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Matrices with Heterogeneous Distribution of the Binding Sites for Immobilization of Biomacromolecules

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ABSTRACT

The coupling of photosensitive reagents has been carried out with the goal of obtaining the predetermined distribution of binding sites either for "surface" or "spacial" immobilization of biomacromolecules. The correlation holds between light intensity and the number of reactant groups emerged in a matrix.

Index Entries: Arylazides; photolysis; photoimmobilization.

INTRODUCTION

Many papers recently appeared that are devoted to nondiscrete biochemical processes and their application in functional blocks of devices of a new generation based on the information-logical principles. One of the promising approaches is the use of enzymatic reactions, which constitute the elements performing simple operations, such as summation, subtraction, multiplication, and so forth (*1–3*). The progress is determined mainly by the possibility of decreasing the size of the elements and their integration into a single block. Therefore, methods of directed immobilization of protein molecules should be developed.

One can formulate the task for obtaining the predetermined distribution of proteins in a matrix as follows: immobilization of an enzyme in the specified locus on a matrix to produce the required local concentration of

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protein molecules. This, in turn, implies variation of the size of the immobilization locus and the concentration of bound protein and reiteration of the immobilization for another protein.

Photoimmobilization (4) is the best method for making "miniprints" of proteins. In brief, preparations with heterogeneous distribution of photoimmobilized enzyme can be obtained as follows. Initially a matrix has no reactive functional groups. Under irradiation by light heterogeneous in both intensity and spatial coordinates, reactive groups emerge on a carrier capable of interaction with functional groups of an enzyme. Subsequent immobilization transforms the spatial modulation of the input signal at the expense of heterogeneous distribution of an enzyme in a carrier. After addition of an enzyme substrate, the heterogeneous distribution of the immobilized enzyme is displayed showing the different amounts of the enzymatic products from the separate parts of the matrix.

We have previously proposed a method of enzyme immobilization with the use of a photosensitive heterobifunctional reagent, arylazide, carrying the *p*-aldehyde group (5). On irradiation, the azide group eliminates the N_2 molecule (6). Nitrenes produced can react with a carrier in both singlet and triplet states, and the interaction with C—H bonds of a polymer is considered the most probable pathway of the reaction. Since the half-life times of the both reactive species are small (10^{-9} and 10^{-4} s, respectively), the nitrene is incorporated into a particular site of a polymeric chain near the place where a quantum of light is absorbed. Uncoupled reagent is washed out from the matrix by a suitable solvent, and an enzyme is further bound chemically to a polymer throughout arreared functional groups. Since proteins are composed from the same amino acids, the photoimmobilization method is nonspecific with respect an enzyme.

In this article, we demonstrate the possibility of creation of a concentration gradient for the protein binding functional groups on a support. The concentration gradient for an enzyme is produced for both the two-dimensional (surface) case by modulation of light intensity over the binding area and the three-dimensional (spacial) case at the expense of superposition of interference patterns over the volume of a carrier.

MATERIALS AND METHODS

p-Azidobenzaldehyde diethylacetal, *p*-azidobenzylidene aniline, and *p*-azidobenzylidene trimethylactate hydrazide were synthesized according to the general procedure (5), and 4,4'-diazidostilbene-2,2'-disodium disulfonate as described by Miyairi (6). 2,4-Dinitrophenyl hydrazide was purchased from Sigma, a chromatography paper FN-4 from Filtrak, and inert photographic gelatine was of A grade, lot 15553, USSR Standart OST-6-17-421-75.

Photolysis of azides in solution was performed at 20°C in a quartz cuvet with stirring by illumination with a DRSh-250 mercury lamp.

The changes in absorption spectra were recorded with a Beckman 35 spectrophotometer.

For sensibilization, cellulose matrix (FN-4 paper) was placed into solution of azide of specified concentrations, and the impregnated material was air-dried at room temperature in the dark. The reflection from the photomodified matrix treated with 2,4-dinitrophenyl hydrazide was measured at λ = 375 nm using a 25R accessory attached to the spectrophotometer. The number of aldehyde groups was calculated using a calibration curve for the model hydrazone as previously described (*7*).

Gelatin layers were applied to the glass plates by casting (*8*), and 4,4′-diazidostilbene-2,2′-disodium disulfonate (5 mg/mL) was added to a gelatin melt before casting.

He-Cd-laser emission (λ = 375 nm) was used for recording holograms. The recording was performed using the symmetric scheme with two laser beams of equal intensities and angles. The thickness of a layer was measured using a Linnik interferometer MII-4. Other parameters (change in a phase of the passed beam and the diffraction efficiency equal to a ratio between intensities of the passed and the fallen beams) were estimated by procedures specified in ref.(*9*).

RESULTS AND DISCUSSION

p-Azidobenzaldehyde has previously been used for photoimmobilization of enzymes (*5*). This was because aldehyde groups appeared in a matrix during photodecomposing of initial arylazide, and amino groups of proteins react with high rates under mild conditions (*10*). Unfortunately *p*-azidobenzaldehyde shows a tendency to polymerize owing to a reaction between azide and aldehyde functions, which are produced simultaneously during photoactivation (*5*). Several reagents were synthesized that have no aldehyde group but generate it in a hydrolytic process after photoinitiated binding to a matrix. Among these are *p*-azidobenzaldehyde diethylacetal, *p*-azidobenzylidene aniline, and *p*-azidobenzylidene trimethylacetate hydrazide. We also used 4,4′-diazidostilbene-2,2′-disodium disulfonate, which binds with amino groups of proteins through either aldehyde groups produced by permanganate oxidation of the stilbene double bond or by carbodiimide activation of the sulfonate groups.

Table 1 shows kinetic data on the photolysis of azides in solution. A protection of the aldehyde group by different means changes substantially the rate of photolysis. It should be noted, however, that the effectiveness with which photoimmobilization proceeds in a matrix is determined not only by the rate of formation of nitrene, but also by the interaction of the latter with matrix. Figure 1 shows that the use of *p*-azidobenzaldehyde diethylacetal is preferred in comparison with trimethylacetate hydrazide, especially with respect to aniline derivatives under the same conditions of irradiation.

Table 1
Photolysis of Arylazides in Solutions at 20°C[a]

Entry	Azide	Spectral sensitivity, nm	k,[b] s^{-1}
1	*p*-Azidobenzaldehyde diethylacetal	200–320 λ^{max} = 250	$5.8 \cdot 10^{-3}$
2	*p*-Azidobenzilidene trimethylacetate hydrazide	200–350 $\lambda^{max,1}$ = 223 $\lambda_{max,2}$ = 300	$1.75 \cdot 10^{-2}$
3	*p*-Azidobenzilidene aniline	200–350 $\lambda^{max,1}$ = 223 $\lambda^{max,2}$ = 310	0.144
4	4,4′-Diaziodostilbene-2,2′-disodium disulfonate	200–375 λ^{max} = 335	0.2

[a]Compounds 1–3 are dissolved in ethanol, 4 in water.
[b]DRSh-250 full emission spectrum.

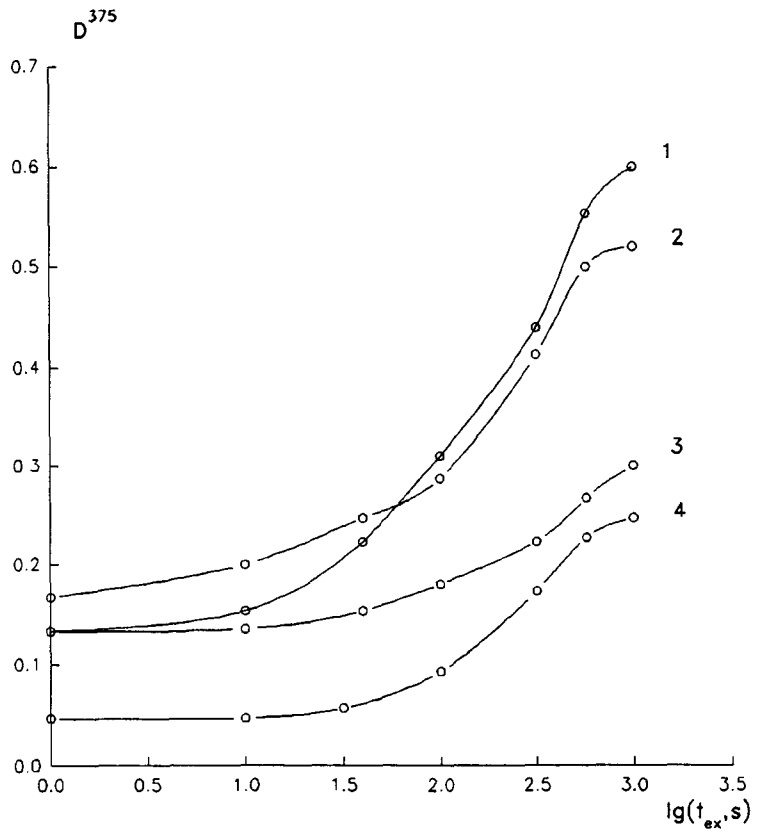

Fig. 1. Dependence of reflection of light from matrices treated with 2,4-dinitrophenyl hydrazide vs irradiation. Matrices are photomodified with: 1—*p*-azidobenzaldehyde diethylacetal; 2—*p*-azidobenzilidene trimethylacetate hydrazide; 3—*p*-azidobenzilidene aniline; 4—4,4′-diazidostilbene-2,2′-disodium disulfonate.

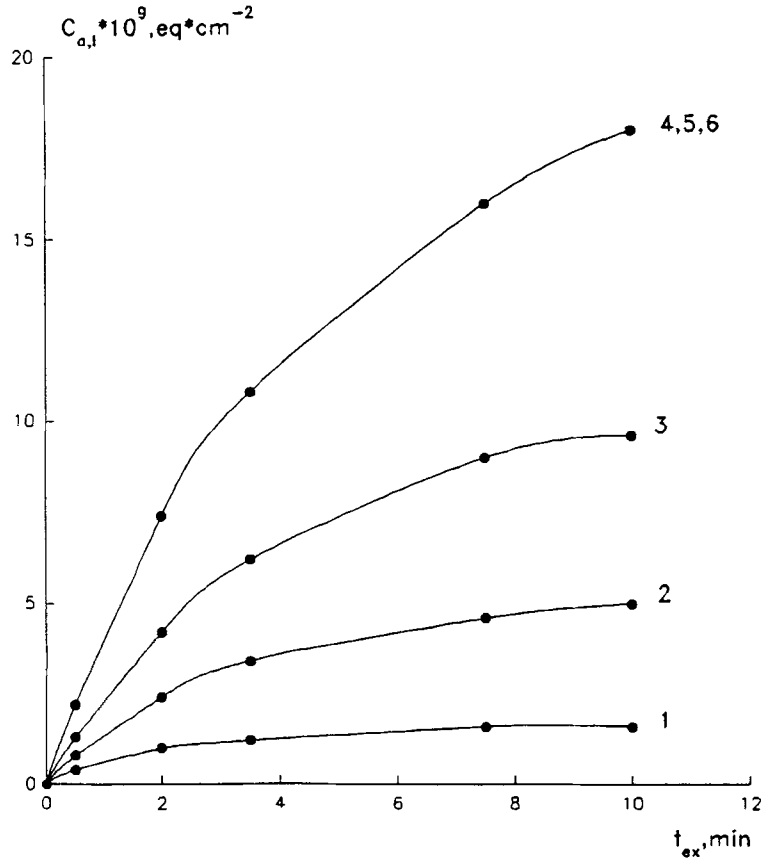

Fig. 2. Kinetics of photobinding of *p*-azidobenzaldehyde diethylacetal with FN-4 matrix irradiated by DRSh-250 mercury lamp as determined by a change of surface concentration of aldehyde groups ($C_{a,l}$) in a layer. The content of photoreagent in the layer in mol/cm^{-1}: 1–$2\cdot10^{-8}$; 2–$6\cdot10^{-8}$; 3–$2\cdot10^{-7}$; 4–$5\cdot10^{-7}$; 5–$1\cdot10^{-6}$; 6–$2\cdot10^{-6}$.

Aldehyde groups can also be generated in a matrix sensibilized with 4,4′-diazidostilbene-2,2′-disodium disulfonate, as described above. This reagent is very promising because of a high solubility in water. Hence, organic solvents that are often added to increase solubility of azides and usually denature enzymes are excluded from the reaction system.

Figure 2 shows the accumulation of aldehyde groups on the FN-4 cellulose matrix sensibilized with *p*-azidobenzaldehyde diethylacetal. We assumed that photochemical reactions proceed solely in the 60-μm thick surface layer because of light scattering and absorption in the matrix. Figure 3 shows the plot of the maximal yields of aldehyde groups (the values derived from Fig. 2) vs the concentration of photoreagent in the layer. One can see that at concentrations higher than $6\cdot10^{-7}$ mol·cm^{-2}, the quantity of aldehyde groups in the matrix is constant. The efficiency of photocoupling is, however, decreased from 6 to 1% owing to increased probability of nitrene molecules to react with each other or with azide

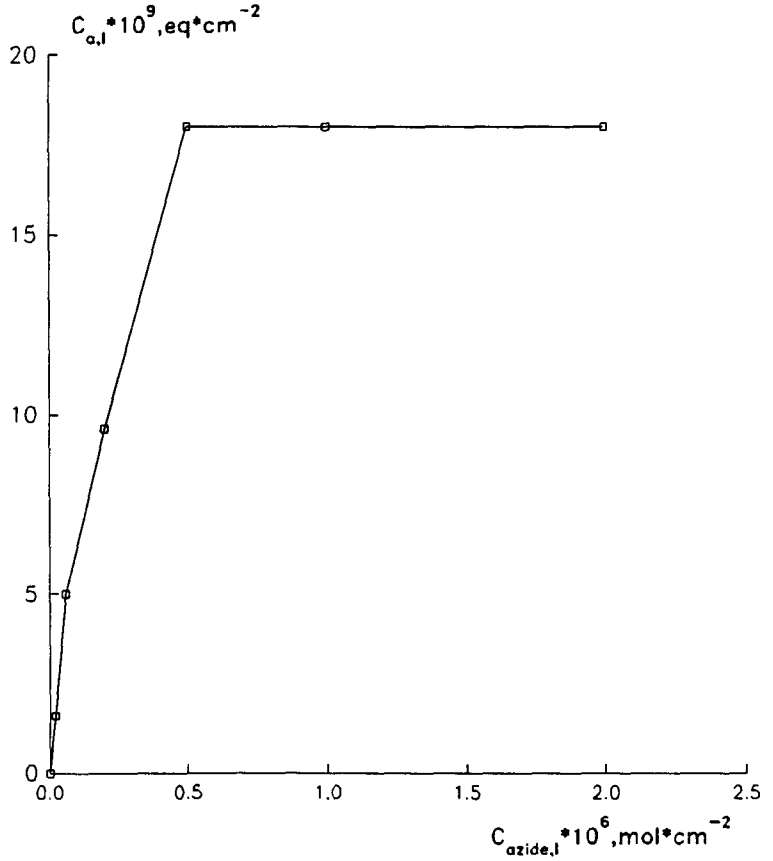

Fig. 3. Dependence of the yields of aldehyde groups ($C_{a,1}$) in a layer on the content of *p*-azidobenzaldehyde diethylacetal ($C_{azide,1}$) in the layer; for irradiation conditions, *see* legend to Fig. 2.

molecules. As a result, azocompounds or polymers of unknown composition are usually produced (*11,12*).

To study the possibility of the spatial modulation of the number of binding sites for an enzyme in the matrix, we used the volume-phase holographic recording technique. This method is based on a photoinduced formation of additional crosslinks in a matrix. In the system under study, 4,4′-diazidostilbene-2,2′-disodium disulfonate can crosslink two adjacent molecules of a polymeric matrix after activation by two photons. Consequently, functional groups for protein binding (sulfo-groups of the diazide in our case) emerge in the volume of the matrix in the particular sites of photocrosslinking. The holographic technique gives a possibility of visualization of crosslink distribution, i.e., the distribution of the centers for the following protein binding.

In terms of the volume-phase holography, at humidities of gelatin layers, W, higher than 20% (highly elastic state of the layer), the expression for a phase change of a deflected light, Φ, is given by the following equation (*13*):

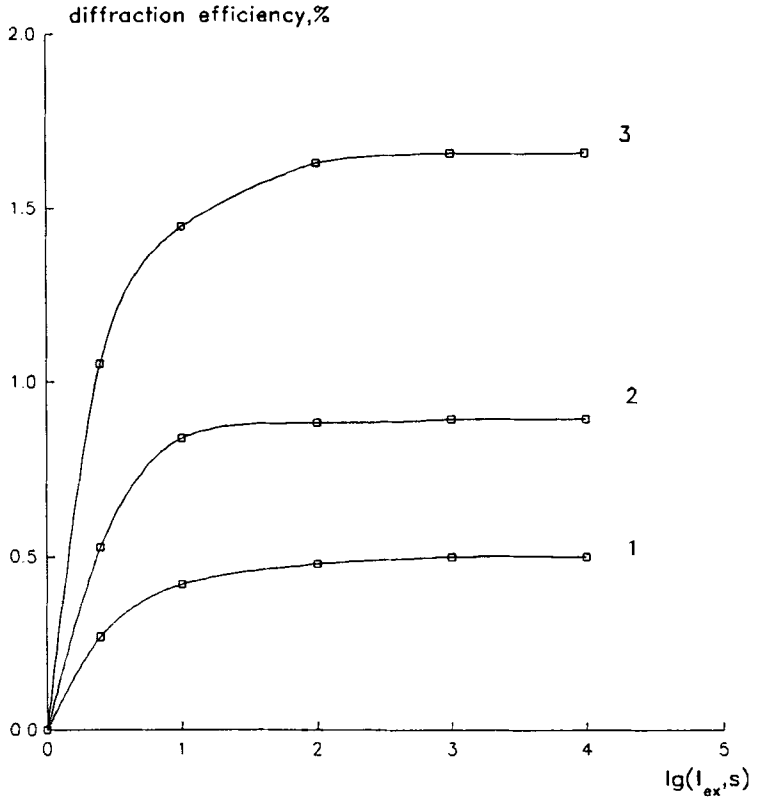

Fig. 4. The increase of diffraction efficiency of the latent image (η_{LI}) during recording at different humidity of the layer, W: 1-30%; 2-60%; 3-80%.

$$\Phi = (\pi/\Lambda)\,[\Delta n \cdot h + \Delta h \cdot (n - 1)] \qquad (1)$$

where λ is the wavelength of irradiation, n is the refractive index, h is the thickness of the layer, and Δn and Δn are the amplitude of refractive index and the variation of the thickness, respectively. Thus, the mechanism of signal transformation in the layer is unequivocally determined by the dependence of Φ on n and h.

Photochemical conversion of 4,4′-diazidostilbene-2,2′-disodium disulfonate in sensibilized gelatin layer led to changes in optical parameters of the layer under the action of laser emission. As a result, a signal of the latent image (LI) was directly observed in the course of the recording process during 0.1-10 s (*see* Fig. 4) with the diffraction efficiency η_{LI}. In the model system used, the ratio $\eta_{LI}/\eta'_{LI} = (\Phi/\Phi')^2$ is close to the ratio $(h/h')^2$ for the layers of different humidity (Fig. 5). Thus, the following expression for Φ derived from Eq. (1) holds:

$$\Phi = (\pi/\lambda) \cdot \Delta n \cdot h \qquad (2)$$

Such relationships are typical of the structure-planar mechanism of modulation of the refractive index. In other words, photohardening by

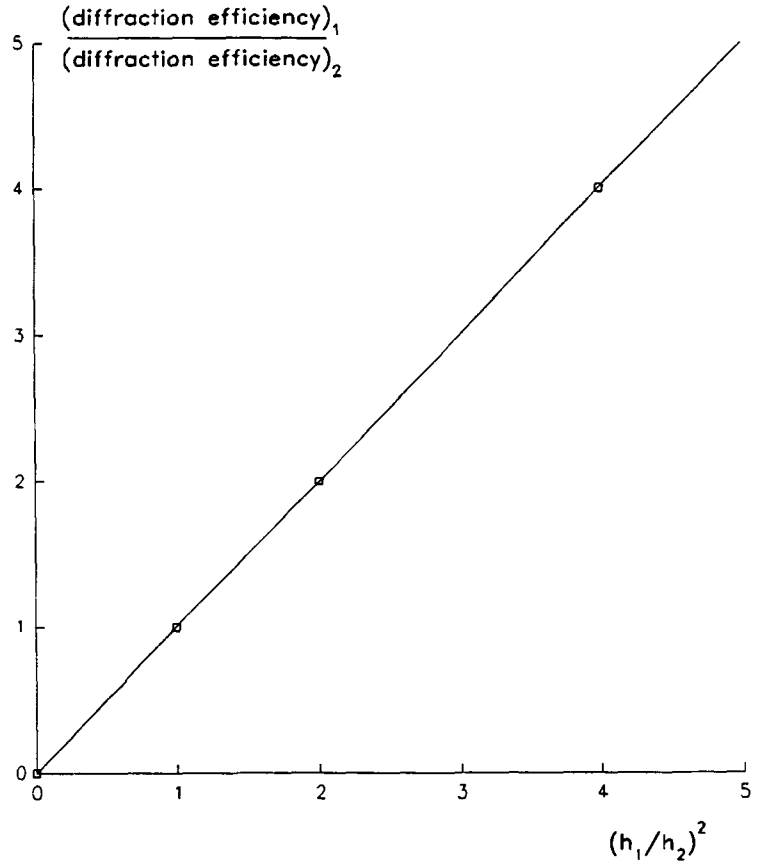

Fig. 5. Dependence of the ratio of diffraction efficiencies of LI-networks vs the square of the ratio of layer thickness for LI-networks recorded in the layers of different humidity.

diazide results only in formation of additional interchain covalent crosslinks without any changes in the three-dimensional network of the gelatin layers preformed in the absence of irradiation. This fact together with the absence of relief in the undeveloped networks of LI gives evidence that a superposition of a holographic signal over diazide-sensibilized polymer layers results in spatial modulation of intermolecular crosslinks. Hence, the modulation of the distribution of binding centers (SO_3-groups in our case) for immobilization of enzymes can be obtained.

CONCLUSIONS

Using arylazides, the matrices are produced with heterogeneous distribution of the binding sites for immobilization of biomacromolecules both on the surface and in the volume of the matrix. There are several reports on protein photoimmobilization devoted to nonspecific sorption in connection with the properties and composition of the matrix. In par-

ticular, subtilisin (*5*), alkaline phosphatase (*14*), and macroglobulin (*15*) were immobilized by using *p*-azidobenzaldehyde diethylacetal; *p*-azidotetrafluorobenzaldehyde was used for immobilization of alkaline phosphatase (*16*); peroxidase was immobilized by using *N*-hydroxysuccinimide-functionalized perfluorophenyl azides (*17*).

The method proposed is applicable for production of matrices heterogeneous in the amount of binding sites. Immobilization of enzymes, antibodies, and so on, in such matrices leads to preparations with the heterogeneous distribution of protein species both on the surface and in the volume of the matrix.

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