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Preparation of avidin-labelled gelatin nanoparticles as carriers for biotinylated peptide nucleic acid (PNA)

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Abstract

The possibility of preparing uniform nanoparticles consisting of proteins such as gelatin followed by covalent linkage of avidin was investigated. Gelatin nanoparticles were prepared by two step desolvation. Functional groups at the surface of the particulate system were quantified with site-specific reagents. The surface of the nanoparticles was thiolated and avidin was covalently attached to the nanoparticles via a bifunctional spacer at high levels. Biotinylated peptide nucleic acid (PNA) was effectively complexed by the avidin-conjugated nanoparticles. Avidin-conjugated protein nanoparticles should prove as potential carrier system for biotinylated drug derivatives in antisense therapy.

Keywords: Nanoparticles; Gelatin; Covalent linkage; Avidin; Peptide nucleic acid (PNA)

In the past different particle materials were used for the preparation of nanoparticles, leading to a variation in surface properties. Most often acrylic acid derivatives were used and drugs were adsorbed on the particle surface, bound by ionic interaction or were entrapped in the matrix of the nanoparticles. An alternative approach for a stable drug binding is the covalent linkage of compounds to the carriers surface. This concept requires functional groups on the surface of the nanoparticles which can easily be used for site-specific chemical modification. Already Molday et al. (1975) described the binding of molecules containing primary amino groups to cross-linked latex spheres by the carbodiimide, cyanogen bromide, and glutaraldehyd methods. In the field of protein based nanoparticulate systems this principle can easily be realized because of the multitude of established protein-modifying reagents. First attempts were undertaken by Akasaka et al. who prepared immunospecific bovine serum albumin (BSA) nanospheres by binding them with rabbit antihuman IgG (Akasaka et al., 1988). Kissel and Roser prepared surface modified BSA nanoparticles by quenching residual aldehyde functionalities with primary amines and demonstrated the stability of the surface-modification in different media (Roser and Kissel, 1993).

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In the present study the possibility of preparing uniform nanoparticles based on gelatin, the introduction of sulfhydryl groups on the surface of the particles as well as the covalent linkage of avidin was investigated. An attempt was made to establish a universal nanoparticulate carrier system for a multitude of biotinylated compounds such as biotinylated antibodies, fluorescent dyes or drugs. The binding of an antisense-compound was focused on, namely a biotinylated peptide nucleic acid (PNA) for the therapy of HIV-infections.

The gelatin nanoparticles were prepared by the two step desolvation method (Coester et al., 1999). After the crosslinking of the gelatin particles, 500 mg of cysteine (Sigma, Germany) were added to quench the remaining free aldehyde groups of the bifunctional glutaraldehyde. After a reaction time of 12 h the particles were purified by centrifugation and redispersion. The coupled cysteine was determined by measuring the free SH-groups with Ellman's reagent.

Five mg NeutrAvidin™ were dissolved in 800 μl PBS pH 7.0 and 200 μl aqueous solution of 0.8% *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (sulfo-MBS) were added. The reaction mixture was incubated for 60 min at 25°C. The activated avidin was purified on a D-Salt™ Dextran Desalting column. The fractions containing the protein were pooled and used for the conjugation step with the thiolated nanoparticles.

A total of 1.0 ml of the thiolated nanoparticle suspension was added to 1.0 ml of maleinimide-activated avidin solution. The mixture was stirred for 2 h. The unreacted maleinimide-activated avidin was removed by centrifugation of the mixture at $12\,000 \times g$ for 10 min and redispersion of the particles in water. The supernatant was used for determination of uncoupled avidin by a standard BCA protein assay.

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). A total of 50.0 μl of a 400 μM aqueous 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 at $14\,000 \times g$ over 10 min. The supernatant was analysed by reversed-phase HPLC using a 120×4 mm ID Eurosil Bioselect 300 C18 (5 μm) column with a 12 min linear 3.5–45.5% acetonitril gradient in 0.1% phosphoric acid/water.

Reagents that bind sequence specifically to DNA are of major interest in the therapy of genetic diseases, viral infections or tumor therapy. Besides the major field of oligonucleotides PNA as a new basis for gene-targeted drugs are of growing interest and therefore were used as a model drug for this study. PNAs are DNA analogues in which the nucleobases are attached via methylenecarbonyl linkers to a polyamid backbone and have been demonstrated to bind strongly and sequence-specifically to DNA (Nielsen et al., 1994). However, PNAs undergo negligible transport across cell membranes (Hanvey et al., 1992) and therefore require appropriate drug carrier systems. As PNAs are very poorly adsorbed to the surface of nanoparticles, in the present study avidin was bound to the surface of the nanoparticles by covalent linkage and a biotinylated PNA derivative was attached by spontanous complex formation.

The gelatin NP were prepared by a two step desolvation process and further surface modified by quenching the free aldehyd groups of glutaraldehyd with an excess of L-cysteine. The results of the SH-group determination showed that about 18 μmol of cysteine per g gelatin was bound to the surface of the prepared gelatin NP. Because of the low stability of free SH-groups in suspension during the 3 month storage the SH-group content was reduced to about 9 μmol SH-groups per g gelatin.

For the conjugation between the thiolated nanoparticles and avidin, the heterobifunctional crosslinker *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide (sulfo-MBS) was employed, which reacts in a first step with primary amine groups of the avidin derivative. The BCA protein assay revealed that between 29.5 and 46.0% (= 6.1–9.6 μM) of the avidin was covalently attached to the surface of the gelatin nanoparticles (Table 1). The unbound NeutrAvidin™ was removed by a repeated washing of the final conjugate.

Table 1
Summary of the physicochemical characterization of the avidin-labelled gelatin nanoparticles (three different batches)

Parameter	Gelatin NP 1	Gelatin NP 2	Gelatin NP 3
Particle size by PCS (nm)	277.0	288.0	291.0
Particle content (mg/ml)	16.7	16.7	10.0
Total avidin content (μM)	20.8	20.8	18.8
Avidin bound to NP (μM) (%)	6.1 (29.5)	9.6 (46.0)	–[a]
PNA content (μM)	20.0	20.0	10.0
PNA bound to NP (μM) (%)	12.3 (61.5)	10.0 (50.0)	9.6 (96.0)

[a] Not determined

For the drug loading experiments 20 μM biotinylated PNA was added to the avidin-modified nanoparticles. After the incubation period the unbound PNA was determined in the supernatant of the carrier system (Fig. 1). Beside the biotinylated compound the chromatogram of the PNA solution revealed the presence of minor impurities in the substance. After drug loading to the avidin-modified nanoparticles the peak of the biotin–PNA exhibited a distinct decrease whereas the peak areas of the impurities remained unchanged. This lays stress to the fact that only the biotinylated compound was bound to the proposed carrier system by avidin–biotin interaction. This was supported by loading experiments in which biotin–PNA and nanoparticles without avidin modification were incubated and revealed no binding of the antisense compound to the carrier (results not shown).

In conclusion, the present study shows that gelatin nanoparticles can easily be surface modified with sulfhydryl groups which can be used for the covalent attachment of proteins such as avidin. As the formation of the interaction between biotin and avidin is very rapid and stable a highly effective carrier system for biotinylated compounds is achieved. In comparison to drug binding by adsorption or ionic interactions the proposed carrier system holds promise for an effective loading with biotinylated compounds. Further studies are under evaluation to determine the antiviral activity of the PNA in HIV-infected monocytes/macrophages and should prove such avidin-modified nanoparticles as potent drug delivery systems for antisense therapy.

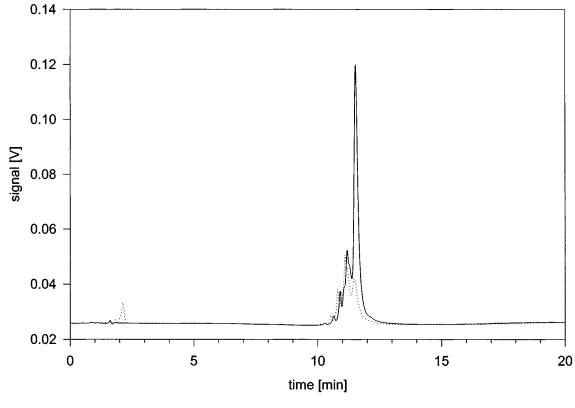

Fig. 1. HPLC-chromatogram of a biotin–peptide nucleic acid (PNA) solution (—) vs. the supernatant of biotin–PNA bound to nanoparticles (· · ·).

References

Akasaka, Y., Ueda, H., Takayama, K., Machida, Y., Nagai, T., 1988. Preparation and evaluation of bovine serum albumin nanospheres coated with monoclonal antibodies. Drug Des. Deliv. 3 (1), 85–97.

Coester, C., Langer, K., von Briesen, H., Kreuter, J., 1999. Gelatin nanoparticles by two step desolvation — a new preparation method, surface modifications and cell uptake. J. Microencapsulation (accepted).

Hanvey, J.C., Peffer, N.J., Bisi, J.E., Thomson, S.A., Cadilla, R., Josey, J.A., Ricca, D.J., Hassman, C.F., Bonham, M.A., Au, K.G., Carter, S.G., Bruckenstein, D.A., Boyd, A.L., Noble, S.A., Babiss, L.E., 1992. Antisense and antigene properties of peptide nucleic acids. Science 258, 1481–1485.

Molday, R.S., Dreyer, W.J., Rembaum, A., Yen, S.P., 1975. New immunolatex spheres: visual markers of antigens on lymphocytes for scanning electron microscopy. J. Cell Biol. 64 (1), 75–88.

Nielsen, P.E., Egholm, M., Buchardt, O., 1994. Peptide nucleic acid (PNA). A DNA mimic with a peptide backbone. Bioconj. Chem. 5 (1), 3–7.

Roser, M., Kissel, T., 1993. Surface-modified biodegradable albumin nano- and microspheres. Eur. J. Pharm. Biopharm. 39 (1), 8–12.