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Note

Preparation of avidin-labeled protein nanoparticles as carriers for biotinylated peptide nucleic acid

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Abstract

The possibility of preparing protein nanoparticles followed by covalent linkage of avidin was investigated. Free sulfhydryl groups were introduced onto the surface of protein nanoparticles either by aldehyde quenching with cysteine or reaction of free amino groups with 2-iminothiolane. The number of primary amino groups and sulfhydryl groups on the surface of the resulting particles was quantified with site-specific reagents. Avidin was attached to the surface of the thiolated nanoparticles via a bifunctional spacer which reacted in a first step with amino groups of avidin and in a second step with the sulfyhdryl groups introduced onto the surface of the nanoparticles. Biotinylated peptide nucleic acid (PNA) as a model compound for biotinylated drugs was effectively coupled to the nanoparticles by complex formation with the covalently attached avidin. Since the formation of the interaction between biotin and avidin is very rapid and stable a highly effective drug carrier system for biotinylated compounds such as PNAs was achieved.

Keywords: Human serum albumin nanoparticles; Gelatin nanoparticles; Covalent linkage; Avidin; Peptide nucleic acid

1. Introduction

In the past different materials such as acrylic acid derivatives and poly(lactic acid) were employed for the preparation of nanoparticles. Drugs were adsorbed onto the particle surface, bound by ionic interaction or were entrapped in the matrix of the nanoparticles. For the binding of proteins to nanoparticles it was shown that monoclonal antibodies were well adsorbed onto the surface of acrylic particles, but that serum compounds led to a competitive displacement [1]. The same was true for the influence of ionic buffer compounds on the displacement of charged drugs bound to the surface of nanoparticles with ion-exchange characteristics [2]. An alternative approach to stabilize drug binding is the covalent linkage of compounds to the surface of the carriers. In 1974 Molday et al. [3] described the binding of proteins to cross-linked latex spheres using the aqueous carbodiimide and cyanogen bromide reaction. With protein based nanoparticles the same strategy can easily be adapted because of the variety of established protein modifying reagents. First attempts in this direction were undertaken by Akasaka et al. [4] who prepared immunospecific bovine serum albumin (BSA) nanospheres by attaching rabbit anti-human IgG by the glutaraldehyde method.

In the present study the possibility of preparing nanoparticles based on gelatin and human serum albumin (HSA), the introduction of sulfhydryl groups onto the surface of the particles as well as the covalent linkage of avidin was investigated. We made an attempt to develop a universal nanoparticulate carrier system for the attachment of a variety of biotinylated compounds. For proof of concept, the binding of a biotinylated 15-mer peptide nucleic acid (PNA) for the therapy of HIV-infections was performed.

2. Materials and methods

2.1. Reagents and chemicals

Gelatin type A (175 bloom), glutaraldehyde 8%, human serum albumin (fraction V), L-cysteine hydrochloride and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma (Steinheim, Germany). 2-Iminothiolane, 5,5′-dithio-bis-(2-nitrobenzoic acid), NeutrAvidin™, D-Salt™

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10 ml Dextran Desalting columns and BCA protein assay kit were purchased from Pierce (Rockford, USA).

2.2. Preparation of protein nanoparticles containing free sulfhydryl groups

Gelatin nanoparticles were prepared as described previously by Coester et al. [5]. 500 mg of L-cysteine were added to quench the remaining free aldehyde groups of the bifunctional cross-linker glutaraldehyde. After a reaction time of 12 h the particles were purified by centrifugation at 20 000 × *g* over 10 min and redispersed in acetone/water (30/70). Purification was repeated until no free cysteine was detected in the supernatant using 5,5′-dithio-bis-(2-nitrobenzoic acid). After the last redispersion the acetone was evaporated on a waterbath at 50°C.

For the preparation of human serum albumin nanoparticles 500 mg HSA were dissolved in 5.0 ml water and desolvation was performed by dropwise addition of 10 ml ethanol under stirring at 500 rev./min. Crosslinking of the protein was achieved by addition of 87 μl aqueous 8% glutaraldehyde solution. Ethanol was evaporated under stirring at RT. The protein concentration of the resulting suspension was determined by gravimetry. For the thiolation, 2 ml of the nanoparticle suspension were diluted with 2 ml nitrogen saturated Tris–buffer pH 8.5 (5 mM EDTA) and then incubated with 1 ml of a 3% 2-iminothiolane solution (Traut's reagent) in water for 120 min. The unreacted 2-iminothiolane was removed by repeated centrifugation of the mixture at 12 000 × *g* for 5 min and redispersion of the particles in ethanol/water (20/80).

The particle size was determined by photon correlation spectroscopy (PCS) with a BI-200 SM Goniometer Version2 (Brookhaven Instruments, Holtsville, USA).

2.3. Determination of the number of amino groups on the particle surface

The number of amino groups on the particle surface was determined as described by Weber et al. [6].

2.4. Determination of the number of sulfhydryl groups on the particle surface

To 35.0 μl of a solution of 0.4% 5,5′-dithio-bis-(2-nitrobenzoic acid) in water, 175.0 μl nanoparticle suspension and 1750.0 μl phosphate buffer pH 7.5 (1mM EDTA) were added and the reaction mixture was shaken at 500 rev./min for 15 min at 25°C. Four hundred and fifty microliters of the reaction mixture were transferred to Microcon 30 microconcentrators (Amicon, Witten, Germany) followed by centrifugation at 6500 × *g* for 30 min. The filtrates were assayed spectrophotometrically at 412 nm for 2-nitro-5-thiobenzoic acid. The number of sulfhydryl groups on the particle surface was calculated relative to a standard curve prepared from L-cysteine solutions.

2.5. Avidin activation and conjugation to thiolated nanoparticles

NeutrAvidin™ (5 mg) was dissolved in 800 μl PBS pH 7.0 and 200 μl of an aqueous 0.8% m-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (sulfo-MBS) solution were added. The reaction mixture was incubated at 600 rev./min for 60 min at 25°C. After equilibration of a D-Salt™ column with the conjugation buffer, the sample mixture was applied onto the column and eluted with 1.0 ml aliquots of the buffer. The NeutrAvidin™ elution was monitored by absorbance at 280 nm. The fractions containing the maleinimide-activated avidin were pooled and used for the conjugation step with the thiolated nanoparticles.

The thiolated nanoparticle suspension (1.0 ml) containing between 2 and 5% (w/v) nanoparticles was added to 1.0 ml of maleinimide-activated avidin solution. The conjugation mixture was incubated for 2 h under stirring at RT. The unreacted maleinimide-activated avidin was removed by centrifugation of the mixture at 12 000 × *g* for 10 min and redispersion of the particles in water. The supernatant of the centrifugation step was used for determination of uncoupled avidin by a standard BCA assay.

2.6. Peptide nucleic acid loading of the carrier systems

A biotinylated 15-mer peptide nucleic acid (biotin-TGT-CTT-CTT-TTT-TAT-Lys-$CONH_2$ = biotin-PNA) was synthesized by TIB-Molbiol (Berlin, Germany). Fifty microliters of an aqueous 400 μM solution of biotin-PNA was added to 950.0 μl suspension of the avidin-conjugated nanoparticles. The mixture was stirred over a period of 1 h followed by centrifugation of the mixture at 14 000 × *g* over 10 min. The supernatant was analyzed by reversed phase HPLC. A 120 × 4 mm ID Eurosil Bioselect 300 C18 (5 μm) column was used with a 12 min linear 3.5–45.5% acetonitrile gradient in 0.1% phosphoric acid/water and PNA was detected at 260 nm.

3. Results and discussion

There have been many studies describing the preparation of nanoparticles in combination with drug loading by adsorption procedures. In order to circumvent the problem of desorption of the bound drug, in the present study avidin was covalently attached to the nanoparticles enabling the binding of a variety of biotinylated drugs by complex formation.

Gelatin nanoparticles were prepared by a double desolvation procedure [5]. In order to introduce free sulfhydryl groups onto the surface of the particles, the residual aldehyde functionalities of the glutaraldehyde used for crosslinking of the nanoparticles were quenched with L-cysteine. The preparation procedure resulted in gelatin nanoparticles with a narrow size distribution (285.8 ± 16.4 nm; polydispersity index 0.0722 ± 0.0278; $n = 4$). As an alternative protein

material for nanoparticle preparation human serum albumin (HSA) with its defined primary structure [7] was used. As a consequence the number of functionalities available for protein modifications can be calculated stoichiometrically. After desolvation of the HSA the obtained nanoparticles were stabilized by addition of glutaraldehyde. Under the assumption that the bifunctional aldehyde predominantly reacts with the ε-amino groups of lysine the chosen amount of glutaraldehyde was by far to low (molar ratio glutaraldehyde/lysine: 1:7) to react and cross-link all of the lysine residues. Free amino groups remained on the surface of the HSA nanoparticles and were calculated after reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS) followed by the determination of unreacted TNBS in the supernatant. It was found that 10.8 $\pm$ 3.3 mol/mol HSA ($n = 4$) amino groups were present on the surface of the nanoparticles. These free amino groups were used subsequently for the thiolation of the particle surface with a 70-fold molar excess of 2-iminothiolane (Traut's reagent), that reacts with primary amines and introduces sulfhydryl residues to the surface of HSA nanoparticles. The result of the thiolation was determined by the use of 5,5′-dithio-bis-(2-nitrobenzoic acid), that reacts highly specifically with sulfhydryl groups and produces 2-nitro-5-thiobenzoic acid, a quantitatable yellow colored product. Up to 60% of the free amino groups were converted to sulfhydryl groups by Traut's reagent. The particle size of the HSA nanoparticles after thiolation was 407.2 $\pm$ 7.6 nm (polydispersity index 0.1597 $\pm$ 0.0267). With increasing storage time of the thiolated particles the amount of sulfhydryl groups decreased probably because of an oxidative formation of disulfide cross-links. Four weeks after the thiolation the content of free sulfhydryl groups was reduced to 3.3 mol/mol HSA. For this reason, further conjugation steps based on thiolated nanoparticles should be performed immediately after particle preparation.

The objective of this study was the preparation of a polymer carrier system that enables the binding of compounds by the avidin-biotin system. The bond formation between avidin and biotin is the strongest known non-covalent, biological interaction between protein and ligand. The dissociation constant of the egg white avidin-biotin-complex was reported to be $K_D = 0.6 \times 10^{-15}$ M at pH 7 [8]. The formation of the interaction is very rapid and stable. A lot of compounds are available in their biotinylated form and therefore enable the binding to the proposed nanoparticles. In this study, avidin was bound to the surface of the nanoparticles by covalent linkage and only the biologically active principle was in the biotinylated form. In comparison to biotinylated nanoparticles, the advantage of the proposed strategy is that there should be no particle aggregation after addition of avidin.

The following part the discussion will focus on HSA nanoparticles, but in principle also applies to the avidin conjugation to the gelatin nanoparticles (Table 1). For the conjugation between the thiolated nanoparticles and avidin, the modified avidin derivative NeutrAvidin™ with a molecular weight of approximately 60 kDa was used. NeutrAvidin™ was activated with the heterobifunctional cross-linker m-maleimidobenzoyl-*N*-hydroxysulfosuccinimide (sulfo-MBS), which reacted in a first step with the primary amino groups of the protein. For this reaction sulfo-MBS was used in 50-fold molar excess over NeutrAvidin™. After a reaction time of 1 h unreacted sulfo-MBS was separated from maleinimide-activated NeutrAvidin™ by size exclusion chromatography. The activated NeutrAvidin™ was then coupled to the nanoparticles via a thioether linkage between the sulfhydryl groups on the nanoparticle surface and the sulfhydryl reactive maleinimide group of the activated NeutrAvidin™. The final conjugation sample contained between 10 and 25 mg/ml HSA nanoparticles corresponding to HSA concentrations between 154 and 385 μM and 1.25 mg/ml (= 20.8 μM) NeutrAvidin™. The BCA assay revealed that between 10.8 and 22.0% (= 2.3–4.6 μM) of the avidin was covalently attached to the surface of the HSA nanoparticles. An increase in the excess of sulfo-MBS over NeutrAvidin™ from 50- to 100-fold (Table 1: sample HSA NP2 vs. HSA NP 1) led to an increase of the avidin binding to 62.2% (= 12.9 μM) but diminished the PNA binding. The particle size of the conjugate was about 410 nm. The complete manufacturing process for the surface modified HSA nanoparticles is presented in Fig. 1.

A peptide nucleic acid (PNA) as a new concept for gene-targeted drugs was used as a model compound in order to

Table 1
Summary of the physicochemical characterization of the avidin-labeled protein nanoparticles[a]

Parameter	Gelatin NP 1	Gelatin NP 2	Gelatin NP 3	HSA NP 1	HSA NP 2	HSA NP 3
Particle size by PCS [nm]	277.0	288.0	291.0	401.8	401.8	412.6
Particle content [mg/ml]	16.7	16.7	10.0	24.0	24.0	10.0
Total avidin content [μM]	20.8	20.8	18.8	20.8	20.8	25.0
Avidin bound to NP [μM] (%)	6.1 (29.5)	9.6 (46.0)	–*	4.6 (22.0)	12.9 (62.2)	–*
Total PNA content [μM]	20.0	20.0	10.0	20.0	20.0	10.0
PNA bound to NP [μM] (%)	12.3 (61.5)	10.0 (50.0)	9.6 (96.0)	15.6 (78.0)	10.1 (50.5)	7.4 (74.0)

[a] Three batches of gelatin and HSA based nanoparticles (NP) were prepared, respectively; the batches were compared concerning particle size, particle content, avidin binding and PNA binding of the final preparations; *not determined.

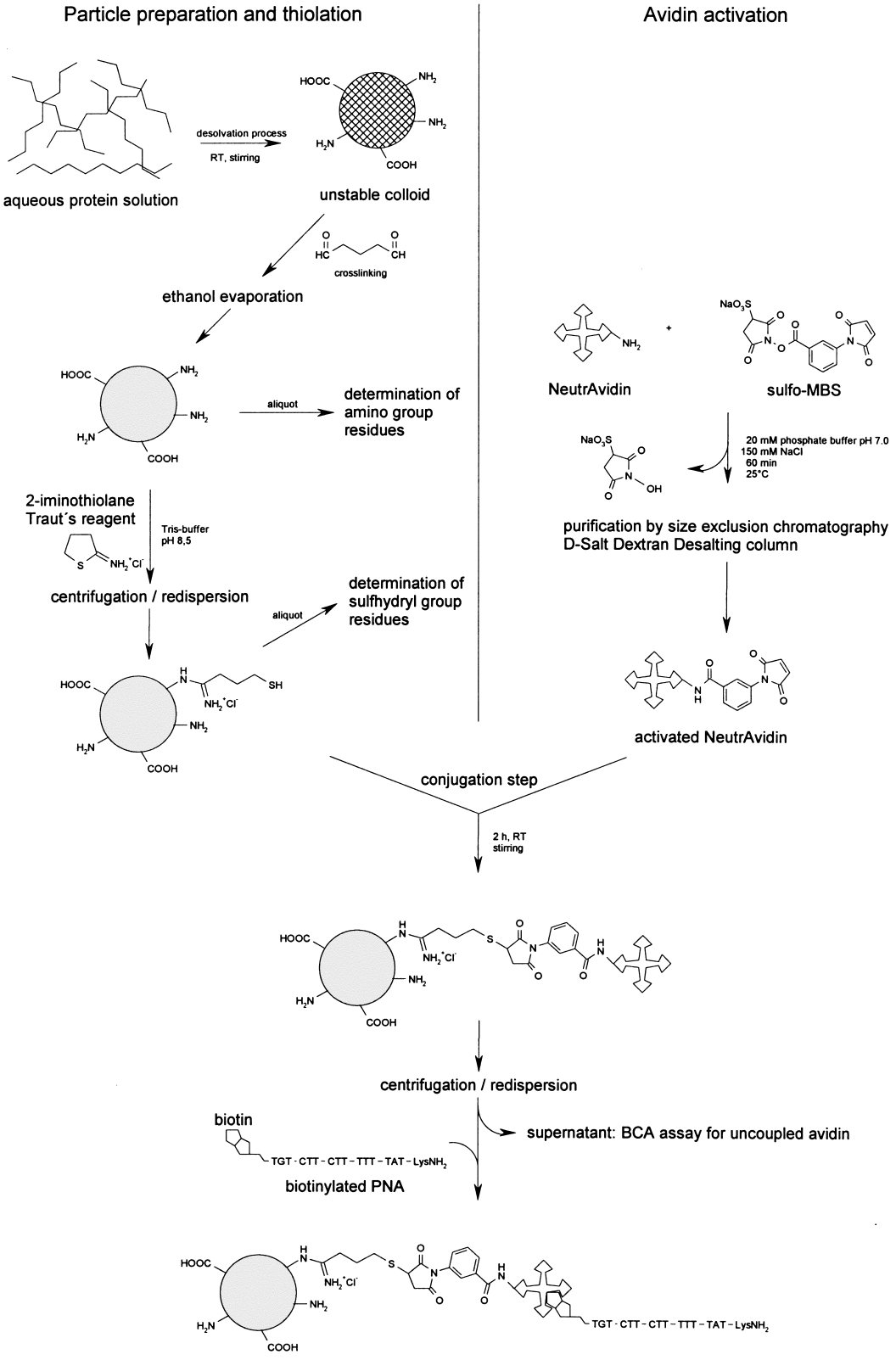

Fig. 1. Complete manufacturing process of the avidin-modified HSA nanoparticles and biotin-PNA binding by complex formation.

test the suitability of the proposed carrier system. PNAs are DNA analogues in which the nucleobases are attached to a *N*-(2-aminoethyl)glycine backbone. They have been demonstrated to bind strongly and sequence-specifically to double-stranded DNA [9] and possess an enhanced resistance to nucleases. However, PNAs undergo negligible

transport across cell membranes. The PNA used in the present study is a 15-mer, wherein the amino terminus was biotinylated and represents an antisense to the pol gene of human immunodeficiency virus type 1 and 2.

For the drug loading experiments the biotinylated PNA was added to the avidin-modified nanoparticles at a 20 μM concentration level. After incubation and ultracentrifugation, the unbound PNA was determined in the supernatant of the carrier system by reversed phase HPLC (Fig. 2). In addition to the biotinylated PNA, the HPLC chromatogram revealed the presence of minor impurities in the substance. After drug loading of the avidin-modified carrier system, the peak of the biotin–PNA exhibited a distinct decrease whereas the peak areas of the impurities remained unchanged. This demonstrates that only the biotinylated compound was bound to the proposed carrier system by specific avidin-biotin interaction. This was supported by loading experiments in which biotin–PNA and nanoparticles without avidin modification were incubated and no binding of the antisense compound to the carrier was observed (results not shown). It should be expected that the efficiency of drug binding is influenced by the amount of avidin bound to the nanoparticles and therefore by the activation step of NeutrAvidin™ with sulfo-MBS. However, no clear correlation between the extent of avidin binding and PNA binding to the nanoparticles was observed (Table 1). For example, an increase in the excess of sulfo-MBS over NeutrAvidin™ in the activation step from 50- to 100-fold (sample HSA NP2 vs. HSA NP 1) led to a distinct

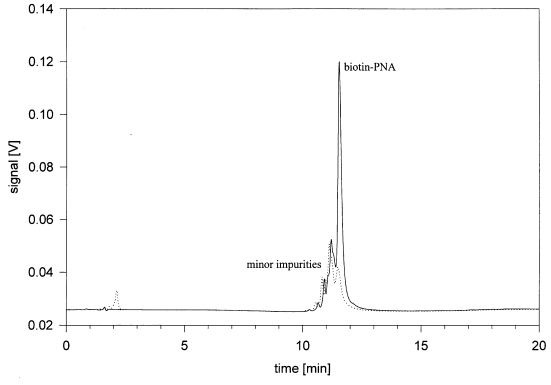

Fig. 2. HPLC-chromatogram of a biotin–PNA solution (—) vs. the supernatant of biotin–PNA bound to HSA-nanoparticles (··· ···). Only the biotinylated compound was bound to avidin-modified nanoparticles whereas the amount of the minor PNA impurities remained unchanged.

increase of the avidin binding but simultaneously, diminished the PNA binding from 78.0 to 50.5% of the 20 μM PNA concentration. Probably, this has to be attributed to structural changes in the avidin molecule by the excess of the cross-linker sulfo-MBS leading to a reduction of the biotin-binding activity. This is in accordance with earlier studies on streptavidin, which showed that the modification of an average of 3 mol tyrosine/mol subunit inactivated completely the biotin-binding activity of the protein [10].

In conclusion, the present study shows that protein based nanoparticles can easily be surface modified with sulfhydryl groups which can be used for the covalent attachment of proteins. Since the formation of the interaction between biotin and avidin is very rapid and stable a highly effective carrier system for biotinylated compounds such as PNAs was achieved. Further studies are under way to further characterize the proposed carrier system and to determine its antiviral activity in HIV-infected monocytes/macrophages.

References

[1] L. Illum, P.D.E. Jones, J. Kreuter, R.W. Baldwin, S.S. Davis, Adsorption of monoclonal antibodies to polyhexylcyanoacrylate nanoparticles and subsequent immunospecific binding to tumour cells in vitro, Int. J. Pharm. 17 (1983) 65–76.

[2] K. Langer, F. Stieneker, G. Lambrecht, E. Mutschler, J. Kreuter, Methylmethacrylate sulfopropylmethacrylate copolymer nanoparticles for drug delivery, Part II: arecaidine propargyl ester and pilocarpine loading and in vitro release, Int. J. Pharm. 158 (1997) 211–217.

[3] R.S. Molday, W.J. Dreyer, A. Rembaum, S.P.S. Yen, Latex spheres as markers for studies of cell surface receptors by scanning electron microscopy, Nature 249 (1974) 81–83.

[4] Y. Akasaka, H. Ueda, K. Takayama, Y. Machida, T. Nagai, Preparation and evaluation of bovine serum albumin nanospheres coated with monoclonal antibodies, Drug Design Deliv. 3 (1988) 85–97.

[5] C.J. Coester, K. Langer, H. von Briesen, J. Kreuter, Gelatin nanoparticles by two step desolvation – a new preparation method, surface modifications and cell uptake, J. Microencapsulation (2000) in press.

[6] C. Weber, C. Coester, J. Kreuter, K. Langer, Desolvation process and surface characteristics of protein nanoparticles, Int. J. Pharm. 194 (2000) 91–102.

[7] D.C. Carter, J.X. Ho, Structure of serum albumin, Adv. Protein Chem. 45 (1994) 153–203.

[8] N.M. Green, Avidin and streptavidin, Methods Enzymol. 184 (1990) 51–67.

[9] P.E. Nielsen, M. Egholm, R.H. Berg, O. Buchardt, Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide, Science 254 (1991) 1497–1500.

[10] G. Gitlin, E.A. Bayer, M. Wilchek, Studies on the biotin-binding sites of avidin and streptavidin Tyrosine residues are involved in the binding sites, Biochem. J. 269 (1990) 527–530.