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APPLICATION OF 1,1′-CARBONYLDIIMIDAZOLE-ACTIVATED MATRICES FOR THE PURIFICATION OF PROTEINS

III. THE USE OF 1,1′-CARBONYLDIIMIDAZOLE-ACTIVATED AGAROSES IN THE BIOSPECIFIC AFFINITY CHROMATOGRAPHIC ISOLATION OF SERUM ANTIBODIES

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SUMMARY

The characteristics of ligands immobilised onto 1,1′-carbonyldiimidazole-treated cross-linked agarose have been further evaluated. Since the intermediate activated matrix (an imidazolyl carbamate–agarose) is susceptible to nucleophilic attack by free amino groups, but is relatively stable to oxygen nucleophiles, ligands ranging from simple organic primary amines, amino acids, proteins and other biological substances, which contain amino group functionality, can be bound to the agarose via a N-alkylcarbamate (urethane) linkage. This covalent linkage has been found to exhibit good chemical stability to mildly acidic and basic elution conditions. The use of 1,1′-carbonyldiimidazole-activated agarose in the biospecific affinity chromatography of immunoglobulins, present in normal and pathological sera, is described.

INTRODUCTION

Biospecific affinity chromatography is based on the ability of water-soluble biological substances to bind specifically and reversibly to complementary ligands which have been immobilised generally onto insoluble, inert polymers. As a technique for the specific isolation of a particular protein, biospecific affinity chromatography is probably the most powerful procedure currently available. The interaction of antibodies with specific antigens, enzymes with specific substrates, lectins with specific

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carbohydrates and hormones with specific receptors have all found expression in the numerous variants of this technique[1]. The most popular form of ligand immobilisation is to covalently attach an appropriate ligand via its functional groups, such as amino, carboxylic or sulphydryl groups, to a suitable support matrix which has been chemically modified by the incorporation of compatible reactive groups. Precise control over the degree of activation of the insoluble matrix and the multiplicity of site attachment of the ligand molecules are essential requirements for reliable affinity supports. To be fully effective in a chromatographic separation the ligand must be covalently attached to the support matrix, either directly or via a spacer, by a chemical linkage which does not restrict the physical accessibility or abolish the specific binding interactions between the ligand and the biological substances under investigation. In addition, the covalent linkage must be stable to the conditions required for the elution of the absorbed molecules.

The reaction of polysaccharide gels with cyanogen bromide (CNBr) at high pH has become the most widely used method for the activation of these insoluble supports. However, all ligand immobilisation procedures based on CNBr activation of polysaccharide gels suffer from the limited stability of the positively charged N-substituted isourea linkage formed between the ligand and the matrix, relatively low activation levels and difficulties in handling the CNBr reagent despite recent refinements in experimental technique[2–4]. These features of the CNBr method have prompted several groups to develop alternative methods of activation of polysaccharide gels, including the oxirane approach pioneered by Sundberg and Porath[5], and the use of substituted *sec.*-triazines[6], pyrimides[7] and benzoquinones[8] as activating reagents. Recently, we reported[9,10] that 1,1′-carbonyldiimidazole (CDI) and related heterocyclic carbonylating reagents can be used to activate in an easily controlled manner, insoluble cross-linked polysaccharide gels, agarose–polyacrylamide copolymers and glycophase-controlled pore glasses. The intermediate activated matrix was found[11] to couple in good yields with N-nucleophiles to give a non-basic urethane linkage, devoid of additional charge groups due to the activation procedure in contrast to the CNBr approach[12,13]. This paper reports additional studies on the characteristics of ligands immobilised onto cross-linked Sepharose CL-6B via the CDI procedure, including the use of affinity matrices for the purification of antibodies present in normal and pathological sera.

EXPERIMENTAL

Materials

Cross-linked agaroses (Sepharose CL-4B and CL-6B) were purchased from Pharmacia (Uppsala, Sweden) and the polyacrylamide-agarose copolymer Ultrogel AcA-44 was from LKB (Bromma, Sweden). CDI was obtained from Pierce (Rockford, IL, U.S.A.) as was CDI-activated 6% cross-linked agarose (Reacti-Gel (6X), activation level, 20–25 μmoles/ml gel). [U-^{14}C]Glycine and ^{125}I (carrier-free) were obtained from The Radiochemical Centre (Amersham, Great Britain). Chloramine T was from May and Baker (Dagenham, Great Britain). All other chemicals were AnalaR reagent grade.

Human thyroglobulin was extracted from human thyroids by the method of Salvatore *et al.*[14] and fractionated by gel filtration on Sepharose CL-4B using a 10

m*M* Tris–HCl–150 m*M* NaCl, pH 8.0, buffer as described previously[15]. Human and rabbit immunoglobulin preparations were obtained by ammonium sulphate fractionation of the appropriate sera, followed by DEAE-cellulose anion-exchange chromatography using established procedures. Specific antisera to the proteins used in this study were raised in this laboratory using New Zealand white rabbits or outbred strain guinea pigs and goats; other antisera were purchased from Hoechst-Behringwerke (Frankfurt, G.F.R.).

Methods

Sepharose CL-6B and Ultrogel AcA-44 were activated with CDI in dioxan using the solvent exchange procedure as described elsewhere[9,10]. The number of active groups present on the matrix was determined by titration analysis using a Radiometer pH titrator (TTT2). In a typical experiment treatment of the agarose matrix with CDI at the level of 300 μmoles reagent/g gel gave an activated matrix containing *ca.* 120–150 μmoles active groups/g gel. CNBr activation of Sepharose CL-6B was carried out using the procedure of March *et al.*[2]. Proteins were generally immobilised onto the activated matrices at a solution concentration of 2–10 mg/ml using a 0.5 *M* sodium chloride–1 *M* sodium carbonate (pH 10.0) buffer for 12–18 h at 4 C. For protein couplings below pH 8.0 a 0.5 *M* sodium chloride–0.1 *M* sodium phosphate buffer was employed. Affinity chromatography was generally carried out at 18 C using 10–100 ml of the appropriate affinity adsorbent packed into glass columns of 12–20 mm I.D. The biospecific ligand–matrix supports were equilibrated in 150 m*M* NaCl–25 m*M* NaH_2PO_4, pH 7.2 or 20 m*M* Tris–HCl, pH 7.2, prior to an affinity separation. The sample proteins to be separated were pumped slowly onto the appropriate affinity column, over a period of 1–2 h, and the non-bound components eluted with the initial column equilibration buffer. Following desorption of the bound components, the eluates were immediately adjusted to pH 7.0 and desalted by gel filtration on short Sephadex G-25 columns, by rapid dialysis or by ultrafiltration through Millipore PM10 membranes (Millipore, Bedford, MA, U.S.A.). Lyophilisation of dialysed solutions containing affinity purified antithyroglobulin antibodies resulted in considerable loss of antibody binding capacity with some preparations. Protein concentrations in the eluates was monitored by the absorbance at 254 and 280 nm and in other samples by the Bradford assay[16]. Haemagglutination assays were carried out using the tanned red cell procedure[17] and antibody binding capacity was evaluated from a specific double antibody "sandwich" technique[18]. Immunodiffusion and immunoelectrophoresis were carried out in 1% agarose in a barbital buffer. Protein iodinations with ^{125}I were carried out as described previously[18].

RESULTS AND DISCUSSION

When the affinity between the interactive substances is high, as in the case with polypeptide hormone–receptor or antigen–antibody interactions, or when the abundance of a specific component under purification is very low, the major limitation of the use of biospecific affinity chromatography is the propensity of the coupled ligand to leak from the support under the eluting conditions. Our previous studies[10,11] on the purification of trypsin and several other proteins using a CDI-activated matrix, led us to conclude that the N-alkylcarbamate linkage formed by the coupling reaction

was reasonably stable at room temperature over the pH range 2.5–10. In order to elucidate more closely the leakage of ligands coupled to agarose via the CDI activation procedure, the rate of release of [^{125}I]thyroglobulin and [U-^{14}C]glycine from their respective specific sorbents was investigated at different times in buffers of different pH. Representative data obtained in these experiments are shown in Fig. 1. These and related experiments with immobilised labelled immunoglobulins, confirmed that the N-alkylcarbamate linkages, formed between the amino groups of amino acids or proteins and the CDI-activated Sepharose CL-6B, exhibit good stability over the pH range normally used in affinity chromatography. Extended periods of time at low or high pHs, *e.g.* treatment of the ligand-matrix with chaotropic eluents at pHs below 3.0 or above 11.0, result in enhanced ligand release and or denaturation of the protein ligands examined in this study. A practical consequence of the low level of thyroglobulin release over the range pH 3–8.5 from the affinity supports prepared via CDI methods was that immunoadsorbents could be prepared in bulk and stored in neutral buffers containing an anti-microbrial agent for more than six months prior to use. Tesser *et al.*[19,20] have studied the liberation of a variety of ligands from sorbents prepared by means of the CNBr activation procedure and found, for example, that the rate of liberation of the ligand from a cAMP-spacer-agarose matrix (2–5 μmoles cAMP substituted/ml gel) in a 5 m*M* Tris–HCl–15 m*M* NaCl, pH 8.0, buffer was *ca.* 6 pmol/ml gel/min. Compared to the date reported in these and other studies[21,22] on the ligand stability of the N-substituted isourea linkage formed by the reaction between amino groups and CNBr-activated agarose, ligands bound to agarose gels by the urethane linkage formed in the CDI procedure are detached more slowly, *i.e.* they are *ca.* 20 fold more stable at comparable pHs[10]. The urethane immobilised ligands

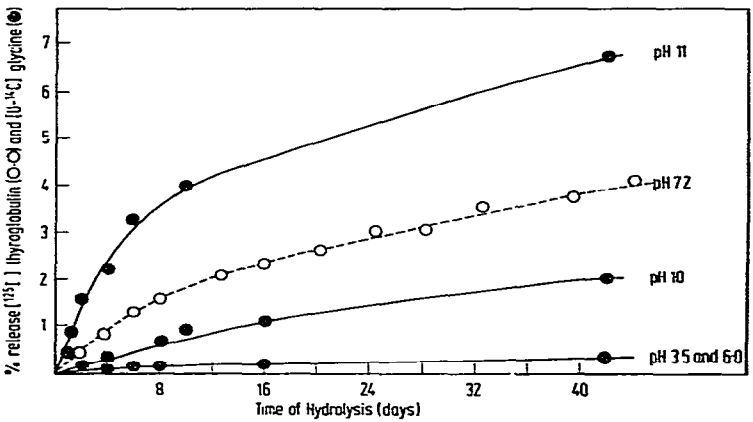

Fig. 1. Comparison of the rates of solvolytic detachment for [U-^{14}C]glycine immobilised onto CDI-activated Sepharose CL-6B (*ca.* 100 μmoles active groups/g moist gel) and for human [^{125}I]thyroglobulin immobilised onto CDI-activated Sepharose CL-6B (*ca.* 300 μmoles active groups/g moist gel) as a function of pH and incubation time at 4°C. The following 100 m*M* buffer solutions were employed: potassium hydrogen phthalate (pH 3.5); potassium hydrogen phosphate (pH 6.0); Tris–HCl (pH 7.2); potassium carbonate (pH 10.0) and disodium hydrogen phosphate (pH 11.0). The residual ligand attached to the matrix at different time intervals was determined for the [U-^{14}C]glycine matrix by digestion with 10 *M* HCl at 60°C and measurement of the cpm of a neutralised 1:10 aliquot and for the human [^{125}I]thyroglobulin-matrix by direct measurement of the cpm of an aliquot, compensated for the spontaneous loss of ^{125}I.

TABLE I

COMPARATIVE COUPLING YIELDS FOR ACTIVATED SEPHAROSE CL-6B

Protein	*CNBr-activated matrix**	*CDI-activated matrix***
Human thyroglobulin	70	75
Human IgG	92	93
Human myeloma IgG	94	83
Rabbit anti-human serum immunoglobulins	–	98
Bovine thyrotrophin	89	67
Ovine thyroid binding proteins***	82	85
Human thyroid binding proteins***	82	80
Human serum proteins	64	40

* Activation level *ca.* 35 μmoles active groups/g moist gel.

** Activation level *ca.* 100 μmoles active groups/g moist gel.

*** A partially purified thyroid membrane glycoprotein fraction which exhibits binding activity for thyroid stimulating autoantibodies.

exhibit stabilities at intermediate pHs similar to ligands coupled to epoxy-activated agarose[5] and hydrazine-activated polyacrylamide[20] gels.

In Tables I and II are shown comparative results for the coupling of human thyroglobulin and several other proteins to the CDI- and the CNBr-activated agarose matrices. These results demonstrate that this group of proteins can be coupled to CDI-activated cross-linked and copolymer polysaccharide gels with coupling yields similar to those found with CNBr-activated polysaccharides. Highly activated agaroses are know to lead to multiple point attachment of protein ligands to the gel support. This can result in impaired biological binding properties of the immobilised protein, attributed to induced conformational changes[23]. However, with the thyroglobulin immunoadsorbent prepared by the CDI approach, no significant loss of binding activity of this antigen for its corresponding antibodies was apparent even with

TABLE II

COMPARATIVE COUPLING YIELDS FOR CDI-ACTIVATED POLYSACCHARIDE GELS

	*Sepharose CL-6B**		*Ultrogel AcA-44***	
	*Coupling yield (%)****	*Concentration of protein on matrix (μmoles/g)* §*	*Coupling yield (%)****	*Concentration of protein on matrix (μmoles/g)* §*
Human thyroglobulin	62	0.34	41	0.15
Human IgG	92	2.0	62	1.4
Bovine thyrotrophin	80	2.4	69	2.0
Ovine thyroid binding protein	91	3.6	52	2.0

* Activation level *ca.* 100 μmoles active groups/ml gel.

** Activation level *ca.* 60 μmoles active groups/ml gel.

*** Based on percentage of added substance.

§ Expressed as μmoles of coupled proteins per gram of dry polymer.

very highly activated (up to 300 μmoles active sites/ml gel) su0ports. In fact, when the coupling conditions were varied to favour a small number of binding sites per protein molecules, *e.g.* by using a slightly acidic coupling buffer and a gel with a low degree of activation, the protein ligand still coupled in satisfactory yield but the resultant support was not very effective as an affinity matrix. In this case, the corresponding antibodies bound very tightly to the immobilised thyroglobulin and this necessitated the application of more forcing elution conditions (3.5 *M* KCNS, pH 11.0). This in turn, resulted in deterioration of the affinity support due to enhanced leakage of the ligand as well as denaturation of the immobilised antigen molecules.

In order to study the binding capacity of the human thyroglobulin immunoadsorbent (CDI method), increasing amounts of a human serum from a patient, L1, with thyroid autoimmunity were added to a standard quantity of the immunoadsorbent. Antibody activity in the serum and the eluate was measured by passive haemagglutination using the tanned red cell procedure[17] and by a double antibody capacity binding assay[18]. The results, summarised in Fig. 2, show that the maximal capacity of the immunoadsorbent was reached for this serum with a loading of *ca.* 900 μl serum/ml gel, equivalent to an immunoadsorbent capacity of *ca.* 2.4 mg bound antibody/ml gel. The column binding capacity was similar when an immunoglobulin fraction purified on DEAE-cellulose, was used in place of whole serum.

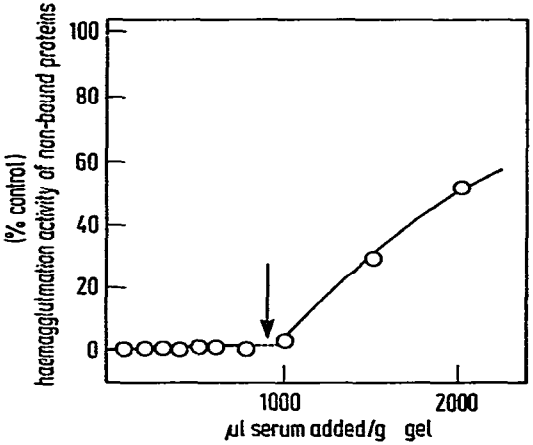

Fig. 2. Capacity of immobilised human thyroglobulin immunoadsorbent (*h*-thyroglobulin–Sepharose CL-6B) in relationship to amount of serum antithyroglobulin autoantibodies applied. The serum in aliquots of 100 μl was applied to equilibrated columns containing 2.75 g moist gel cake of the immunoadsorbent. The haemagglutination activity of the non-bound peak is expressed as a percentage of the activity for the control experiment using columns packed with Sepharose CL-6B alone. The immunoadsorbent was prepared from CDI-activated Sepharose CL-6B (*ca.* 180 μmoles active groups/g moist cel cake).

Rabbit anti-bovine thyroglobulin antibodies have been purified using conjugated polyaminopolystyrene or *p*-amino benzylcellulose matrices[24]. More recently, human antithyroglobulin autoantibodies have been fractionated from other serum components using immunoadsorbents prepared from glutaraldehyde insolubilised supports[25] and CNBr-activated agarose[26,27]. In the present study with CDI-activated matrices, it was found that desorption of the bound autologous or heterologous

antibodies from the *h*-thyroglobulin matrix could be achieved under a variety of conditions including 2 *M* sodium iodide and 2–3.5 *M* sodium thiocynate, but most successfully with 200 m*M* glycine HCl, pH 2.8 (Fig. 3). Elution recovery of the binding activity of the antibodies purified by these affinity chromatographic procedures were evaluated by haemagglutination and double antibody techniques and compared to the binding capacity of the original serum. In several instances, very good recoveries of binding activities for autologous and heterologous antibodies were obtained *e.g.* serum L1 gave an elution recovery of specific autoantibodies greater than 80%, although generally the recoveries were in the region of 30–50% for the 200 m*M* glycine HCl (pH 2.8) or 2 *M* NaCNS (pH 9.0), desorption buffers and 10–20% with 3 *M* NaCNS (pH 10.0) 5 *M* urea or 5 *M* guanidine hydrochloride (pH 3.0) desorption eluents. Similar results have been noted[26,27] in earlier studies on anti-thyroglobulin antibodies purified by means of CNBr-activated gels.

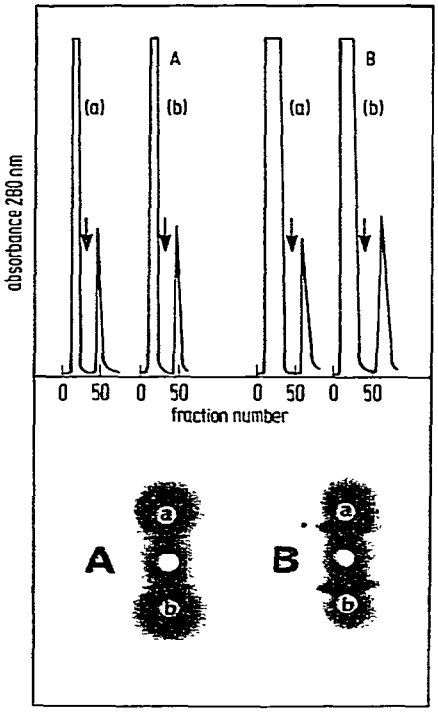

Fig. 3. A, Chromatography of human sera containing antithyroglobulin autoantibodies and B, chromatography of rabbit sera containing heterologous antithyroglobulin antibodies on columns containing a human thyroglobulin–Sepharose CL-6B immunoadsorbent, prepared by the CDI-activation procedure. The equilibration and washing buffer was 150 m*M* NaCl–20 m*M* Tris–HCl (pH 7.2). For the experiments shown in A and B the same columns were used for 10 consecutive affinity separations, runs 1 and 5 are shown in (a) and (b), respectively. Sample loadings: A, 1.5 ml serum; B, 5.0 ml serum. Specific desorption was performed with a 200 m*M* glycine–HCl (pH 2.8) buffer, added at the points indicated. Column size: A, 2.8 ml gel; B, 3.0 ml gel.

A further example of the use of a biospecific adsorbent derived from CDI-activated agaroses is shown in Fig. 4. Using a human IgG–Sepharose CL-6B conjugate, goat and rabbit anti-human IgG antibodies were fractionated from other

serum components using step elution conditions. The antibody capacity of the immunoadsorbent prepared for these experiments was *ca.* 6 mg goat/rabbit anti-human IgG antibodies/ml agarose gel. In Fig. 4A is shown the elution profile for the biospecific separation of goat antihuman IgG antibodies usihg different NaI concentrations (0.5, 2.0, 3.0 and 4.0 *M*) as the desorption reagent for the specific antibodies. Similar desorption patterns have been noted previously[28,29] in the affinity chromatographic separation of mixed populations of antibodies with different affinities for a specific ligand.

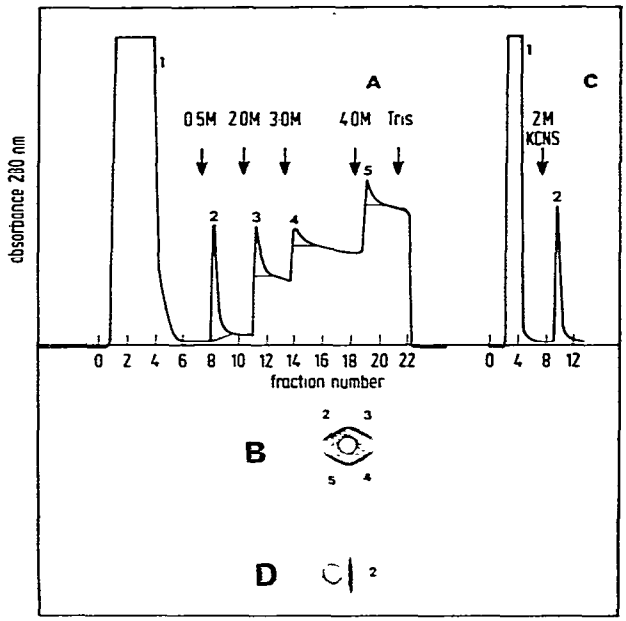

Fig. 4. Biospecific affinity chromatography of goat (A) and rabbit (C) anti-human IgG serum on human IgG–Sepharose CL-6B immunoadsorbents. The human IgG-gel conjugates were prepared from CDI-activated Sepharose CL-6B, 100 μmoles active groups/g moist gel cake. The equilibration and washing buffer was 150 m*M* NaCl–20 m*M* Tris–HCl (pH 7.2). For the experiment shown in A goat anti-human IgG serum (20 ml) was applied to the immunoadsorbent column (1.6 g gel, *ca.* 2 μmoles immobilised human IgG/g gel). After washing with 30 ml of buffer, elution of specific antibodies was affected with NaI at various concentrations (arrows). The elution profile is uncorrected for base-line absorbance changes due to the high NaI concentrations. Immunodiffusion patterns of the recovered fractions *versus* human IgG are shown in B. For the experiment shown in C, rabbit anti-human IgG serum (1 ml) was applied to the immunoadsorbent column (2.1 g gel, *ca.* 2 μmoles immobilised human IgG/g gel). After washing with 30 ml buffer, elution of specific antibodies was affected with 2 *M* KCNS (pH 9.5) (arrow). The immunodiffusion of the recovered fraction *versus* human IgG is shown in D.

In conclusion, the above experiments illustrate that the CDI-activation procedure can be used to produce effective immunoadsorbents for the fractionation of antibodies by biospecific affinity chromatography. Under suitable coupling conditions, the amounts of protein ligands which can be immobilised onto CDI-activated polysaccharide supports compare favourably with those obtained via the CNBr method. Under similar coupling conditions the CNBr-activated matrices appear to be more reactive than the corresponding CDI-activated gels. In view of the precise

control over the level of activation which can be achieved with the CDI reagent and the good stability of ligands immobilised onto insoluble gels activated by this method, CDI-activated agaroses provide a useful alternative to existing methods for the preparation of ligand–polysaccharide conjugates. With polysaccharide supports activated by CDI to very high substitution levels a decrease in the swollen bed volume of the ligand-matrix occurs, indicative of progressive cross-linking and a modified pore structure. Under high pH coupling conditions the ligand coverage on the matrix tends to approach limiting values with these highly activated supports, possibly due to steric restrictions. During the present study, immunoadsorbents prepared with polysaccharide gels, previously activated with CDI up to 300 μmoles active groups/ml gel, were found to be suitable in the purification of autologous and heterologous immunoglobulins with specificities directed against human thyroglobulin and human IgG. In studies involving the immobilisation of less robust protein ligands, lower levels of matrix activation may be required[3,23,30]. In these cases, it should be possible to tailor the level of CDI activation to ensure that optimal binding activity of the ligand is preserved whilst still permitting a mode of interaction between the ligand and the substances under purification such that desorption can be achieved under mild elution conditions.

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