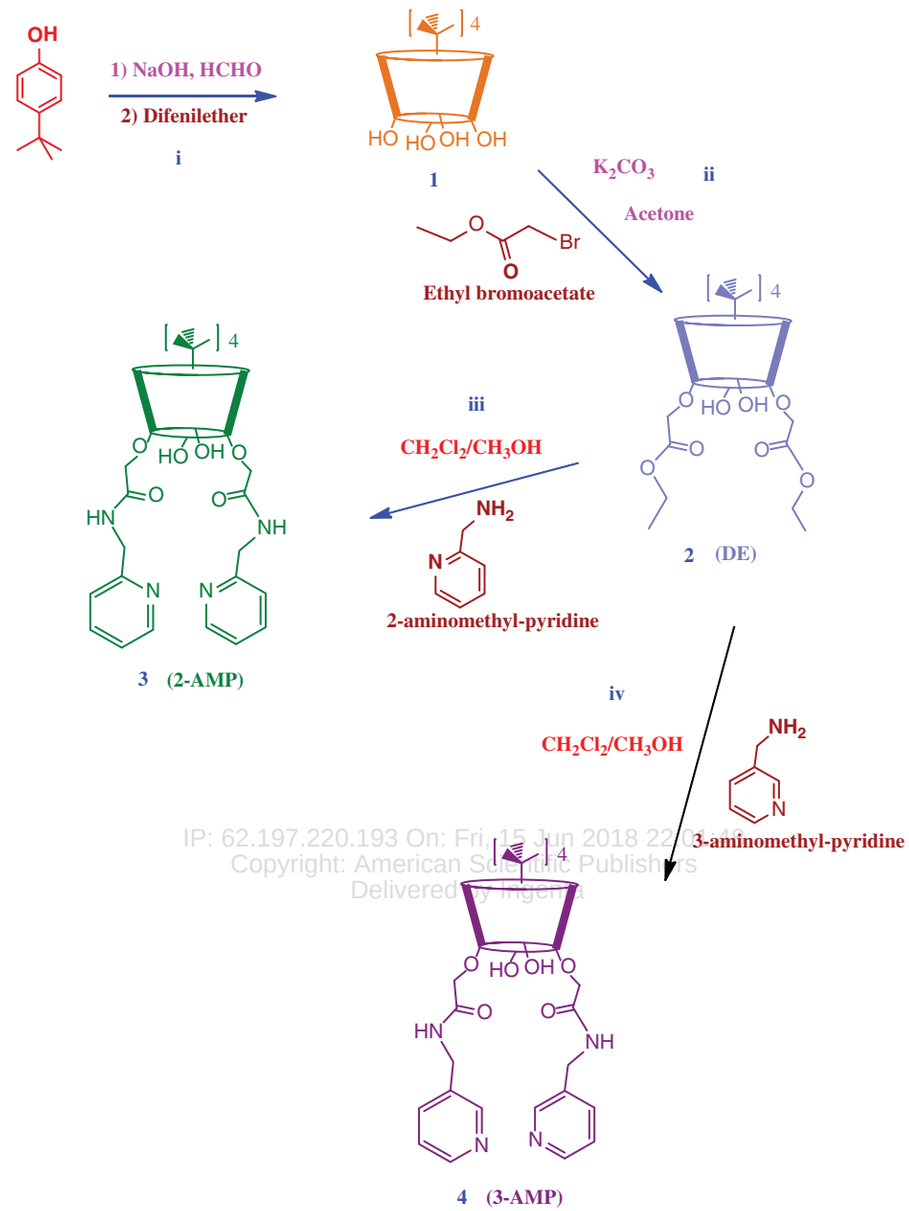
1. INTRODUCTION

Nanotechnology is a growing science that is extensively used for synthesis of inorganic and organic materials, have unique physical, chemical and biological functions.[1, 2] The materials produced at the nanoscale level have attracted increasing attention in the fields such as drug delivery, protein affinity, diagnostic, biomedical imaging and engineering by development of nanotechnology.[3–8] A large amount of the nanomaterials based on polymers, chitosan, graphene, gold and magnetic nanoparticles had been investigated due to their unique properties such as good biocompatibility, a large specific surface area and pore volume, controllable particle size. Another advantage of nanoparticles which is pore size ranging from 2 nm to 50 nm are excellent candidates for biological applications.[9–12] Nanoparticles based nanoscience and biotechnology have shown great promise especially in biomedical applications such as drug/gene delivery, biosensing, bioimaging and photothermal therapy.[13–15] Nanocarriers have been designed as platforms to integrate therapy and diagnostics, which are emerging directions in biomedical.[16, 17]

Recently, nanoparticles (NPs) systems based on proteins including hemoglobin, globulin, lysozyme, gelatin, collagen, casein, soy and whey protein have been studied for encapsulation duet to their enhanced properties of bioavailability, absorbability and low toxicity.[18] Protein-nanoparticles systems have been extensively investigated *in vitro* and *in vivo* drug delivery and encapsulation of bioactive compounds because of its capability to form self-assembled NPs and more importantly, its capability for sustained drug release.[19, 20] Due to the binding affinity to neutral polymers and proteins via hydrogen bonding and hydrophobic interactions, metal ions for immobilized metal affinity, protein nanoparticles systems were utilized to form robust nanocarrier film or platform.[21, 25] Some limitation of the proteins such as low stability and high cost synthesis have limited widespread applications of the proteins. Therefore, immobilization protein onto ideal substrate materials with desirable properties such as available particles size, surface area and functionalization with different group, is necessary. There are different strategies for protein immobilization, such as cross-linking, covalent attachment, adsorption on solid supports or entrapment in silica/polymer matrices.[26]

*Author to whom correspondence should be addressed.

 1533-4880/2018/18/5292/007 doi:10.1166/jnn.2018.15381



Scheme 1. Schematic illustration of the synthesis of *p*-tert-butyl-calixarene derivatives. (i) Formaldehyde, NaOH, diphenyl ether, (ii) bromoethylacetate, Acetone, reflux, 48 h, (iii) 2-(aminomethyl)pyridine, CH_2Cl_2/methanol (1:1) at room temperature, (iv) 3-(omethyl)pyridine.

Calixarenes are widely used in supramolecular chemistry. Due to having a cyclic structure, the calixarenes which can be functionalized easily with polar and apolar groups are good carrier for cations, anions and neutral molecules. The calixarenes can be used in many biological applications because of the easy-functionalized and having large surface area.[27–29] In recent years, the usage of the nanofibers fabricated by electrospun method is getting increased.[30] In literature, polymers having large molecular weight are available to produce as nanofibers.[31] The calixarene which is also one of the key members of chemistry has recently begun to combine with polymers in fabrication of the nanofibers. In the report of Chen et al., polyacrylonitrile (PAN) was prepared as 670 nm dia fiber via electrospun method by using different derivatives of *p-tert*-butyl-calix[8]arene. These *p-tert*-butyl-calix[8]arene derivatives were immobilized on PAN. Then, resulted fibers were used in absorption of congo red.[31] A study of polyacrylonitrile polymers (PAN) by pulling the nanofibers was on electrospinning method. In another study, C-Methylcalix[4] resorcinarene was immobilized onto the PAN nanofibers with 10–40 nm of diameter by using dipping method.[32] Derivatives of different *p*-tert-butyl-calix[4]arene has also been functionalized by using PAN additive nanofibers for removal of toxic anions.[33]

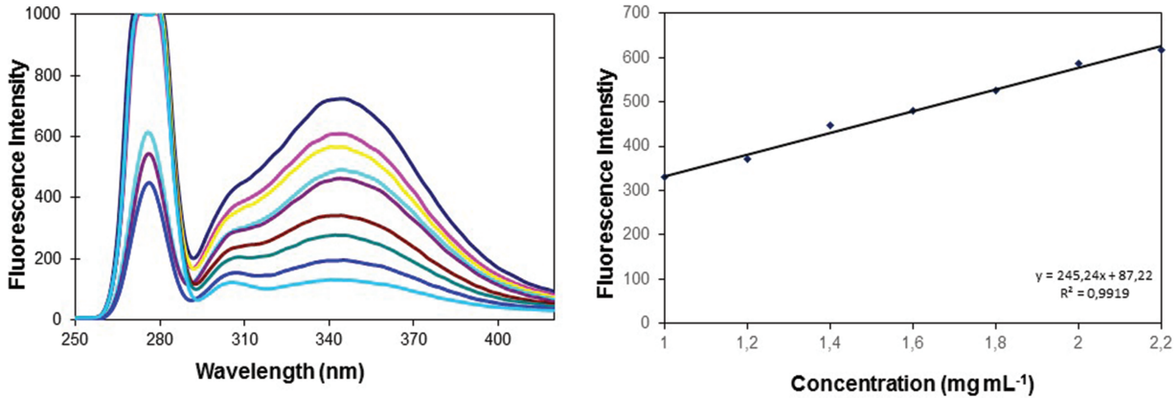


Figure 1. Fluorescence spectra of human serum albumin at 280 nm of excitation wavelength.

In this paper, it is the first time that calixarene-albumin nanofibers have been prepared. The new nanofiber systems were developed by electrospin coating with three different calixarene to evaluate protein binding. Human serum albumin (HSA) was used as a model protein to examine the binding characteristics of calixarene nanofibers for tissue engineering.

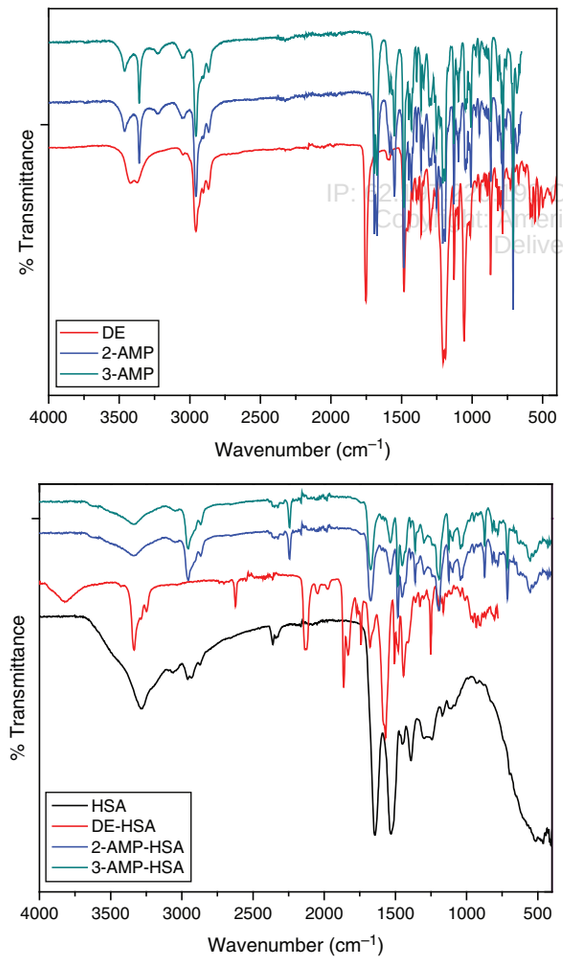


Figure 2. FTIR spectra of nanofibers and protein immobilized nanofibers DE, 2-AMP and 3-AMP; HSA, DE-HSA, 2-AMP-HSA and 3-AMP-HSA.

2. EXPERIMENTAL DETAILS

All of the reagents used in this study were obtained from Merck (Germany) or Sigma Aldrich (USA) and used without further purification. CH_2Cl_2 was distilled from $CaCl_2$/MeOH over Mg and stored over molecular sieves. Anions were used as their sodium salts. Thin layer chromatography (TLC) was performed using silica gel on glass TLC plates (silica gel H, type 60, Merck). Solvents were dried by storing them over molecular sieves (Aldrich; 4_A, 8e12 mesh). All aqueous solutions were prepared with deionized water that was passed through a Millipore Milli-Q Plus water purification system. Melting points of the molecules were determined on a Gallenkamp apparatus. ^1H and ^{13}C NMR spectra were obtained using a Varian 400 MHz spectrometer operating at 400 MHz. The prepared nanofiber mats were characterized by using a Bruker Vartex 70 ATR-FTIR, instrument in the range 4000–400 cm^{-1}. A Zeiss EVO LS10 model scanning electron microscope was used to take SEM images. EDX analysis was done with 123 eV Bruker detector on Zeiss EVO LS10 electron microscope. Inovenso basic system electrospinner was used for nanofiber production. Fluorescence measurement was done with using Perkin Elmer LS-55 Fluorescence spectrophotometer.

The calixarenes such as 5,11,17,23-Tetra-tert-butyl-25,26,27,28-tetrahydroxycalix[4]arene **1**,5,11,17,23-Tetra-tert-butyl-25,27-bis methoxycarbonylmethoxy) 26,28 dihydroxycalix[4]arene **2**(**DE**), 5,11,17,23-Tetra-tert-butyl-25,27-bis(2 aminomethylpyridineamido)-26,28 dihydroxy calix[4]arene **3**(**2-AMP**) 5,11,17,23-Tetra-tert-butyl-25,27-bis(3-aminomethylpyridineamido)-26,28 dihydroxycalix[4] arene **4**(**3-AMP**), (Scheme 1) were synthesized according to the reported literatures Scheme 1.[34–36]

In this study, calixester **DE** and amides **2-AMP** and **3-AMP** nanofibers were produced by electrospinning of

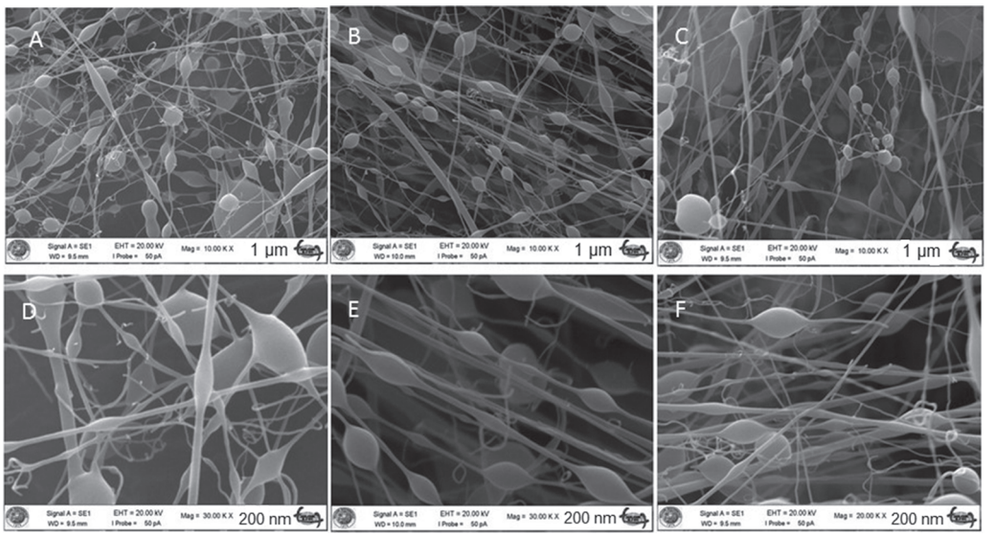


Figure 3. SEM images of nanofibers (a) DE (b) 2-AMP, (c) 3-AMP at 1 μm and 200 μm.

100–120% (w/v) solution in DMF. The electrospinning process was conducted under a fixed electrical voltage of 22–26 kV, while the distance between the needle tip and the collector was kept as 16 cm. Flow rate was set at 5.5–8.5 mL/h, and the process was performed continuously. The materials were collected on an aluminum foil-coated square plate collector as nanofiber mats.

1 mg mL^{-1} of human serum albumin in 20 mM× Tris, pH 7.4 was mixed for 2 h at 4 °C at a surface area of cm^{-2} of all calixarene nanofibers.[23] Solution including unbounded protein were separated by pipet from the piece of nanofiber. The pieces of the nanofiber were washed three times with 20 mM× Tris, pH 7.4. The nanofibers were washed with the Tris buffer for chemical

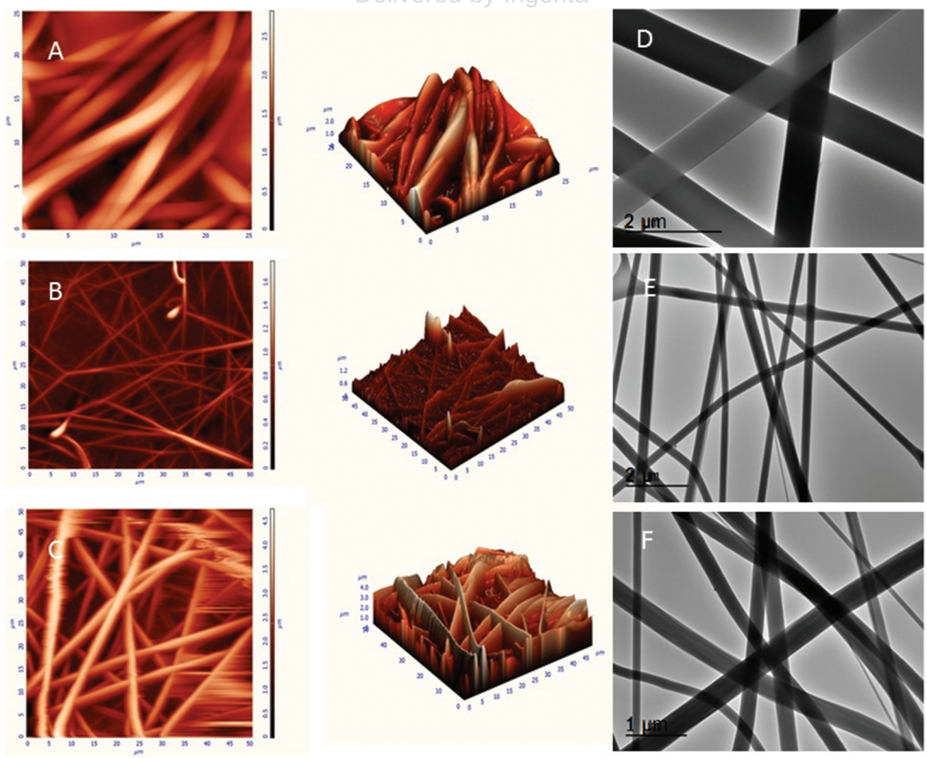


Figure 4. AFM images of nanofibers (A) DE (B) 2-AMP, (C) 3-AMP and TEM images of nanofibers (D) DE (E) 2-AMP, (F) 3-AMP.

characterization. Solution was used to determine the amount of bounded protein via calibration curve drawn at 280 and 342 nm of excitation and emission wavelengths by fluorescence spectroscopy. The regression equation of calibration curve of albumin in 20 mM× Tris, pH 7.4 was found to be as $y = 154.4x + 27.513$, $r = 0.99$.[23]

3. RESULTS AND DISCUSSION

In this study, three different types of calixarene nanofibers abbreviated as DE, 2-AMP and 3-AMP were synthesized as shown Scheme 1. The calixarenes were synthesized with different functional groups as *p-tert*-butyl-Calix[4]arene ester and amides. Calixarene nanofibers were produced by electrospin coating. Then, the effect of functional group was studied for evaluation of protein binding.

The surface characteristics such as physical and chemical properties would be effective on binding of biological molecules like albumin. For this aim, albumin from human serum was immobilized on these three calixarene based nanofibers. The binding amount of albumin was measured by using Fluorescence Spectroscopy. Albumin has an emission spectra at 280 nm and 342 nm of an excitation and emission wavelengths (Fig. 1). After protein was bounded onto the nanofibers, the remaining solution was analyzed at these wavelengths. According to the results, binding amount of albumin was found to be as 91.45 mg cm^{-2} for DE, 148.55 mg cm^{-2} surface for 2-AMP and 177.85 mg cm^{-2} for 3-AMP. This showed that amide bond is more effective for protein binding when compared with DE and other two calixarene nanofibers including amide bonds. In addition to amide bond, pyridine ring also contributes to the interaction of protein with nanofibers. But, when 2-AMP and 3-AMP nanofibers were compared, 3-AMP exhibited higher affinity than 2-AMP due to nitrogen in the ring more closer to surface, led to increase in amount of protein binding to the nanofiber surface. This interaction may also be non-covalent interaction including hydrogen bond, Van der Waals and hydrophobic interaction.[24, 37, 38]

Expected binding mechanism of calixarene nanofibers to albumin was given at Scheme 1.

Table I. Atom weights of elements in the nanofibers related to the EDX results.

Nanofiber	Carbon (%)	Nitrogen (%)	Oxygen (%)	Sulfur (%)
DE	48.1	28.5	23.3	–
DE-HSA	58.2	22.8	17.4	1.4
2-AMP	45.0	30.2	24.8	
2-AMP-HSA	49.6	26.3	22.9	1.1
3-AMP	45.4	24.6	29.9	
3-AMP-HSA	55.1	16.5	21.7	10.8

Figure 2 shows that the ATR FT-IR spectra of the particles DE, 2-AMP and 2-AMP. The aliphatic C–H stretching of CH, CH_2 and CH_3 groups from DE were observed at 2956–2867 cm^{-1}. The bands at 3429 cm^{-1} and 1750 cm^{-1} belong to stretching vibrations of –OH groups and ester, respectively. The bands of aliphatic C–H stretching of CH, CH_2 and CH_3 groups observed at 2956–2867 cm^{-1} and the bands belonged to the amide bond at 1676 cm^{-1} were overlapped in the spectra of DE, 2-AMP and 3-AMP nanofibers.

Figure 2 also shows the ATR FT-IR spectra of the particles HSA, DE-HSA, 2-AMP-HSA and 2-AMP-HSA. In order to characterize binding of albumin on calixarene nanofibers, starting materials were compared with albumin bounded ones. New peaks at around 2250 cm^{-1} and 580 cm^{-1} related to amide bond were observed in the spectra of the three nanofibers. Also, the new band at around 1800 cm^{-1} indicated that albumin bounded to 2-AMP and 3-AMP. However, a large amount of peaks shifted and increased in transmittance which is indicated the binding of protein to all starting materials.[39, 40]

Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM) verified the structure of produced calixarene nanofibers (Figs. 3 and 4(A–F)). According to the data, nanofiber diameters of DE, 2-AMP and 3-AMP were measured as 75–100 nm, 250–350 nm and 180–250 nm, respectively. Scanning Electron Microscopy (SEM) allowed the verification of the morphological differences between nanofibers which were DE, 2-AMP and 3-AMP and nanofibers-protein. As viewed from Figures 5(A)–(C), after protein immobilization on the nanofibers, the morphology greatly changed. Protein binding results in big differences on the view of each

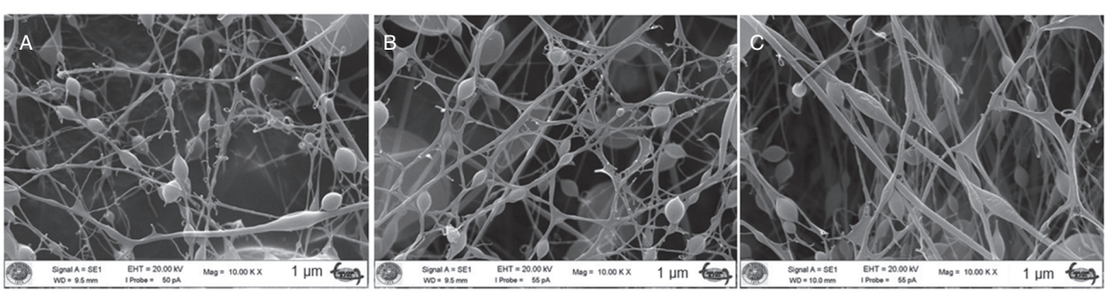


Figure 5. SEM images of nanofibers (A) DE-HSA (B) 2-AMP-HSA, (C) 3-AMP-HSA at 1 μm.

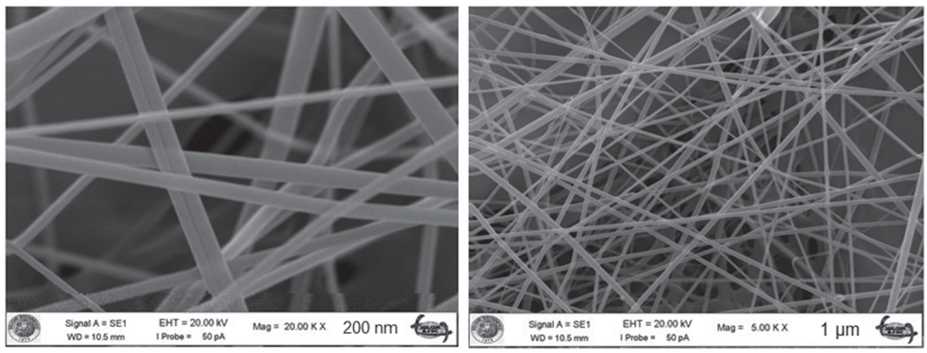


Figure 6. SEM graphs of albumin-nanofibers with 3-AMP.

calixarene nanofibers when compared with bare ones. Reticulation structures on linear calixarene nanofibers appeared on the surface due to binding of the proteins (Figs. 5(A–C)).[23, 24]

EDX analysis was obtained from SEM images of the nanofibers. EDX results related to the atom weights (%) of the each elements in the nanofibers were shown at Table I. According to the results, protein bounded calixarene nanofibers have sulfur elements which introduced to the nanofiber structure due to sulfur elements in the structure of cysteine and methionine residues in the protein. In addition, the differences in ratio of the carbon, oxygen, nitrogen and sulfur indicated that the binding amount of albumin to calixarene nanofibers.[24]

As a result, it is the first time albumin nanofiber prepared by electrospinning method to form a stable shape in order to use synthetic organ or tissue. According to the obtained results from the study, 3-AMP was selected to produce albumin nanofiber. The calixarene and albumin were spattered together to electrospin coating system in order to prevent time consuming instead of protein binding to nanofiber. The produced nanofiber was viewed on Scanning Electron Microscopy to confirm protein binding. Surprisingly, new structure of the albumin nanofiber was completely different than the prepared three calixarene based albumin nanofibers. This new structure obviously was more symmetrical as linear at 1 μm and 200 nm of scale (Figs. 6(A–B)). This is clearly an opportunity for biomedical research and application, especially in tissue engineering.

4. CONCLUSION

Nanofiber has recently focused on the synthesis and characterization by using calixarenes with different functional groups. This functionalization is very effective on biological applications. When considered about protein binding, interaction of protein with functionalized nanofiber is more effective via bioavailable ligands or groups. Developing a new albumin-calixarene nanofiber system with stable structure was useful for biological applications. This is the first time that we developed an available calixarene-protein nanofiber system with smooth shape and surface to evaluate in biomedical applications such as tissue engineering.

Acknowledgment: We would like to thank The Research Foundation of Selcuk University (BAP) for financial support of this work.

References and Notes

1. S. Kazemi-Bagsangani, M. Jamili, and S. Zavareh, *Inter. J. Pharm.* 497, 228 (**2016**).
2. F. Song, X. Li, Q. Wang, L. Liao, and C. Zhang, *J. Biomed. Nanotechnol.* 11, 40 (**2015**).
3. J. Shi, A. R. Votruba, O. C. Farokhzad, and R. Langer, *Nano. Lett.* 10, 3223 (**2010**).
4. S. Lanone and J. Boczkowski, *Curr. Mol. Med.* 6, 651 (**2006**).
5. S. K. Sahoo and V. Labhasetwar, *Drug. Discov. Today* 8, 1112 (**2003**).
6. O. C. Farokhzad and R. Langer, *Adv. Drug Deliv. Rev.* 58, 1156 (**2006**).
7. W. J. Mulder, G. J. Strijkers, G. A. Van Tilborg, D. P. Cormode, Z. A. Fayad, and K. Nicolay, *Acc. Chem. Res.* 42, 904 (**2009**).
8. S. D. Caruthers, S. A. Wickline, and G. M. Lanza, *Curr. Opin. Biotechnol.* 18, 26 (**2007**).
9. Y. Wang, Q. Zhao, Y. Hu, L. Sun, L. Bai, T. Jiang, and S. Wang, *Int. J. Nanomed.* 8, 4015 (**2013**).
10. K. K. Karthikeyan and P. Biji, *Micropor. Mesopor. Mater.* 224, 372 (**2016**).
11. I. I. Slowing, B. G. Trewyn, S. Giri, and V. Y. Lin, *Adv. Funct. Mater.* 17, 1225 (**2007**).
12. Y. Wang, Q. Zhao, N. Han, L. Bai, J. Li, J. Liu, E. Che, L. Hu, Q. Zhang, T. Jiang, and S. Wang, *Nanomed. Nanotech. Biol. Med.* 11, 313 (**2015**).
13. C. Liu, P. Zhang, X. Zhai, F. Tian, W. Li, J. Yang, Y. Liu, H. Wang, W. Wang, and W. Liu, *Biomater.* 33, 3604 (**2012**).
14. M. Liu, M. Li, S. Sun, B. Li, D. Du, J. Sun, F. Cao, H. Li, F. Jia, and T. Wang, *Biomater.* 35, 369 (**2014**).
15. E. Maltas, S. Malkondu, P. Uyar, and M. Ozmen, *Mater. Sci. Eng. C* 48, 86 (**2015**).
16. M. Li, H. Yu, T. Wang, N. Chang, J. Zhang, D. Du, M. Liu, S. Sun, R. Wang, and H. Tao, *J. Mater. Chem. B* 2, 1619 (**2014**).
17. H. Peng, X. Liu, G. Wang, M. Li, K. M. Bratlie, E. Cochran, and Q. Wang, *J. Mater. Chem. B* 3, 6856 (**2015**).
18. J. Xie, Y. Cao, M. Xia, X. Gao, M. Qin, J. Wei, and W. Wang, *Adv. Healthcare Mater.* 2, 795 (**2013**).
19. H. Liang, Q. Huang, B. Zhou, L. He, L. Lin, Y. An, Y. Li, Y. Chen, and B. Li, *Mater. Chem. B* 3, 3242 (**2015**).
20. R. Paliwal and S. Palakurthi, *J. Contr. Release* 189, 108 (**2014**).

21. I. Erel-Unal and S. A. Sukhishvili, *Macromol.* 41, 3962 (**2008**).
22. A. Shukla, J. C. Fang, S. Puranam, F. R. Jensen, and P. T. Hammond, *Adv. Mater.* 24, 492 (**2012**).
23. E. Maltas, A. N. Kursunlu, G. Arslan, and M. Ozmen, *App. Surf. Sci.* 349, 811 (**2015**).
24. E. Tamyurek, E. Maltas, S. Z. Bas, M. Ozmen, and S. Yildiz, *Int. J. Biol. Macromol.* 73, 76 (**2015**).
25. S. Z. Bas, E. Maltas, B. Sennik, and F. Yilmaz, *Coll. Surf. A: Physicochem. Eng. Aspec.* 444, 40 (**2014**).
26. R. Goyal, L. K. Macri, H. M. Kaplan, and J. Kohn, *J. Contr. Release* 240, 77 (**2016**).
27. A. Ikeda and S. Shinkai, *Chem. Rev.* 97, 1713 (**1997**).
28. C. D. Gutsche, Calixarenes, Revisited, RSC, Cambridge (**1998**), pp. 32–40.
29. C. D. Gutsche, M. Iqbal, and D. Stewart, *J. Org. Chem.* 51, 742 (**1986**).
30. F. Ozcan, S. Ertul, and E. Maltas, *Mat. Lett.* 182, 359 (**2016**).
31. M. Chen, C. Wang, W. Fang, J. Wang, W. Zhang, G. Jin, and G. Diao, *Langmuir* 29, 11858 (**2013**).
32. M. Kaestner and I. W. Rangelow, *Microelec. Eng.* 97, 96 (**2012**).
33. M. Bayrakci, F. Ozcan, and S. Ertul, *Tetrahed.* 71, 3404 (**2015**).
34. C. D. Gutsche and K. C. Nam, *J. Am. Chem. Soc.* 110, 6153 (**1988**).
35. A. Yilmaz, B. Tabakci, E. Akceylan, and M. Yilmaz, *Tetrahed.* 63, 5000 (**2007**).
36. S. Sayin, F. Ozcan, and M. Yilmaz, *Desal.* 262, 99 (**2010**).
37. S. Z. Bas, *Anal. Met.* 6, 7752 (**2014**).
38. S. Z. Baş, H. Gülce, S. Yıldız, and A. Gülce, *Talanta* 87, 189 (**2011**).
39. A. Barth, *Biochim. Biophy. Acta* 1767, 1073 (**2007**).
40. J. Kong and S. Yu, *Acta Biochim. Biophy. Sinica* 39, 549 (**2007**).

Received: 30 August 2017. Accepted: 23 October 2017.