Determination of polyurethane-grafted peptide (GSGREDVGSG) using bicinchoninic acid (BCA) assay

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The goal in the presented study was to develop a simple, fast and accurate method for measuring the surface density of a short peptide sequence bound to a polymeric substrate. We analyzed polyurethane samples chemically modified with acrylic acid and polyurethane-grafted peptide (GSGREDVGSG) peptide and investigated the possibility of using the bicinchoninic acid (BCA) assay to determine surface density of the solid-supported peptide. We set the conditions (temperature, time) under which the test should be conducted. We also studied the interaction of the BCA reagent with polyurethane substrate and the effect of drying conditions as well as material type and form on the test response. We have proposed potential factors that might interfere with the BCA assay and chosen the proper control materials.

Introduction

The goal of the presented research was to develop a simple, fast and accurate method for measuring the surface density of a short peptide sequence bound to a polymeric substrate.

Estimating the surface density of a protein/peptide bound to the substrate is a difficult and demanding task. Methods proposed so far can be divided into those requiring the use of advanced and specialized equipment and those based on simple colorimetric tests. The first group of methods includes techniques such as XPS analysis and TOF-SIMS. More information on the use of these techniques in the analysis of solid-supported peptides can be found elsewhere [1,2].

One of the simpler methods based on colorimetric determination is measuring the concentration of sulfoNHS, which is released during peptide attachment. However, this method, as an indirect method, has a large measurement error. An interesting solution is the use of immuno-enzymatic methods, including use of peptides conjugated with fluorochrome [3] or ELISA

[4,5]. The ELISA test is based on the use of monoclonal antibodies directed against the designated peptide. The huge advantage of the test is simultaneous information on the surface density and biological activity of the peptide being determined. Unfortunately, there is a high probability of nonspecific antibody–polymer interactions, which often overstate the obtained values or give false-positive results even in the case of a control (peptide-free) material. During our research we met with the described problem, and it was not possible to eliminate it despite the use of various blockers (please refer to the Supporting Information for details).

Colorimetric assays are widely used to quantify proteins in solution and solid substrates due to their simplicity [3]. One of the commonly used tests is based on bicinchoninic acid (BCA) [6,7]. The BCA method eliminates difficulties of the Lowry method, making it easy to perform, with high sensitivity [8–11]. Briefly, the BCA assay is based on two reactions. First, amino acids form a complex with Cu⁺ ions. In the next step, BCA creates a colored complex with the previously formed Cu⁺ complex [12–14]. This reaction is temperature-dependent –

the test is more sensitive at higher temperatures [9].

The BCA test offers the possibility to estimate the concentration of chemical substances containing peptide bond and/or the following amino acids: cysteine, cystine, tyrosine and tryptophan. This assay has been successfully used in the determination of RGD on polycaprolactone substrates [6,12].

The aim of the presented study was to estimate the surface density of a ten-amino acid peptide (GSGREDVGSG) chemically linked to polyurethane (PU). The REDV sequence has been shown to exhibit selectivity for selected cell types [15–18], so its use is highly desirable for biomaterials that induce a specific biological response from surrounding tissues.

We performed several microBCA assays to quantify the short peptide sequence grafted on the PU film. The PU film was modified with acrylic acid and short peptide sequence as described previously [19]. First, analysis of the BCA reagent interaction with PU substrates was carried out. The polymer substrates were prepared under different drying temperature and in different forms –

METHOD SUMMARY

We proposed a simple method based on the bicinchoninic acid (BCA) test to estimate the surface density of a solid-supported peptide.

in pellets, cut pellets, pellets with scraped outer surface, and PU film. This experiment allowed us to examine the interactions of the solid background with the BCA reagent and whether the potential residual solvent in polymer film is important. After determining these interactions, we examined the surface density of PU-grafted peptide.

Materials and methods

Influence of BCA reaction conditions

The reaction of analyzed peptide with BCA reagent has been performed under different incubation temperature (IT) and time (T). The following variants were tested: IT = 37°C, T = 2 h; IT = 60°C, T = 0.5 h; IT = 60°C, T = 1 h; and IT = 60°C, T = 2 h. BCA working solution was prepared according to the supplier protocol. The peptide solution was prepared by dissolving GSGREDVGSG (custom made, Novazym, Poznan, Poland) in the sodium phosphate buffer (0.1 M, pH = 8); concentrations of 0, 0.5, 5, 10, 20 and 30 µg/ml were used. 150 µl of the peptide solution was mixed with 150 µl of BCA reagent and reacted in the dark under given IT and T. After the reaction, the sample was cooled down to room temperature and the absorbance at 562 nm was read. The standard curves were created by plotting net absorbance (calculated as a difference between the absorbance recorded for the sample and for the blank) versus total amount of peptide present in the sample. QuantiProBCA assay (Sigma-Aldrich, Poznan, Poland), which gives a linear response for peptide concentration from 0.5 to 30 µg/ml, was used in all experiments.

Influence of polymer substrate

In order to investigate the influence of polymer substrate on BCA reagent, two sets of materials were prepared and tested. The first set involved materials dried under different drying conditions. The PU foil was prepared as follows: ChronoFlex (AdvanSource Biomaterials, MA, USA) granules washed with 5% ethanol, dried to constant weight and dissolved in N,N-dimethylacetamide (DMAc, Sigma-Aldrich) to a final concentration of 20% (w/v). The solution was poured onto the clean glass and dried in the incubator at different temperatures (40, 70 and 100°C) for 48 h under normal pressure. After the drying, part of the foil was subjected to an additional drying process under low pressure. The additional drying

was performed in a freeze-dryer under the following parameters: pressure 0.1 mbar, temperature 40°C, time 72 h. After drying, round-shaped samples were cut off from the foil and contacted with BCA reagent (1 h, 60°C).

The second set of materials differs in terms of sample type. Pristine, nonmodified PU was analyzed in the form of granules as received from the supplier. The following variants were tested: granules without any intervention (G), granules cut in half (CG), granules washed with pro-pan-2-ol (WG) and granules mechanically cleaned with scalpel (SG). Three different types of PUs were used: ChronoFlex, Elastolane and Estane. A given amount of granules (80 mg ± 6 mg) was contacted with BCA reagent (1 h, 60°C) and the absorbance was measured according to the supplier protocol.

Density of peptide chemically grafted to PU

Pristine, non-modified PU (PU) and peptide-grafted PU (PU-REDV) were analyzed with BCA assay. Polymer samples were cut off from foil (the foil was prepared according to the description in 'The influence of drying conditions', under the following drying conditions: 40°C, 24 h, normal pressure). Next, polymer samples were modified in a two-step reaction (Figure 1). In the first modification step, samples were coupled with spacer molecule (acrylic acid [AA]). Briefly, PU discs were immersed in a 2.3% solution of HNO₃ in distilled water and heated to 35°C. After reaching this temperature, ammonium cerium (IV) sulphate dihydrate (0.1% w/v) and AA (5% v/v) were added to the solution. The mixture was left covered and stirred continuously for 1.5 h. After that time, discs were washed with 0.1% sodium dodecyl sulfate (SDS) solution and rinsed three times with distilled water.

PU discs modified with AA were placed in 0.05 M 2-(N-morpholino)ethanesulfonic acid buffer (MES) for 1 h. Then, samples were transferred to the 5 mM N-hydroxysulfosuccinimide (sulfoNHS) and 2 mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) solution for 15 min at room temperature (RT) and washed with MES buffer. Samples after reaction with AA and sulfoNHS/EDC were marked as 'PU-NHS'.

In the next step, discs were placed in the GSGREDVGSG solution (5 mM) in 0.1 M sodium phosphate buffer, pH 8.0 for 4 h at RT. Next, discs were incubated in sodium phosphate buffer, pH = 9 for 1 h to hydrolyze unreacted sulfoNHS groups. Finally, samples were rinsed with PBS. Samples after reaction with peptide were marked as 'PU-REDV'.

Simultaneously to the peptide immobilization, control samples were prepared. After reaction with sulfoNHS and EDC, discs were placed in tris(hydroxymethyl)aminomethane (TRIS) solution (5 mM) in 0.1 M sodium phosphate buffer, pH 8.0, for 4 h at RT, which should lead to TRIS conjugation due to the amide bond formation. Afterward, discs were incubated in sodium phosphate buffer, pH = 9 for 1 h to hydrolyze unreacted sulfoNHS groups. Finally, samples were rinsed with PBS. Samples after reaction with TRIS were marked as 'PU-TRIS'.

Simultaneously, we analyzed samples chemically modified using different concentrations of the linker molecule (1, 2, 3 and 5% v/v of AA) and peptide (0.5, 5 mM). A determination of the peptide containing the RGD sequence (5 mM, GSGRGDGSG, custom made, Novazym) was also carried out. The RGD grafting procedure was analogous to that described above for REDV grafting.

All analyzed samples in the form of discs (with a diameter of 10 mm) were placed in a 24-well plate and contacted with BCA

Figure 1. Scheme presenting types of materials used in the study.

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reagent (500 μ l per sample, 1 h, 60°C). After the reaction, BCA solutions were cooled down to room temperature and transferred to a 96-well plate. The absorbance at 562 nm was read; BCA reagent was used as a blank.

The surface density of the grafted peptide was calculated as follows. The net absorbance used to calculate peptide amount was obtained by subtracting the absorbance value for PU-NHS from the value for PU-REDV. Next, the amount of peptide adsorbed on the sample's surface was calculated based on the standard curve equation ($y = -0.012 \times 2 + 0.170$) obtained for the same incubation conditions (1 h, 60°C). Finally, the surface density of the adsorbed peptide was calculated as a number of peptide moles divided by the sample's surface.

Statistical analysis

Results of BCA analysis were expressed as mean $\pm$ SD. Statistical significance of differences was analyzed using single factor analysis of variance (ANOVA) for $p < 0.05$ with post-hoc Tukey's test (OriginPRO 8.0).

Results & discussion

Influence of BCA reaction conditions

The calibration curve for analyzed peptide (GSGREDVGSG) was prepared under conditions differing in IT and T. The aim of this experiment was to select incubation parameters that give relatively high absorbance values. The obtained curves for four different incubation conditions are presented in Figure 2. The absorbance values obtained for a given concentration of the peptide increased together with an increase in both IT and T. The smallest absorbance values were obtained for IT = 37°C, T = 2 h, whereas the absorbance values obtained for IT = 60°C, T = 2 h were the highest.

Smith *et al.* suggested that during the BCA assay, the BCA-Cu¹⁺ purple-blue complex is produced by two separate reactions [8]. The first one is temperature-independent and it is based on oxidation of cysteine, cystine, tyrosine and tryptophan. In the second mechanism, which is temperature-dependent, Cu¹⁺ is produced as a result of peptide bond oxidation. This process is more effective in higher temperatures (60°C). The analyzed amino acid chain (GSGREDVGSG) does not involve any of the Cu²⁺-reducing amino acids. Thus,

the reaction occurs only due to the presence of a peptide bond and, in our opinion, should be performed at higher temperatures. The obtained standard curves showed that the absorbance values were in fact higher at the higher incubation temperature. This finding was also in line with data obtained by other authors [8,20]. Taking into account these results and optimization of experiment time, we decided to perform all BCA assays under the followed conditions: IT = 60°C, T = 1 h.

Another aspect that should be considered is the linearity of standard curves. According to the information from the supplier, peptide concentrations in the range 0.5 to 30 μ g/ml give a linear response with absorption. Surprisingly, in the case of the GSGREDVGSG peptide, the polynomial curve (Figure 2B) gave better fit of the absorption values than the linear curve (Figure 2A). Smith *et al.* [8] showed that the linearity of standard curves in the BCA test strongly depends on the type of protein

Figure 2. Standard curves resulting from the reaction of GSGREDVGSG with BCA reagent under different incubation time and temperatures. (A) Linear fitting; (B) second-order polynomial fitting; data are presented as MV $\pm$ SD (n = 8).

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being analyzed and on the incubation parameters. In the case of GSGREDVGSG all curves presented a slightly parabolic shape. The standard curves became less linear at higher incubation temperatures and longer incubation times. Based on these findings, we considered $IT = 60^{\circ}\text{C}$ and $T = 1\text{ h}$ to be the optimal incubation parameters. These results remain in accordance with the studies by Tylanakis *et al.* [21]. They proved that many solid-supported substances required 1 h of incubation at 60°C to reach plateau absorbance values.

Influence of polymer substrate

As already mentioned, the purpose of the presented work was to estimate the surface density of a peptide chemically bound to a PU substrate. First, we aimed to check if the base material (PU) interferes with the BCA reagent. Wiechelman *et al.* showed there is a number of substances containing functional groups similar to those present in cysteine, cystine, tryptophan and tyrosine that react with the BCA reagent [20]. In the case of PU, the positive result during BCA assay might come from (i) polyurethane bonds; (ii) the solvent used during processing granulate into film; and (iii) chemical residues present on the surface/inside the granules supplied from the manufacturer.

To verify these hypotheses, a number of tests were carried out. The BCA reagent was first contacted with a chemically unmodified PU film. The results showed that the material had reacted with BCA and gave a positive response. The list of interfering substances provided by the PU manufacturer indicates that DMAc interferes with BCA. Thus, the possibility of DMAc residues remaining in the materials was verified. For this purpose, materials were dried in various conditions. DMAc is a low volatile solvent (boiling point = 165°C under normal pressure), and therefore drying under low pressure was used to increase the efficiency of the drying process. Namely, the following drying conditions were applied: drying in 40°C under normal pressure (40); drying in 40°C under normal pressure followed by drying under low pressure (40L); drying in 70°C under normal pressure followed by drying under low pressure (70L); and drying in 100°C under normal pressure followed by drying under low pressure (100L).

The obtained results are presented in Figure 3. In each PU sample, the obtained absorbance value was higher than the absorbance value for blank ($p < 0.05$). At the same time, the values were similar for all PU samples dried under different temperatures and pressures ($p > 0.05$). On this basis,

we concluded that the solvent has been successfully removed from the polymer and the positive BCA test response was not derived from DMAc.

Another potential BCA interfering factor may be the urethane bond. The structure of this bond is similar to the peptide bond. However, the BCA test carried out with pure PU granulate gave a negative result (Figure 4). A similar result was obtained for three different types of PU; OD560 values were comparable to the values of a blank. On this basis, we concluded that the polyurethane bond was not an interfering agent.

As a next step, the hypothesis regarding the presence of chemical residues inside the PU granules was verified. Such residues may diffuse to the surface of the film in the process of dissolving and processing granules and cause a positive material response during the BCA test. The results showed a significant increase ($p < 0.05$) in the absorbance value for BCA contacted with cut granules (Figure 4). The OD560 value was significantly higher compared with whole (non-cut) granules. What is more, OD560 values depended on the type of PU analyzed. On this basis, we concluded that the positive response of the PU foil during BCA assay is the result of the presence of chemical additives used in the granule production process.

Density of peptide chemically grafted to PU

To assess the surface density of the peptide, a number of materials were prepared and incubated with BCA reagent. As the control material, the unmodified PU film was reused (PU). The analyzed material was a PU film with a chemically attached peptide GSGREDVGSG (PU-REDV). The modification process was performed in several stages: (i) activation of the material; (ii) attachment of the spacer molecule terminated with carboxylic groups; (iii) activation of carboxyl groups with the use of sulfoNHS/EDC; and (iv) reaction with the peptide. The experiment also applied two reference materials. The first one (PU-NHS) was subjected to subsequent modification stages (1–3) but omitting the last stage – peptide attachment (4). The second reference material (PU-TRIS) was subjected to subsequent modification stages (1–3), and in the last stage a control molecule (TRIS) was attached to the surface of the material. The material modification

Figure 3. Results of BCA assay obtained for pristine polyurethane dried under different conditions. Drying in 40°C under normal pressure (40), drying in 40°C under normal pressure followed by drying under low pressure (40L), drying in 70°C under normal pressure followed by drying under low pressure (70L), drying in 100°C under normal pressure followed by drying under low pressure (100L); data are presented as $MV \pm SD$ ($n = 8$).

Figure 4. Results of BCA assay obtained for pristine polyurethane of different types and different forms. Granules (G), cut granules (CG), granules washed with alcohol (WG), granules cleaned with scalpel (SG); data are presented as MV $\pm$ SD (n = 8).

scheme is presented in Figure 1. As can be seen, all modified materials were contacted with sulfoNHS in order to attach peptide/control molecule. Vashist *et al.* showed that

sulfoNHS interferes with BCA assay [22]. To remove any unreacted sulfoNHS groups from the surface of the material, all tested materials were hydrolyzed (1 h, pH = 9)

Figure 5. Results of BCA assay obtained for non-modified polyurethane (PU), polyurethane modified with peptide (PU-REDV) and control materials (PU-NHS, PU-TRIS). Data are presented as MV $\pm$ SD (n = 8).

ANOVA: Analysis of variance.

before the BCA test. According to the literature, the half-life of sulfoNHS is only a few minutes at pH > 8 [23].

The results of the BCA assay obtained for nonmodified PU as well as the results for PU with chemically grafted peptide (PU-REDV) are presented in Figure 5. Pristine PU (PU) reacted with BCA reagent, giving the absorbance value of OD562 = 0.365. After the first step of modification absorbance decreased and reached the value of 0.274 (PU-NHS). The difference between absorbance values for PU and PU-NHS was statistically significant ($p < 0.05$). On the other hand, the value obtained for PU-NHS was comparable to the absorbance value of blank. This indicates that (1) the process of hydrolysis was effective and the unreacted sulfoNHS groups were absent on the material; and (2) the interfering substance that was present on the surface of the PU film was covered by the modification layer. Such a material can be successfully used as a reference material in the assessment of the peptide surface density. In this case, the peptide surface density is directly proportional to the absorbance value subtracting the background value (the absorbance value obtained for PU-NHS).

As expected, after coupling the peptide (PU-REDV) the absorbance value increased to the highest value compared with other tested materials (OD562 = 0.543). On this basis, it can be concluded that the reaction of peptide grafting was successful. Incubation of the BCA reagent with control material (PU-TRIS) generated a lower absorbance value, comparable to the value obtained for PU-NHS (OD562 = 0.269; $p > 0.05$ vs PU-NHS).

Figure 6 presents the results obtained for various densities of surface-grafted peptide. The final surface density of the grafted peptide is influenced by both the concentration of the linker molecule solution as well as the concentration of the peptide solution. In the study, we used four different concentrations of the linker and two different concentrations of peptide, resulting in eight different surface densities of the peptide. PU-NHS and PU-TRIS were used as control materials.

As expected, all control materials generated similar absorbance values (for all tests AA concentration). As can be seen in the Figure 6, the absorbance values obtained for materials modified with 3 and 5% AA were significantly higher than those of the control materials. In the case of 1

Figure 6. Results of BCA assay obtained for materials differ in surface density and type of grafted peptide. PU-REDV 0.5 mM – materials modified with 0.5 mM solution of GSGREDVGSG, PU-REDV 5 mM – materials modified with 5 mM solution of GSGREDVGSG, PU-RGD 5 mM – materials modified with 5 mM solution of GSGRGDGSG, control materials: (PU-NHS, PU-TRIS). Data are presented as MV $\pm$ SD (n = 8).

and 2% AA the resulting peptide surface densities were too low to be determined (values comparable to those obtained for PU-NHS). These results were observed for both PU-REDV and PU-RGD.

When determining the surface density of a peptide, the response obtained for the reference material should be taken into account. In our case, the reference material presented the same surface structure, only without the peptide (PU-NHS). The OD560 value obtained for PU-NHS was subtracted from the OD560 value obtained for PU-REDV/PU-RGD and then the surface density was determined using a standard curve. The values of peptide surface density calculated for various materials are presented in Table 1. The applied method allowed the peptide density in the range of 1.2 to 5.7 nmol/cm² to be determined. It

has been shown that this density is sufficient to induce a biological response. What is important, a specific biological response of a cell is highly correlated with an optimal peptide surface density. Therefore, in our opinion, the development of a fast, simple and effective technique measuring the surface density of solid-supported peptides is crucial in the study of bioactive materials.

As a result of the conducted studies, we concluded that polymer substrate (PU) might interfere with the BCA reagent. The interfering agents are probably the chemical additives used in the specific type of PU's granule production process, while the polyurethane bond itself is not an interfering agent.

Studies have also shown that drying conditions applied during processing of PU samples did not influence the assay

response. To summarize, surface density of REDV peptide chemically grafted to PU can be determined; however, the appropriate control material must be chosen with great care (Table 1).

Author contributions

BBR: the conception and design of the study, analysis and interpretation of data, drafting the article; AK: performing all experiments, analysis and interpretation of data, drafting the article.

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Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2018-0004

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Table 1. Density of surface-grafted peptide (nmol/cm²) for different concentration of linker molecule (AA) and peptide. Data are presented as MV $\pm$ SD (n = 8).

	AA (%)			
	1	2	3	5
PU-REDV 0.5 mM	n/a	n/a	1.2 $\pm$ 0.2	1.9 $\pm$ 0.8
PU-REDV 5 mM	n/a	n/a	1.6 $\pm$ 1.8	4.8 $\pm$ 1.1
PU-RGD 5 mM	n/a	n/a	1.7 $\pm$ 1.5	5.7 $\pm$ 1.9

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