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Cationized bovine serum albumin with pendant RGD groups forms efficient biocoatings for cell adhesion

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Abstract: Cationized bovine serum albumin (cBSA-147) has been modified by attaching the cyclic pentapeptide cRGDfK to its surface through linkers of different length. Coatings of these bioconjugates on glass surfaces were studied for their ability to stimulate cell adhesion. These chemically modified albumins combine a high number of positive charges which facilitate the initial cell adhesion to the surface with multiple Arg-Gly-Asp groups which enable focal adhesion of fibroblast cells by specific interactions with cell-surface receptors. The biocoatings are easily prepared within a few minutes by simple incubation from a dilute solution of the modified albumin. This constitutes a convenient approach for preparing surfaces for cell adhesion. Excellent focal adhesion of NIH 3T3 fibroblast cells on the biocoatings was observed. About 75% of the seeded cells attached to the cRGDfK-cBSA-147 coated surfaces, and 97% of them underwent focal adhesion. Adhering cells were able to grow and proliferate on the coated surfaces, confirming the outstanding biocompatibility of these biocoatings. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 99B: 282–290, 2011.

Key Words: biocoating, cell adhesion, RGD peptide, cBSA-147, protein modification

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INTRODUCTION

Focal adhesion of cells to the extracellular matrix or to neighboring cells is crucial for maintaining tissue integrity, function, migration and proliferation.[1,2] The Arg-Gly-Asp (RGD) sequence is perhaps the most commonly found effective short peptide sequence capable of stimulating cell adhesion to surfaces.[3] This tripeptide sequence interacts with integrins of the cell surface and initiates a complex series of signaling events which lead to remodeling of the cytoskeleton, cell spreading, and stabilization of focal adhesion.[4] The recognition of specific integrins depends on the amino acids flanking the RGD sequence and on the conformation of the peptides.[3,5] Cyclic derivatives of the RGD sequence, for example, cRGDfK, recognize $\alpha v\beta 3/\alpha v\beta 5$ integrins based on the insertion of D-phenylalanine into their cyclic structure.[6,7] Compared with linear RGD sequences, this cyclic peptide shows improved resistance to degradation and represents a potent ligand to interact tightly with the integrin receptor.[3,7,8] Interaction of cRGDfK with integrins is governed by the accessibility of the RGD loop. RGD peptides can act as an integrin receptor antagonist and will inhibit cell adhesion if they are applied in solution.[9] Therefore, efficient immobilization of the peptide represents a key concern in the preparation of cell adhesion stimulating surfaces.

Covalent grafting of these peptides to, for example, polymer surfaces or nanoparticles is an approach commonly used to improve biocompatibility and to promote cell adhesion on synthetic materials.[10–17] The length of the spacer plays an important role to allow the immobilized peptide to access the binding site of the integrin. A detailed review of this topic has been done by Hersel et al.[3] Beer et al. observed that a spacer length of approximately 11–32 Å was sufficient to interact with the majority of the binding sites of integrins, and interaction with all receptors was achieved with spacer lengths of about 46 Å. Longer spacers were reported to yield significantly reduced cell binding.[18] This result was in agreement with the optimum spacer length of 35 Å reported by Kessler's group.[7] It was suggested that optimum spacer lengths are essential for cells to undergo tight binding to surfaces.[19,20] However, Massia and Hubbel[21] demonstrated that excellent cell adhesion is possible in the absence of any spacer between the RGD peptide and the surfaces. The importance of spacer length is still discussed controversially and represents a topic of current research to achieve improved biocoatings for efficient cell adhesion.

Engineered surfaces that promote cell adhesion are attractive for a broad range of applications such as *in vitro* growth of tissues, the preparation of cell-based sensors, and the study of the mechanisms of cancerogenesis.[2,22,23] For engineering of tissue implants, biodegradable polymers like poly(glycolic acid) and poly(L-lactic acid) have been used

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extensively due to their excellent mechanical properties and biological affinity.[24–26] However, the absence of reactive groups on these polymers makes it difficult to impart additional functionalities to them.[27] An interesting approach to functionalize such materials has recently been described by Dong et al.[28] and You et al.[29] They rationalized that the enzymes that are involved in the biosynthesis and metabolism of polyhydroxyalkanoates (PHA) have to be able to bind to these hydrophobic molecules. They, therefore, genetically engineered a chimeric PHA granule binding protein that was fused to the RGD motif and expressed it in an *Escherichia coli* expression system. This material was found to significantly improve the adhesion of chondrocyte like cells on scaffolds made from PHA. The application of more readily functionalizable bio-polymers such as poly-L-lysine was limited due to non-specific cell adhesion. Cytotoxicity and abnormal cell spreading were frequently observed.[30] Grafting of RGD containing peptides onto native bovine serum albumin (nBSA) to obtain a coating material for cell adhesion has been demonstrated previously.[31–33] This approach attracted attention because serum albumin is a biocompatible macromolecule which presents many functional groups on its surface, thereby allowing further chemical modifications.[34,35] However, a coating of RGD-modified nBSA is merely based on physical adsorption. Preparing these coatings requires extended incubation times between 1 and 14 h, but the adhesion to the surface is easily impaired under mechanical stress.[3,36] Improved cell adhesion has been reported for coatings that facilitated non-specific electrostatic interactions with cells caused by the presence of multiple positive charges.[37–39] Recently, Lai et al.[40] introduced poly-lysine trimers as a positively charged linker to cRGDfK, and they observed a strong cooperative effect on the focal adhesion of fibroblast cells due to the presence of both non-specific (ionic) and specific RGD-based interactions. Research by Geiger et al.[41–44] had shown that hyaluronan in the pericellular coat at the cell surface plays an important role in the initial stages of cell adhesion. This material carries negative charges under physiological conditions, and a positively charged surface may therefore be conducive for adhesion.

In this article, we present our efforts toward tailored albumin-biocoatings that promote cell adhesion by long range non-specific electrostatic interactions as well as RGD mediated adhesion. Cationized bovine serum albumin cBSA-147 which carries multiple positive charges represents an ideal carrier for grafting RGD containing peptides because several primary amino groups are accessible for chemical coupling reactions.[45] Previously, cBSA-147 has been studied as a vehicle for cell transfection, and no significant cytotoxicity has been found at the effective concentrations.[35,45] The chemical stability and high charge density of cBSA-147 were also essential in its application as a promoter for the adsorption of a biocatalyst (whole cells of recombinant *E. coli*) onto the inner walls of silica capillary micro-channels.[46] With this immobilized-cell micro-reactor, the synthesis of a chiral alcohol proceeded in continuous flow over several days without cell loss. This result confirmed that incubation with cBSA-147 leads to a strongly adsorbed protein layer at the surface which is able to withstand mechanical stress.

We now prepared cBSA-147 which was decorated with different numbers of the cRGDfK using linkers of varying chain lengths. These cRGDfK-grafted cBSA-147 proteins have been assessed with respect to their cytotoxicity and their ability to promote cell adhesion. Our results pave the way toward tailored biocoatings for demanding medicinal applications such as coating of stents or implants.

MATERIALS AND METHODS

Synthesis of cRGDfK and the isothiocyanate-functionalized linker

cRGDfK was prepared according to a procedure reported previously by Kalinina et al.[47] Detailed experimental procedures, including all intermediates and purification protocols, are provided in the supplementary information. Solid-phase peptide synthesis with a 2-chlorotrityl chloride polystyrene resin and Fmoc-protected amino acids was used together with hydroxybenzotriazole/*o*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate coupling chemistry. A 20% piperidine was used in all cases to deprotect the Fmoc group. Cyclization of the linear RGDfK with diphenylphosphoryl azide/sodium bicarbonate was carried out after cleavage of the peptide from the resin. Diisothiocyanate was used to functionalize the lysine residue of the cyclic peptide after deprotection of the carbobenzyloxy protective group. After functionalization, cRGDfK was deprotected using trifluoroacetic acid (TFA)/triisopropylsilane/H_2O to afford the isothiocyanate-functionalized cRGDfK (**1**). Longer linkers were constructed by incorporating one and two ε-aminohexanoic acid (Ahx) groups between the peptide and the terminal isothiocyanate function. The peptides with these linkers are referred to as Ahx-cRGDfK (**2**) and Ahx-Ahx-cRGDfK (**3**), respectively. The synthesis strategy for compounds (**1**), (**2**), and (**3**) is illustrated in Figure S1 (Supplementary Information). Progress of the reactions was monitored by electrospray ionization mass spectrometry.

Synthesis of cBSA-147

cBSA-147 was prepared via the same procedure as described previously.[35,45] In short, the acid functionalities of the glutamic acid and aspartic acid present in native BSA were randomly converted into amino groups by exhaustive reaction with an excess of ethylenediamine and 1-ethyl-3-(3′-dimethylamino-propyl)-carbodiimide hydrochloride as coupling agent at pH 4.75. The resulting product was washed by repetitive ultrafiltration [Pierce concentrators, 20 kDa molecular weight cut-off (MWCO)], dialyzed against milli-Q water (Slide-A-Lyzer Dialysis Cassette G2, 20 kDa MWCO) and subsequently lyophilized.

Synthesis of cRGDfK-grafted cBSA-147

A 100 μL cBSA-147 in milli-Q water (10 mg/mL) was diluted with 100 mM potassium phosphate buffer at pH 7.4 to a concentration of 1 mg/mL. The respective peptide (40 equi., unless otherwise stated) was dissolved in DMSO to a concentration of 8 mg/mL, and the solution was added

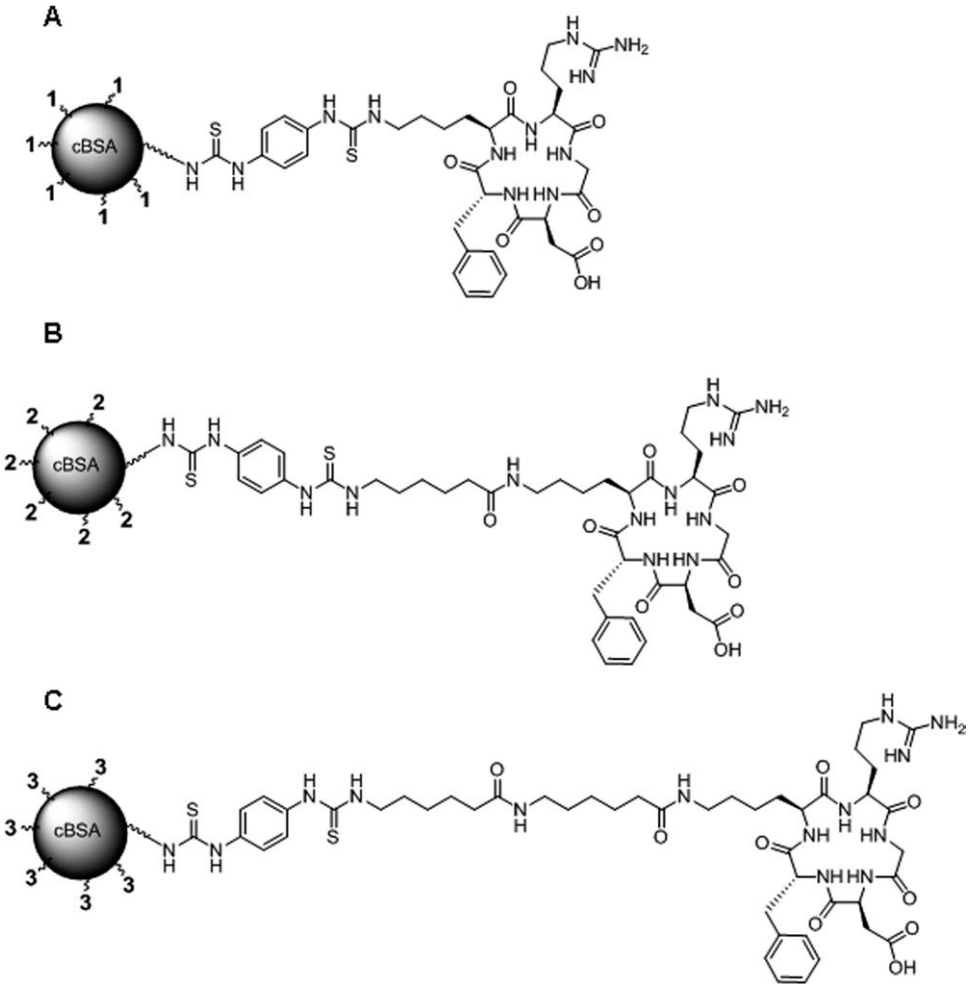

FIGURE 1. Structure of cBSA-147 grafted with cRGDfK using spacers of different length. (a) cBSA-147-cRGDfK (**4**), (b) cBSA-147-Ahx-cRGDfK (**5**), and (c) cBSA-147-Ahx-Ahx-cRGDfK (**6**).

dropwise to the cBSA-147 solution under cooling in an ice bath. The final concentration of DMSO was always kept below 6 vol %. The solution was stirred for another 5 min at ice bath temperature and further stirred at room temperature for 3 h. Subsequently, the solution was concentrated and purified using a Pierce concentrator, 20 kDa MWCO. The concentrated protein solution was further washed 5 times with milli-Q water by centrifugation and the resulting protein solution was lyophilized. Three different cBSA-147 conjugates with cRGDfK substituents of varying spacer lengths (Figure 1) were isolated as white solids: cBSA-147-cRGDfK (**4**, 88%), cBSA-147-Ahx-cRGDfK (**5**, 95%), and cBSA-147-Ahx-Ahx-cRGDfK (**6**, 90%).

Preparation of peptide coated glass surface

Standard microscope slides (Corning, 75 mm × 25 mm) were pretreated with 1 *N* NaOH at 80°C for 30 min and washed with milli-Q water. Subsequently the slides were treated with 1 *N* HCl at room temperature for 15 min and stored in milli-Q water. Prior to use, each slide was sterilized with 70% ethanol and dried in a laminar flow hood. A FlexiPerm Micro12 reusable cell culture chamber (Greiner Bio One GmbH, Germany) was fixed onto the slide to form the culture wells (6.5 mm diameter and 10 mm height). A 20 μL of a 10 μM solution of the cBSA-147-cRGDfK conjugate (**4**), (**5**), or (**6**) was incubated in the well for 10 min before the solution was pipetted off and the well was washed with 3 × 100 μL of PBS.

Cell adhesion study

NIH 3T3 mouse embryonic fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM with 4.5 g/L glucose with L-glutamine) supplemented with 1% penicillin–streptomycin mixture and 10% fetal calf serum at 37°C, 5% CO_2. Confluent cultures were detached using trypsin/ethylenediaminetetraacetic acid. The detached cells were spun down and washed once with serum-free medium before they were used for the adhesion test on the surfaces coated with bioconjugates (**4**), (**5**), or (**6**). Cells in serum-free medium were seeded onto the coated glass slides at a cell density of 1×10^5 cells/mL corresponding to 250 cells/mm^2 (unless otherwise stated) and incubated at 37°C, 5% CO_2 for the stated time. The medium was then removed and the wells were washed with 3 × 100 μL of PBS to remove unattached cells. The FlexiPerm Micro12 was removed but a

hydrophobic film created by the FlexiPerm still remained on the slides thus allowing further fixing and staining. The adhering cells on each spot were fixed for 15 min with 50 μL of 4% freshly prepared paraformaldehyde solution in PBS. The spots were washed with 3 × 50 μL of PBS, stained with 0.5% safranin O solution for 5 min, and further washed with 3 × 50 μL of PBS prior imaging with a microscope (Olympus BX 51). For quantitative evaluation of the cell adhesion, attached cells were counted in 10 areas of 0.04 mm^2 per slide, with three independent replicates for every variable tested. The data given in this article are expressed as mean + one standard deviation.

MALDI-TOF mass spectroscopy

Mass spectra were recorded on a Bruker Autoflex II matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) instrument. Sinapinic acid (saturated sinapinic acid in 0.1% TFA:Acetonitrile 2:1) was applied as sample matrix. The laser power used was 37% at 25 Hz with 30 shots per spectrum.

Atomic force microscopy

Measurements were performed on a VEECO dimension 3100 nanoscope IV using the tapping mode. A tip with a force constant of 40 N/m operated at a resonant frequency of 300 kHz was used.

RESULTS

Preparation of cBSA-147-cRGDfK bioconjugates

cBSA-147 was prepared by conversion of the carboxylic acid groups of accessible glutamic acid and aspartic acid side chains into primary amino groups after condensation reactions with excessive amounts of ethylenediamine (Materials and Methods). Native BSA carries 41 aspartic acid, 59 glutamic acid, and 59 lysine residues.[48] After cationization, the total number of available amino groups on the protein surface corresponds to the sum of 59 lysine residues, 1 amino terminus, and the attached ethylenediamine residues. For cBSA-147, the mass increased from about 66 kDa to 69.65 kDa (Figure S2, Supplementary Information) thus yielding cBSA-147 with an average of about 147 primary amino groups. The isolation and characterization of cBSA-147 have been described previously.[35,45]

The conjugation of peptide (**1**) to the cBSA-147 was carried out at different pH (7.4, 8.5, and 9.5) to assess the influence of the pH on the coupling efficiency. The MALDI-TOF spectra (data not shown) indicate no significant differences in the molecular weights of the products. However, protein precipitation occurred when the synthesis was carried out at higher pH. Therefore, all subsequent reactions were performed at pH 7.4. The number of equivalents of (**1**) was varied, and the resulting molecular weights and molecular weight distributions were determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and MALDI-TOF (Figure 2). The product bands (**4a–d**) on the SDS-PAGE appeared at significantly higher molecular weights compared with cBSA-147. This indicates the successful coupling of (**1**) onto cBSA-147, yielding cBSA-147-cRGDfK (**4a–d**). The MALDI-TOF spectrum further confirmed this observation. The maximum of the signals for (**4a–d**) was shifted to a higher molecular weight compared with the educt cBSA-147. The significant broadening and the asymmetry of the peaks indicate that variable numbers of residues (**1**) were attached to the protein through a random reaction with accessible free amino groups at the cBSA-147 surface. The grafting efficiency is estimated based on a deconvolution of the MALDI-spectrum. From 1 to more than 10 peptide chains were attached. A 50% area of the total signal (in the higher mass range) was examined to determine the average degree of peptide substitution. Table I summarizes the estimated grafting efficiency when varying molar ratios of cyclic peptide to cBSA-147 had been used. Generally, the substitution was higher when more equivalents of (**1**) were used. Increasing the length of the spacer for the cRGDfK-substituent resulted in a somewhat lower grafting efficiency.

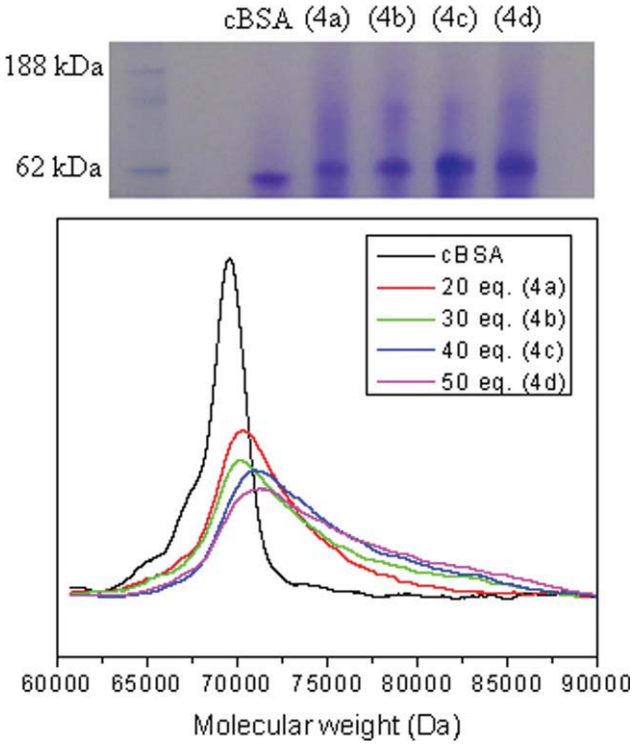

FIGURE 2. MALDI-TOF spectra of cBSA and conjugates (**4a–d**) obtained with increasing equivalents of (**1**). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Similarly, cBSA-147-Ahx-cRGDfK (**5**) and cBSA-147-Ahx-Ahx-cRGDfK (**6**) were achieved by applying 40 equi. of the corresponding peptides (**2**) and (**3**). The maximum in the MALDI-TOF spectrum in Figure 3 shows that in (**5**), molecules carrying two substituents of (**2**) are most abundant, but the width of the peak indicates that 50% of (**5**) contained four or more peptide residues. For (**6**), between one and two peptides were grafted on average to cBSA-147 based on the mass at peak maximum, but 50% of the obtained protein was decorated with two or more substituents of (**3**).

Cell adhesion on glass surfaces with different coatings

cRGDfK is able to recognize αvβ3/ αvβ5 integrins.[6,7] Fibroblasts are known to express αvβ3 on their cell coat[49] and

TABLE I. Grafting Efficiency of cRGDfK to cBSA-147 Yielding (4a–d) with Different Equivalents of (1)

Compound	Equivalents of (**1**)	Molecular Weight (kDa) max. Peak	Number of Grafted (**1**) (50% Peak Area of MALDI-TOF Spectra in Higher Mass Range)
cBSA-147	0 equi.	69.6 (0)	0
4a	20 equi.	70.4 (1)	>1
4b	30 equi.	70.5 (1)	>2
4c	40 equi.	71.3 (1–2)	>4
4d	50 equi.	71.5 (2–3)	>5

The number in brackets represent the estimated number of grafted (**1**) calculated from the MALDI-TOF spectra.

were thus chosen for the cell adhesion experiments. The cell adhesion test was carried out for 1 h of culturing time. Because fibroblasts are able to release fibronectin as an intrinsic adhesion promoter, the efficiency of the coating in promoting the focal adhesion is easier to compare during the initiation of focal complex formation and their maturing into focal contacts.[6] All cell adhesion experiments were carried out under serum-free conditions to avoid stimulation of cell adhesion due to fibronectins from the serum.[50] Typical micrographs and the statistical evaluation of the results are shown in Figures 4 and 5, respectively. Mainly round cells were observed on surfaces coated with nBSA [Figure 4(a)]. When the cationic cBSA-147 was applied, a higher number of cells were attached to the surface most likely due to the presence of the high number of positive charges. However, similar to the observation with nBSA, mainly round cells were found on the coated surface. Cells that showed normal adhesion appear as round spots with a diameter of 20–30 μm, whereas focal adhesion formed elongated cells with diameter in the range of 30–70 μm. For all surfaces coated with (**4c**), (**5**), and (**6**), cell spreading was observed for the majority of the cells, indicating the formation of focal adhesion.

The results of the cell adhesion study on the different biocoatings are depicted in Figure 5. The number of spread fibroblast cells on the surfaces treated with (**4c**), (**5**), and (**6**) was greatly enhanced compared with the control surfaces (blank, nBSA, and cBSA-147). After only 1 h of culturing time, about 75% of the seeded cells were already attached to the surfaces, and 97% of them underwent focal adhesion on the coated surfaces. To further prove that the spreading of the adhering cells on surfaces coated with (**4c**) was mediated via specific interaction with cRGDfK, a control experiment was carried out. The cells were pre-incubated with dissolved cRGDfK before seeding onto a surface coated with (**4c**). As expected, the number of spread cells on surfaces treated with (**4c**) was now greatly reduced. Furthermore, with increasing spacer length of the grafted peptides from (**4c**), (**5**) to (**6**), no decrease in the number of spread cells was observed as shown in Figure 5. Because (**4c**), (**5**), and (**6**) gave similar performance, (**4c**) which was easier to prepare was chosen for all further experiments.

To verify and correlate the relationship between spacer length and cell adhesion, the height of the cRGDfK onto the glass slide was determined using atomic force microscopy (AFM), and the images are shown in Figure 6. nBSA has a overall dimension of (40 × 140) Å.[51] After cationization, the cBSA-147 coated glass surface showed features with average dimension of (50 × 2000) Å. The large value on the lateral axis can be attributed to cluster formation of cBSA-147. The surface coated with (**4c**) displayed higher heterogenicity and agglomeration of the protein was observed. However, the majority of the features had dimensions of (100 × 1500) Å.

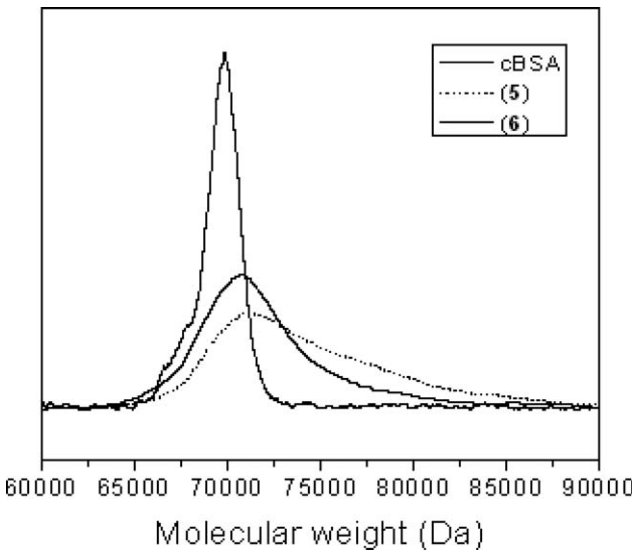

FIGURE 3. MALDI-TOF spectra of cBSA-147and conjugates (**5**) and (**6**).

Optimization of surface coating

Peptide (**1**) was used in different molar ratios from 20 to 50 relative to cBSA-147 to achieve derivatives (**4a–d**) with different numbers of cRGDfK peptides on the surface. The effect of the number of RGD-groups linked to the albumin carrier on the cell adhesion was then investigated and the results are shown in Figure 7. Mostly round cells were observed on the surface coated with (**4a**) prepared from 20 equi. of (**1**). With 20 equi., the density of the peptide on the surface is lower, and fewer cells formed focal adhesions within the culturing period. The results for cBSA-147 functionalized with ≥30 equi. of (**1**) did not differ significantly with respect to the number of adhering cells. Therefore, for the subsequent experiments, (**4b**) prepared from 30 equi. of (**1**) was used.

Different concentrations (2–20 μM) of (**4b**) were used to coat the glass slide for the cell adhesion study. Figure 8 shows the results. With lower concentration of (**4b**), there were more round cells observed due to the lower density of

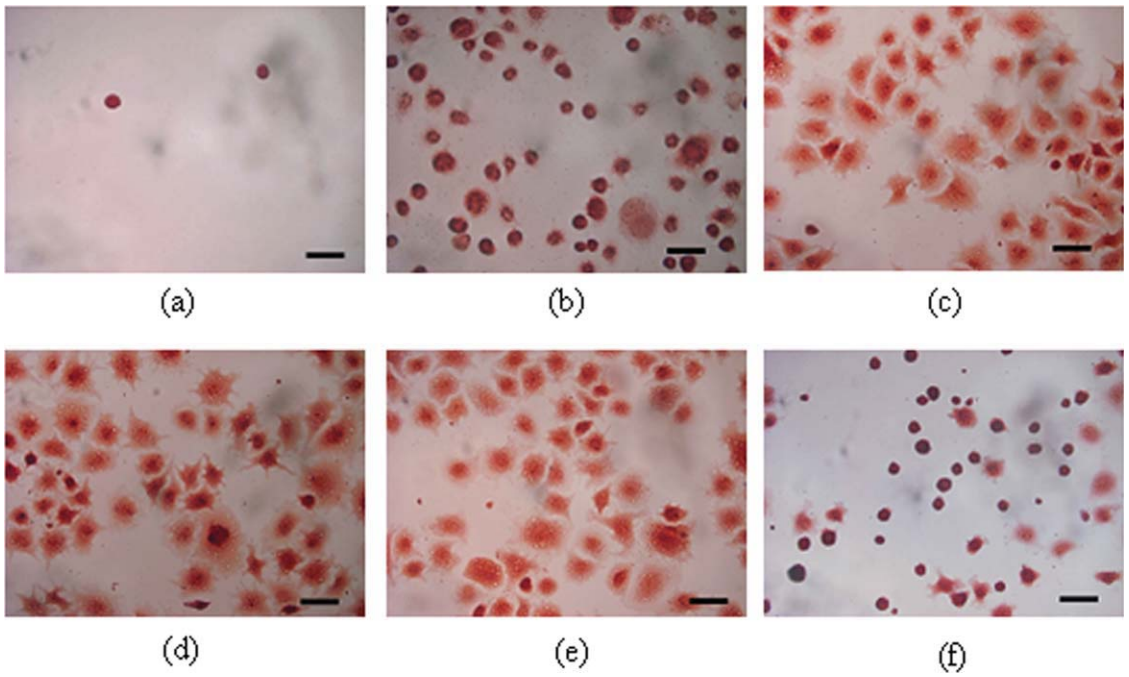

FIGURE 4. Adhesion behavior of NIH 3T3 fibroblast cells on surfaces coated with (a) nBSA, (b) cBSA-147, (c) cBSA-147-cRGDfK (**4c**), (d) cBSA-147-Ahx-cRGDfK (**5**), (e) cBSA-147-Ahx-Ahx-cRGDfK (**6**), and (f) cells exposed to soluble cRGDfK before seeding on cBSA-147-cRGDfK (**4c**); scale bar: 50 μm. Cell seeding density: 575 cells/mm^2 (2.3 × 10^5 cells/mL); 1 h culturing time. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the peptide on the surface. From the experiment, 10 μM was determined as optimum for future experiments.

Proliferation study of NIH 3T3 cells

Figure 9 shows the images of the proliferation of NIH 3T3 cells at different time points. The cells were seeded at low cell density so that the proliferation of the adhered cells can be observed. The cell adhesion test was done under serum-free conditions for the first 4 h. To prove that (**4b**) coated surface can promote initial cell adhesion. It can be seen that the cells spread and formed initial focal adhesion after 1 h incubation time. At 4 h, elongation of cells was clearly seen. To further prove that the adhered cells are capable to proliferate and grow, the samples were taken out from the incubator and unattached cells were washed off with medium. The adhering cells were further cultured in DMEM with 10% fetal bovine serum. The growth and proliferation of adhering cells on surfaces coated with (**4b**) were clearly observed after 24 h and 48 h.

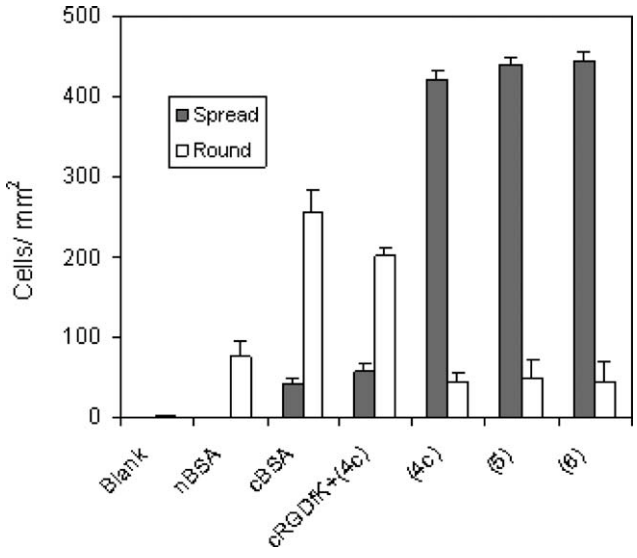

FIGURE 5. Comparison of the number of spread and round NIH 3T3 fibroblast cells on differently pre-treated glass surfaces. Cell seeding density: 575 cells/mm^2 (2.3 × 10^5 cells/mL); 1 h culturing time.

DISCUSSION

In this study, we aimed to develop a biomaterial which combines electrostatic and RGD mediated adhesion to prepare engineered surfaces suitable for cell adhesion in a rapid, simple and mild manner. cBSA-147 was conjugated with the cyclic pentapeptide cRGDfK (f = D-phenylalanine) through linkers of varying chain lengths, to obtain a set of cRGDfK-grafted cBSA-147 proteins (**4**), (**5**), and (**6**). The successful preparation of the bioconjugates was confirmed by SDS-PAGE and MALDI-TOF analysis (Figure 2). The material could be coated on the surface of glass slides by simple incubation within minutes. Cell adhesion tests on these biocoatings showed excellent adhesion of NIH 3T3 fibroblast cells, which was much higher than that of control surfaces (blank, nBSA, and cBSA) as shown in Figure 5. When the cells were pre-incubated with cRGDfK before seeding, the number of spread cells was much reduced (Figure 5). This is because dissolved cRGDfK binds strongly to the integrins and thereby acts as an antagonist for the integrin function. Blocking of the integrins prevents the cells from spreading on the surface. Therefore,

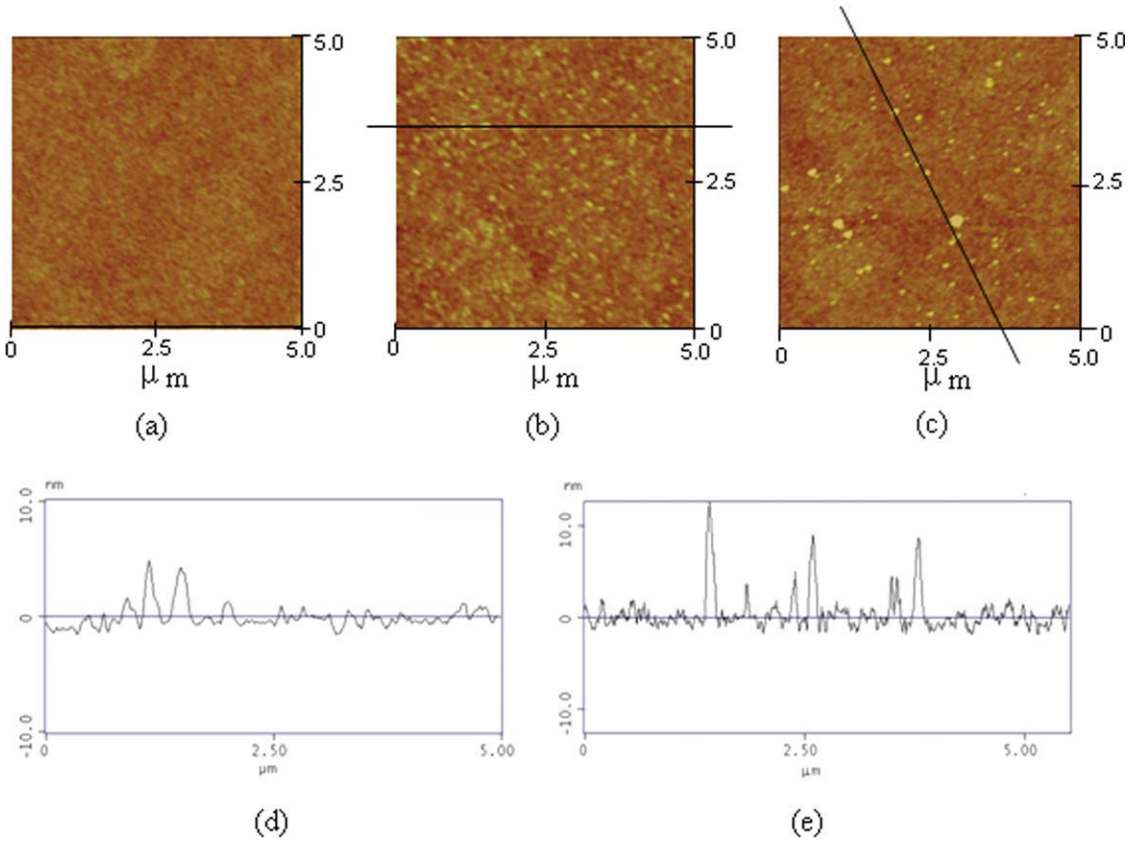

FIGURE 6. Atomic force microscopy images of (a) blank glass surface, (b) cBSA-147 coated surface, (c) cBSA-147-cRGDfK (**4c**) coated surface, and line profiles (d) for cBSA-147 coated surface, and (e) for cBSA-147-cRGDfK (**4c**) coated surface. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the improvement in the focal adhesion of the cells observed on the coated surfaces (**4c**, **5**, and **6**) was obviously mediated by the grafted cRGDfK on cBSA-147.

The excellent adhesion of the cells to the bioconjugate-treated surfaces can be mainly attributed to the fact that positively charged (**4c**), (**5**), and (**6**) are able to attract cells to the coated surfaces via long distance electrostatic interactions which then facilitates the specific interaction with the adhesion promoting RGD peptide attached to the surfaces. The results from our AFM study (Figure 6) further imply

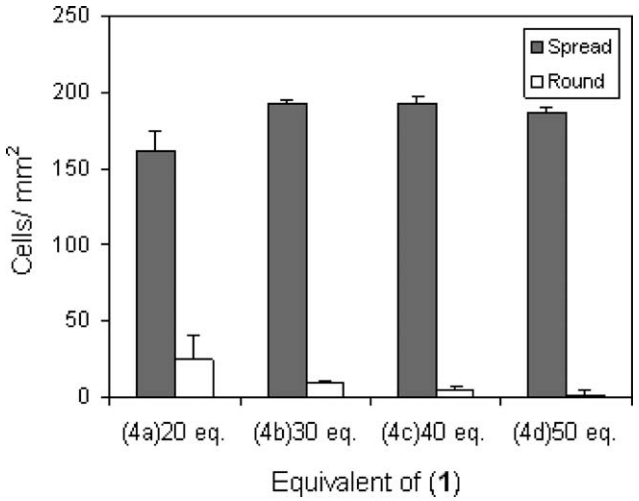

FIGURE 7. Study on the number of spread and round NIH 3T3 fibroblast cells on 10 μM of (**4a–d**) coated glass surfaces; cell seeding density: 250 cells/mm^2 (1 × 10^5 cells/mL); 1 h culturing time.

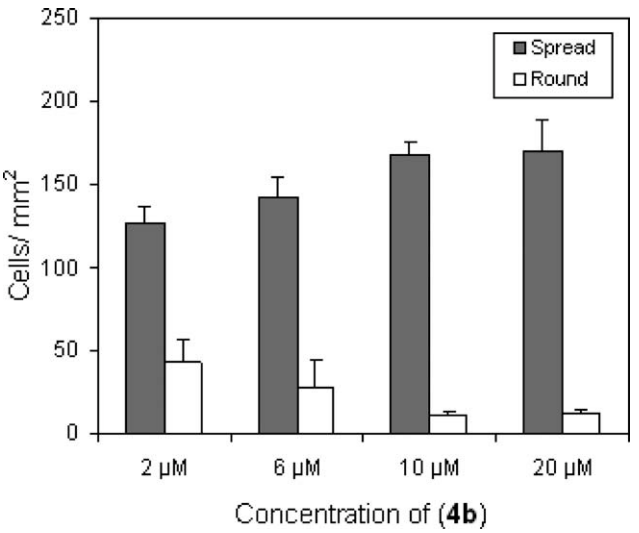

FIGURE 8. Statistical study on the spread and round NIH 3T3 fibroblast cells on glass surfaces coated with different concentrations of (**4b**); cell seeding density: 250 cells/mm^2 (1 × 10^5 cells/mL); 1 h culturing time.

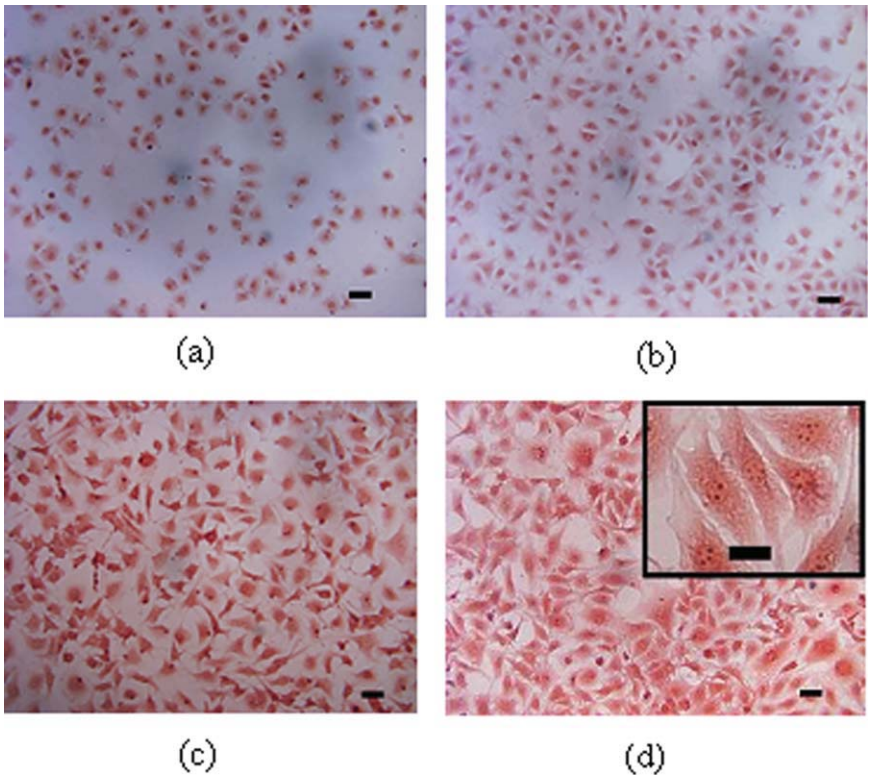

FIGURE 9. Proliferation test for NIH 3T3 fibroblast cells on (**4b**) coated glass surface at (a) 1 h, (b) 4 h, (c) 24 h, and (d) 48 h. Cell seeding density: 500 cells/mm^2 (2 $\times$ 10^5 cells/mL), scale bar: 50 μm. The insert in (d) shows the cells at higher magnification (scale bar: 50 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

that the cRGDfK grafted onto positively charged cBSA-147 could indeed promote tight cell binding even though the distance of the cRGDfK from the glass surface was two times larger than the optimum spacer length reported previously.[7,12] Nevertheless, excellent focal adhesion of the cells on surface coated with (**4c**) was observed. Furthermore, the number of adhering cells on surfaces treated with (**4c**, **5**, and **6**) was similar, irrespective of the increasing spacer length of the grafted peptide used (Figure 5), indicating that in all cases, cRGDfK was readily accessible for binding. Apparently, the size and flexibility of the cBSA used as support is already sufficient to allow for optimal interactions between the grafted RGD-groups and the cell-surface receptors.

After optimizing the biocoating, 10 μM of cRGDfK-cBSA-147 conjugate (**4b**) prepared from 30 equi. of (**1**) was identified as the best bioconjugate for preparing engineered surfaces for cell adhesion. (**4b**) promotes strong and uniform cell adhesion to glass surfaces, whereas displaying low cytotoxicity (Figure 9).

Application of (**4b**) offers a convenient, time saving and efficient approach to obtain surfaces coated with cells in a very uniform fashion. Therefore, (**4b**) provides an attractive platform for the design of tailored biocoatings because additional functional groups such as phosphines or thiol groups can easily be introduced which will facilitate binding to surfaces with different chemical properties. Likewise, antibiotics could be attached to suppress bacterial cell growth. Studies to use these biocoatings for demanding medicinal applications such as coating of stents or implants are currently under way.

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