A new fluorescence-based, hydrophobic photolabeling technique for analyzing membrane-associated proteins

D. Hess[1], G. Isenberg*

Biophysics-Dept. E22, Technical University of Munich, James Franck Str., D-85747 Garching, Germany

Received 17 December 1998; received in revised form 25 January 1999

Abstract We introduce a new, fluorescent and photoactivatable fatty acid derivative (SANU) for hydrophobic labelling of membrane-bound proteins. The technique allows fast and highly sensitive screening of hydrophobically inserting proteins analyzed by SDS-PAGE with a detection limit below 0.1 pmol. A reliable calculation of labelling efficiencies is achieved by simultaneous densitometry of fluorescence and protein staining. We have applied the new technique on the membrane inserting protein talin, G-actin, and, as a negative control, on RNase, which only binds electrostatically to negatively charged lipid interfaces. In several ways superior to radiolabelling, we can recommend this technique for all laboratories under any circumstances.

Key words: Fluorescence; Hydrophobic photolabelling; Membrane-associated protein; Talin; RNase; G-actin

1. Introduction

In previous studies [1,2], we have applied hydrophobic photolabelling using radiolabelled lipid analogues to monitor the association of cytoskeletal proteins with lipid membranes. Here, we present an alternative and efficient technique to label membrane-inserting proteins by a photoactivatable fatty acid derivative which we have coupled to sulforhodamine B (SANU: N^1-(N^2-(sulforhodamine B sulfonyl)aminoethyl, 11-(5-azido-1-naphtoxy))undecanylamide). Fluorescence detection is faster than comparable radioactive methods and at least equally sensitive. The quantification can be achieved with commercially available standard equipment. Simultaneous densitometric quantification of fluorescence and protein staining in SDS-PAGE allows the direct calculation of labelling efficiencies. Thus, our method is superior to techniques employing radiolabelled compounds. To validate our method, we have used the membrane inserting protein talin [1] as a positive control. In order to show that our photolabel partitions mainly into the hydrophobic core of the membrane, we have used RNase, which binds predominantly via electrostatic interactions to lipid interfaces [3,4], as a negative control. For G-actin which has been shown to undergo conformational changes at lipid interfaces [5,6], we obtain a considerable amount of hydrophobic insertion when bound to positively as well as negatively charged membranes. Since fluorescent probes also do not bear any contamination risks or disposal problems, we can recommend this technique to all laboratories under any circumstances.

2. Materials and methods

2.1. Synthesis of SANU

The hydrophobic photolabel was obtained in a one step activation reaction from the succinimidyl ester of 11-(5-azido-1-naphtoxy)undecanoic acid (compound A) and sulforhodamine B sulfonylethylenediamine (= Lissamine sulfonylethylenediamine) (compound B) (both purchased from Molecular Probes, Eugene, OR, USA). In order to prevent the photoactivation of the azido moiety, all steps were performed in the dark or under red light. 20 mg of A were added to 8.5 mg B (stoichiometric ratio 1.1:1), the solvent was water-free *N*,*N*-dimethyl formamide (20 ml). The reaction was stirred for 12 h and its completion checked by thin layer chromatography (TLC). Dimethyl formamide was removed by high vacuum evaporation and the completely dry products were dissolved in chloroform/methanol/water (3:1:1, solvent A). Not reacted compounds and by-products were removed by filtration on a silica gel column (Silica 60 (Merck, Darmstadt, Germany), height 25 cm, bed volume 16 ml) in solvent A. After column purification, the solvent was again exchanged by pure chloroform. Mass spectrometric analysis of the reaction product gave an M_r of 952.0, in good agreement with the relative mass derived from the formula of SANU (952.4: Fig. 1A). A reaction yield of 95% was determined from the total dry mass.

2.2. Proteins and vesicle preparation

Actin from rabbit skeletal muscle was purified [7] with an additional gel filtration step [8]. Talin was prepared from outdated platelets [9], with further purification by gel filtration [10]. RNAse A was purchased from Sigma (Deisenhofen, Germany). Dimyristoyl-phosphatidyl-choline (DMPC), dimyristoyl-phospho-rac-glycerol (DMPG) and dimyristoyl-tetramethylammoniumpropane (DMTAP) were purchased from Avanti (Alabaster, AL, USA). 5 mg of lipids (DMPC, DMPC/DMPG (1:1) or DMPC/DMTAP (1:1)) were mixed with the hydrophobic label (0.16 mol%) in chloroform/methanol (4:1) and dried under a nitrogen flow with subsequent vacuum exsiccation (12 h). The vesicles were swollen from the dry lipid films by addition of 1 ml G-buffer (2 mM Tris-HCl, pH 7.5, 0.2 mM CaCl, 2 mM MgCl, 1.1 mM EGTA, 1.1 mM EDTA, 0.5 mM ATP) under vigorous shaking. Small vesicles (< 200 nm) were obtained by ultrasonication (3 min, 50% time pulses at a power of 35 W) and equilibrated for 15 h at 37°C. The vesicles were then purified by gel filtration on a G-25 desalting column (Pharmacia, Freiburg, Germany). Large unilamellar vesicles (DMPC/DMPG (98:2, 5 mg/ml): SANU (0.16 mol%)) were prepared by electroswelling [11].

2.3. Reconstitution of proteins and analysis of hydrophobic photolabelling

Proteins were added to small vesicles (DMPC, DMPG/DMPC 1:1, DMTAP/DMPC 1:1, 7.4 mM, SANU 12 μM) at concentrations of 1.3 μM (talin) and 4.8 μM (RNase and actin). 500 μl aliquots were incubated for various time periods at 4°C, 25°C or 37°C. The azido moiety of SANU was activated by UV irradiation or flashlight pulses.

*Corresponding author. Fax: (49) (89) 289 12469.

[1]Present address: Roche Diagnostics GmbH, D-82372 Penzberg, Germany.

Abbreviations: DMPC, dimyristoyl-phosphatidyl-choline; DMPG, dimyristoyl-phospho-rac-glycerol; DMTAP, dimyristoyl-tetramethylammoniumpropane; DSC, differential scanning calorimetry; RNase A, ribonucleic acid hydrolase A; SANU, N^1-(N^2-(sulforhodamine B sulfonyl)aminoethyl, 11-(5-azido-1-naphtoxy))undecanylamide; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TLC, thin layer chromatography

PII: S0014-5793(99)00155-6

The labelling of membrane-associated proteins was absolutely dependent on the photoactivation of SANU. Under our experimental conditions, saturation of activation determined from the total amount of protein labelled was reached after 3 min UV irradiation (50 W, distance 2 cm) or 10–11 flashlight pulses (Ultrablitz 38 M Logic (Braun, Germany), distance 2 cm). UV irradiation reduced the fluorescence yield of the label, but not the flashlight activation. We therefore used the latter method throughout all the experiments. Proteins were separated from lipids and excess dye by chloroform/methanol (1:2) precipitation, centrifugation and washing of the pellet with ice-cold methanol. Vacuum-dried samples were separated on SDS-PAGE.

Standards for fluorescence in SDS-PAGE were prepared from G-actin. Briefly, actin (4 mg/ml) was dialyzed against 50 mM potassium phosphate buffer, pH 7.5 for 3 h. Subsequently, the solution was incubated for 30 min with Lissamine sulfonylchloride (Molecular Probes, Eugene, OR, USA) at a molar ratio of 100:1. Excess dye was removed by gel filtration (Superose 6 (bed volume 25 ml), Pharmacia, Freiburg, Germany). The actin concentration in the solution was determined by a colorimetric assay (Bio-Rad, Munich, Germany) using actin as a reference, fluorophore concentration was determined from absorption using an extinction coefficient of 87 500/M/cm. For talin and RNase, an analogous procedure was carried out. The in gel fluorescence intensity of samples and standards was recorded in a Fluor S multiimager system (Bio-Rad, Munich, Germany) equipped with a CCD camera. For excitation, the UV absorption band (311 nm, Fig. 1B) of SANU was excited by broad range UV. The fluorescence was recorded with 3 min exposure time using a 520 nm longpass filter. After fluorometry, proteins were stained by Coomassie blue (Serva, Heidelberg, Germany) and the total protein OD was scanned. Ultracentrifugation experiments for the separation of vesicles from unbound protein were carried out in a Beckmann L7-55 ultracentrifuge equipped with Ti 50 Rotors at 200 000 × g for 20 h (27°C).

3. Results and discussion

3.1. Properties of SANU

The photoactivatable fluorescence label (SANU) can be obtained in a one step reaction from the succinimidyl ester of 11-(5-azido-1-naphtoxy)undecanoic acid and sulforhodamine B

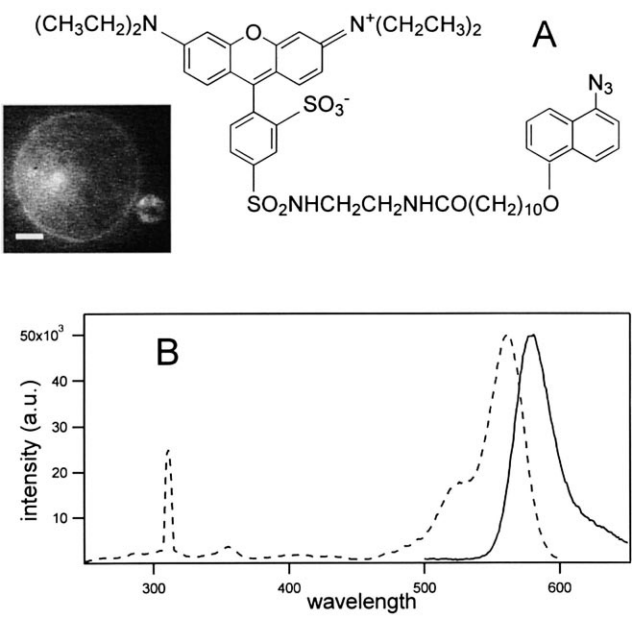

Fig. 1. (A) Structure and formula of SANU; (N^1-[N^2-(sulforhodamine B sulfonyl)aminoethyl, 11-(5-azido-1-naphtoxy)]undecanylamide). Inset: large unilammelar vesicle dosed with 0.16 mole % SANU (Bar length indicates 2 μm). (B) Fluorescence excitation (- - -) and emission (———) spectra of SANU; spectra were recorded in methanol. For UV-excitation, the absorption band at 311 nm was used.

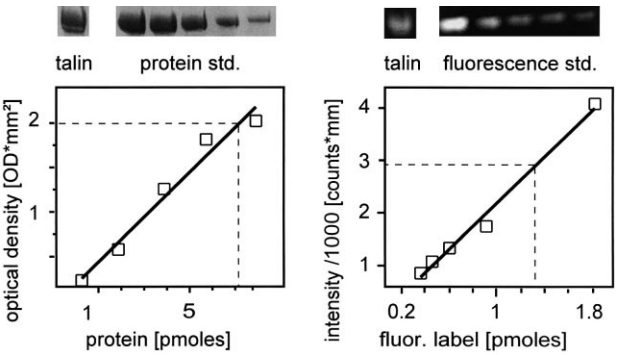

Fig. 2. Talin labeling after insertion into DMPG/DMPC (1:1) vesicles (0.16 mole % SANU). Talin incorporation into lipid vesicles was performed for 4 h at 25°C in 2 mM Tris/HCl, pH 7.5, 0.2 mM CaCl, 2 mM MgCl, 1.1 mM EGTA, 1.1 mM EDTA, 0.5 mM ATP (G-buffer), pH 7.5. Standard lanes (from left to right): 7.8; 5.7; 3.9; 2.0; 0.4 (pmol; protein dye); 1.8; 0.9; 0.6; 0.5; 0.4 (pmol; sulforhodamine fluorescence). Fluorescence and protein bands are quantified densitometrically as illustrated by the corresponding graphs (standards: open squares; talin quantification: (- - - -)).

sulfonylethylenediamine. After silica gel purification, a pure compound was obtained (as judged from TLC). The yield was 95% as shown by weight determination. From mass spectrometry, a molecular mass of 952.0 was determined which corresponds well to the molecular mass deduced from the formula (952.4: Fig. 1A). For the photoactivatable fluorescence label, extinction coefficients (in methanol) of 87 300/M/cm (561 nm) and 17 500/M/cm (311 nm) were calculated from the absorption spectra of known concentrations. The fluorescence excitation and emission spectra are given in Fig. 1B. The spectrum and respective maxima were identical to sulforhodamine B (Lissamine). SANU is insoluble in water, poorly soluble in methanol (<0.5 mg/ml), but readily dissolved in chloroform. The label is incorporated in lipid bilayers of large unilamellar vesicles, as shown by fluorescence microscopy (inset Fig. 1B). When present in vesicle preparations at 0.16 mol%, SANU does not alter phase transitions of lipid mixtures, as demonstrated by differential scanning calorimetry (DSC: data not shown). Taken together, these find-

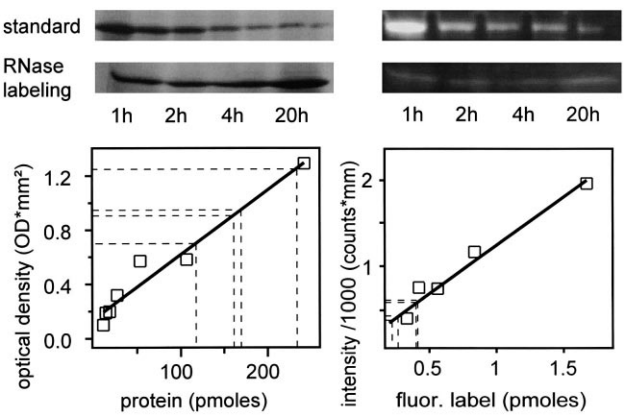

Fig. 3. RNase labeling after adsorption to DMPG/DMPC (1:1) vesicles (0.16 mole % SANU). RNase adsorption was performed from 1 up to 20 h as indicated at 25°C in G-buffer (see Fig. 2), pH 7.5. Standard lanes (from left to right): 247.8; 108.9; 54.3; 39.0; 27.2; 18.4; 14.0; (pmol; protein dye); 1.8; 0.9; 0.6; 0.5; 0.4 (pmol; sulforhodamine fluorescence). In the corresponding graphs: standards (□), RNase quantification: (- - - -).

ings indicate that SANU can be incorporated in lipid bilayers without affecting the lipid conformation significantly. RNase, a protein that binds mainly by electrostatic interactions to lipid interfaces [3,4] is labelled by SANU only to a very limited extent (see Section 3.3). This control experiment indicates that the photoactivatable azidonaphtoxy moiety of SANU partitions mainly into the hydrophobic part of the bilayer.

3.2. Densitometric quantification of labelled proteins

The calculation of hydrophobic labelling ratios was achieved by including fluorescently labelled proteins with known concentrations as a standard in SDS-PAGE. The fluorescence and protein OD after Coomassie staining increased linearly with the concentration (Figs. 2–4) with correlation coefficients between 0.95 and 0.99. Fluorophore and protein concentrations of the samples were calculated from these standard curves. The fluorescence detection was extremely sensitive, with a detection limit below 0.1 pmol (>0.1 μg for Coomassie stain protein OD). In contrast to radiolabelling, our method allows simultaneous in gel detection of fluorescence and protein staining, without the need of gel transfer, band excision, etc. Furthermore, labelling ratios of the samples can be reliably calculated from the standard curves, without having a problem with the background correction (as with scintillation).

3.3. Analysis and quantification of SANU-labelled membrane-associated proteins

We have tested the partitioning of the label into the hydrophobic core of lipid bilayers using the membrane-associated proteins talin and RNase as a positive and a negative control, respectively. Talin is a major constituent of cell-focal contacts and thought to be involved in coupling actin filaments to the cell membrane [12]. By using negatively charged vesicles incorporating SANU (DMPC/DMPG, 1:1, 0.16 mol% label), we obtained a considerable amount of fluorescence labelling (0.18 mol/mol talin, Fig. 2). This finding correlates well with

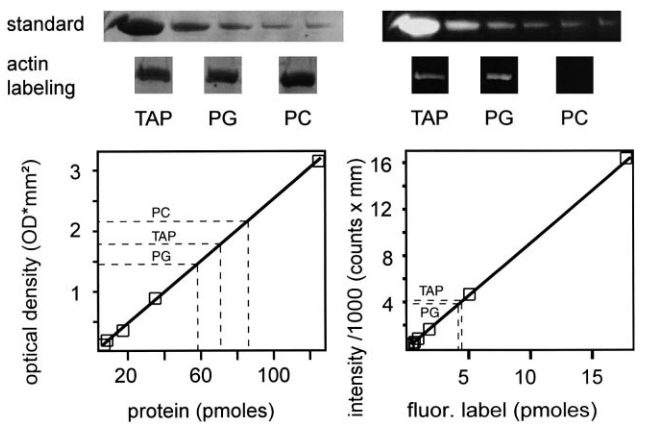

Fig. 4. Actin labeling in the presence of DMTAP/DMPC (TAP, 1:1), DMPG/DMPC (PG, 1:1) and DMPC (PC) vesicles (0.16 mole % SANU). Actin adsorption was performed for 1 h at 25°C in G-buffer, pH 7.5. Standard lanes (from left to right): 126.2; 35.0; 17.4; 8.6; 5.7 (pmol; protein dye); 18.2; 1.8; 0.9; 0.6; 0.5; 0.4 (pmol; sulforhodamine fluorescence). Protein amount per lane (quantification: (- - - -)) as deduced from protein dye standard curve (□) in pmol: 71.4 (TAP, 25°C); 58.6 (PG, 25°C); 85.5 (PC, 25°C). Fluorescence label (pmoles) as calculated from standard curve (□): 4.4 (TAP, 25°C); 4.2 (PG, 25°C).

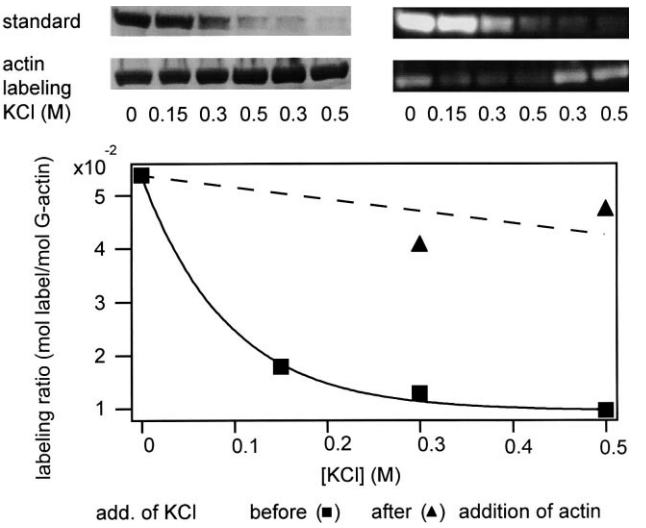

Fig. 5. Actin labeling in the presence of DMPG/DMPC (1:1) vesicles (0.16 mole % SANU) with increasing KCl concentration. Actin adsorption was performed for 4 h at 25°C in G-buffer (see Fig. 2), pH 7.5. KCl was added at concentrations indicated before (▲) and 2 h (■) after addition of actin. Standard lanes (from left to right): 126.2; 83.3; 35.0; 17.4; 8.6; 5.7 (pmol; protein dye); 5.0; 3.3; 1.8; 0.9; 0.6; 0.5 (pmol; sulforhodamine fluorescence).

previous results [1,13] from which insertion of talin into the hydrophobic part of negatively charged lipid bilayers has been inferred. However, we find about 10-fold higher labelling ratios for talin in comparison to previous results obtained by hydrophobic radiolabelling [1]. The reduced radiolabelling efficiency is probably due to quenching effects and problems which result from the background correction after liquid scintillation. Thus, hydrophobic photolabelling with fluorescent probes seems to be superior in comparison to radiolabelling since it allows quantification of fluorescence and protein staining simultaneously. As a negative control for hydrophobic photolabelling with SANU, we used RNase, a small, globular and hydrophilic protein. RNase has been shown to interact primarily by electrostatic interactions with the surface of lipid bilayers [3,4]. In the presence of uncharged DMPC vesicles, RNase shows no detectable fluorescence labelling. In the presence of negatively charged DMPG/DMPC vesicles, RNase is labelled only to a very limited extent (0.002–0.004 mol/mol, Fig. 3), irrespective of incubation time and temperature (25°C and 37°C). On the other hand, we find that RNase can be quantitatively precipitated in ultracentrifugation assays by negatively charged DMPG/DMPC (1:1) vesicles (but not by neutral DMPC vesicles). Our results employing talin as a positive and RNase as a negative control indicate that SANU partitions mainly into the hydrophobic interior of lipid membranes.

Hydrophobic photolabelling experiments of G-actin are shown in Figs. 4 and 5. When compared with RNase, G-actin gets about 20–25-fold stronger labelled by SANU when inserted in either positively or negatively charged vesicles (0.04–0.05 mol label/mol actin). In the presence of neutral DMPC vesicles, we observed a very low level of labelling, comparable to that of RNase (0.002 mol label/mol protein).

In order to measure the effect of ionic strength on the extent of hydrophobic photolabelling of G-actin, KCl (150 mM, 0.3 M, 0.5 M) was added before and after incubation of actin with charged vesicles (Fig. 5). Increasing the ionic strength

prior to addition of actin reduces the labelling efficiency significantly (from 0.05 mol/mol to 0.012 mol/mol). At high salt concentrations (0.5 M KCl), we observed some hydrophobic photolabelling of G-actin (about 20% compared to 0 M KCl: Fig. 5, closed triangles). Addition of salt to preformed actin-vesicle complexes shows a much less inhibition of hydrophobic labelling (Fig. 5, closed squares).

Our data suggest a considerable binding of G-actin to positive and negative lipid interfaces. Although actin bears a net negative charge at neutral pH, its interaction with negatively charged vesicles may be mediated by a positive cluster of amino acids near the N-terminus [14]. For the association of actin with charged vesicles, electrostatic interactions contribute significantly to the binding, as indicated by the inhibition of labelling with increasing ionic strength prior to addition of actin. However, a portion of the actin molecules is labelled in the presence of 0.5 M KCl (Fig. 5, closed triangles). Also, the dissociation of actin already bound to vesicles at low ionic strength (Fig. 5, closed squares) by increasing the salt concentration is much less effective. These results may be explained by hydrophobic insertion after conformational changes. Once attracted to charged lipid interfaces, actin molecules undergo a transition from α-helix to β-sheet, as evidenced from Fourier transformed infrared spectroscopy [5] and DSC [6].

4. Conclusion

We present the characterization of a hydrophobic, photoactivatable and fluorescent compound suitable for fast in gel detection of interaction of membrane proteins with the hydrocarbon part of the lipid bilayer. A reliable quantification of the extent of hydrophobic interaction is possible by simultaneous UV and white light scanning densitometry and comparison with appropriate standards.

Hydrophobic radiolabelling of actin at liposome preparations from various tissues has been reported by one group [15], while others see no labelling [16,17]. We find for G-actin hydrophobic labelling in the presence of positively and negatively charged vesicles in vitro. Although actin is generally believed to be coupled to plasmamembranes via coupling proteins (for a review see [12]), a possible role for lipid-bound actin could be to act as a pool of G-actin directly at the plasmamembrane.

Acknowledgements: We thank Dr. I. Dorn for the help in the chemical preparations, Dr. S. Kaufmann for the preparation of talin, Mrs. H. Hirsch for the preparation of actin and Mrs. M. Rupp for the expert technical assistance. This work was supported by Grants from the Deutsche Forschungsgemeinschaft (SFB 266, C-5 and Is 25/7-2).

References

[1] Goldmann, W.H., Niggli, V., Kaufmann, S. and Isenberg, G. (1992) Biochemistry 31, 7665–7671.
[2] Tempel, M., Goldmann, W.H., Dietrich, C., Niggli, V., Weber, T., Sackmann, E. and Isenberg, G. (1994) Biochemistry 33, 12565–12572.
[3] Jain, M.K. and Zakim, D. (1987) Biochim. Biophys. Acta 906, 33–68.
[4] Bergers, J.J., Vingerhoeds, M.H., van Bloois, L., Herron, J.N., Janssen, L.H.M., Fischer, M.J.E. and Crommelin, D.J.A. (1993) Biochemistry 32, 4641–4649.
[5] Gicquaud, G. and Wong, P. (1994) Biochem. J. 303, 769–774.
[6] Gicqaud, C. (1993) Biochemistry 32, 11873–11877.
[7] Spudich, J.A. and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871.
[8] MacLean-Fletcher, S.D. and Pollard, T.D. (1980) Biochem. Biophys. Res. Commun. 96, 18–27.
[9] Collier, N. and Wang, K. (1982) J. Biol. Chem. 257, 6937–6943.
[10] Kaufmann, S., Pieckenbrock, T.H., Goldmann, W.H., Bärmann, M. and Isenberg, G. (1992) FEBS Lett. 284, 187–191.
[11] Angelova, M.I., Soléau, F., Méléard, P., Faucon, J.F. and Bothorel, P. (1992) Prog. Colloid. Polym. Sci. 89, 127–131.
[12] Isenberg, G. and Niggli, V. (1998) Intern. Rev. Cytol. 178, 73–125.
[13] Dietrich, C., Goldmann, W.H., Sackmann, E. and Isenberg, G. (1993) FEBS Lett. 324, 37–40.
[14] Bouchard, M., Paré, C., Dutasta, J.-P., Chauvet, J.-P., Gicquaud, C. and Auger, M. (1998) Biochemistry 37, 3149–3155.
[15] Rotman, A., Heldman, J. and Linder, S. (1982) Biochemistry 21, 1713–1719.
[16] Bercovici, T. and Gitler, C. (1978) Biochemistry 17, 1484–1489.
[17] Meyer, D.I. and Burger, M.M. (1979) FEBS Lett. 101, 129–133.