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Avidin-biotin technology in targeted therapy

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Importance of the field: The goal of drug targeting is to increase the concentration of the drug in the vicinity of the cells responsible for disease without affecting healthy cells. Many approaches in cancer treatment are limited because of their broad range of unwanted side effects on healthy cells. Targeting can reduce side effects and increase efficacy of drugs in the patient.

Areas covered in this review: Avidin, originally isolated from chicken eggs, and its bacterial analogue, streptavidin, from *Streptomyces avidinii*, have extremely high affinity for biotin. This unique feature is the basis of avidin-biotin technology. This article reviews the current status of avidin-biotin systems and their use for pretargeted drug delivery and vector targeting.

What the reader will gain: The reader will gain an understanding of the following approaches using the avidin-biotin system: i) targeting antibodies and therapeutic molecules are administered separately leading to a reduction of drug dose in normal tissues compared with conventional (radio)immunotherapies; ii) introducing avidin gene into specific tissues by local gene transfer, which subsequently can sequester and concentrate considerable amounts of therapeutic ligands; and iii) enabling transductional targeting of gene therapy vectors.

Take home message: Avidin and biotin technology has proved to be an extremely versatile tool with broad applications, such as pretargeting, delivering avidin gene into cells enabling targeting of biotinylated compounds and targeting of viral vectors.

Keywords: avidin, biotin, gene therapy, pretargeting, streptavidin, targeting, viral vector

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1. Introduction to targeting and radioimmunotherapy

Targeted drug delivery is one of the most important challenges in pharmaceutical research. At the turn of the twentieth century, Paul Ehrlich proposed the idea of 'magic bullets' in targeting a compound to eradicate a disease. It took 50 years to achieve the first implementation of the targeted antibody-conjugated radionuclide. The goal in drug targeting is to concentrate the therapeutic agent in the tissues of interest while reducing its relative concentration in remaining tissues. This improves the therapeutic window and decreases accompanying drug toxicity [1]. Despite encouraging preliminary studies, many applications for cancer treatment are still limited because of their broad range of unwanted side effects on healthy cells, indicating a clear need to develop new methods that would allow more efficient, easy and robust targeted drug therapy [2].

The characterization of disease markers at the molecular level is essential in developing targeting strategies, as are approaches to predict the clinical behavior of the affected tissue. To this end, natural or artificial ligands have been designed that bind to the altered membrane-associated proteins on the cell surface [3]. Sometimes coupling therapeutics to carriers, such as liposomes or synthetic polymers, is needed

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Article highlights.

- Avidin and its bacterial counterpart streptavidin have the highest affinity known in nature towards biotin (Kd ~ 10^{13} – 10^{15} M^{-1}).
- Biotin is small soluble vitamin H, essential to life as it functions as a coenzyme in metabolic reactions.
- High affinity between avidin and biotin has been utilized in pretargeting applications, which can be divided into two- to five-step approaches.
- Pretargeting has been used in several clinical trials to treat various cancer types.
- Another pretargeting application is to display avidin or biotin on the surface of a target cell, which can be achieved using virus-mediated gene transfer or biotinylation of the target tissue.
- Avidin and biotin can function as a bridge between the virus and a targeting moiety.
- Avidin and biotin technology has proved to be an extremely versatile tool with broad applications.

This box summarizes key points contained in the article.

to protect the drug during the transport [4]. Therapeutic agents (e.g., drugs, viral vectors, radionuclides) can be targeted by either direct or indirect methods. In direct methods, the therapeutic agent is linked directly to the targeting ligand (e.g., antibody) [5]. Pretargeting is an indirect method where the target site is first pretargeted, followed by administration of the therapeutic agent, which then accumulates in the pretargeted area (**Figure 1**). Direct targeting is a straightforward one-step approach, whereas pretargeting requires two or more steps but has been shown to enhance target-to-background tissue ratios [1].

Antibodies, commonly immunoglobulin G (IgG), are often used in targeting because of their specific binding activity to antigens on the surface of target cells, usually cancer cells [1]. Indium-131 (^{131}In) and yttrium-90 (^{90}Y) are frequently used radioconjugates in antibody labeling [1]. This approach of tissue targeting is called radioimmunotherapy. Many clinical trials with directly conjugated antibodies are in progress. So far, radioimmunotherapy has given the best results in the treatment of non-Hodgkin's lymphoma [1]. The first approved radiolabeled antibodies for the treatment of cancer were antibodies directed to CD20 labeled either by ^{131}In (Tositumomab; Bexxar, GlaxoSmithKline, London, UK) or by ^{90}Y (Ibritumomab tiuxetan; Zevalin, Biogen Idec, Cambridge, MA, US). In addition to targeting of cancer cells, approaches targeting tumor vessels [6] or the blood–brain barrier [7] have been also developed. Nevertheless, the long serum half-lifes of antibodies yield low tumor-to-normal-tissue ratios and prolong radiation exposure to normal organs and bone marrow, causing toxic effects. One way to overcome this consists of administering the labeled antibody locally [8]. For inaccessible tumors, however, other strategies to expand the therapeutic window between the tumor and healthy organs have been sought. To this end, drug pretargeting has proved superior, as indicated by several preclinical studies [1]. This strategy is discussed in more detail in the following sections.

The extremely high affinity of avidin for biotin (Kd ~ 10^{-15}) [9] has been the main reason behind the wide-ranging use of the system in many kinds of biotechnological applications. Since 1988 this system has been exploited in pretargeting [10] and also used in gene therapy for several viral vector studies, especially for targeting and purification purposes [11,12]. This article reviews the targeting applications where the avidin-biotin system has been used to achieve targeted therapy.

2. Basic characteristics of avidin and biotin

2.1 Avidin and streptavidin

Avidin is found in oviparous vertebrates including various birds, reptiles and amphibians, but no analogous protein has been detected in mammalian species. Chicken avidin, isolated from the hen egg white, and streptavidin, secreted by several species of *Streptomyces*, are functionally [9] and structurally [13,14] analogous proteins. The main biological function of (strept) avidin is to bind biotin, a vital enzymatic cofactor also known as vitamin H [9]. Both proteins share a rather similar secondary, tertiary and quaternary structure forming tetrameric complexes of ~ 60 kDa in which each subunit can bind one molecule of biotin with extremely high affinity (Kd ~ 10^{13} – 10^{15} M^{-1}) [13,14]. This interaction is primarily thought to represent a natural defense mechanism against biotin requiring microbes [15], but also extra roles have been suggested for avidin [16]. In addition to their exceptional ligand binding characteristics, avidin and streptavidin are exceptionally stable against high concentrations of denaturing agents, proteases, and a wide range of pH and temperature [9].

Despite similarities, avidin and streptavidin differ in their primary amino acid sequence (41% similarity), glycosylation, isoelectric point (pI) [9], immunological reactivity and pharmacokinetics. Each avidin monomer has a single oligosaccharide moiety whereas streptavidin is devoid of sugars. Avidin is a basic protein with a high pI 10.5, whereas streptavidin has a mildly acidic isoelectric point of ~ 6. Owing to these differences, streptavidin has the advantage of lower nonspecific binding to lectin-like and negatively charged molecules than avidin. This has been shown further to affect the pharmacokinetics of these proteins, with avidin eliciting a shorter plasma half-life compared with streptavidin [17,18]. The glycosylation is responsible for avidin's tendency to accumulate in the liver, whereas its accumulation in the kidneys is mainly a result of high pI [19]. Modifications such as deglycosylation or neutralization lengthen the circulation time of avidin. Streptavidin remains for a longer time in the circulation as it has lower nonspecific binding to most tissues compared with the avidin, however it too accumulates in the kidneys [17,18]. Avidin and streptavidin are not found in mammalian tissues, providing a good rationale for their use. Both avidin and streptavidin are immunogens, which may prevent their repeated use. However, it appears

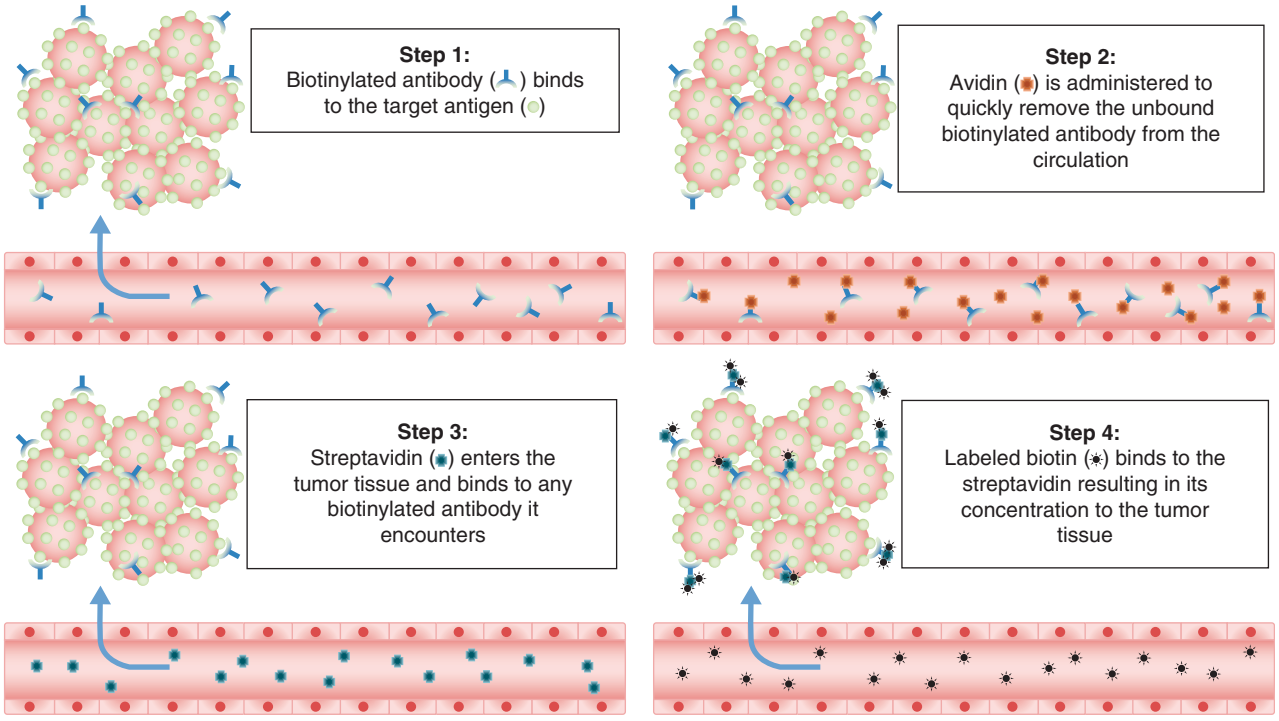


Figure 1. Schematic of the four-step pretargeting procedure. A biotinylated monoclonal antibody is first targeted to the tumor. Avidin, which is glycosylated and quickly cleared by the liver, is injected to remove the biotinylated antibody from the blood. Streptavidin, which is non-glycosylated, then enters the tumor and binds tightly to the biotinylated antibody. Once avidin and streptavidin are cleared from the blood, labeled biotin is administered.

that avidin has a lower immunogenicity compared with streptavidin, thus being more appropriate for pretargeting applications [20,21].

In attempts to improve the usability of the (strept)avidin technology, the proteins involved have been modified both chemically and genetically. Genetic modification of (strept) avidin can change the binding activity to biotin, stability, and alter the physicochemical properties, structure or function of the proteins. Examples of modifications are adjustable pH-dependent binding of avidin to biotin, a monomeric form of avidin, or in order to change binding properties of streptavidin to biotin by conjugating stimuli-responsive polymers to streptavidin (e.g., to respond to light or temperature), reviewed in [22].

2.2 Biotin and biotinylation

Biotin is a small (244 Da) water-soluble vitamin H synthesized by bacteria, yeasts, molds, algae and some plants, but required by all forms of life [23]. Biotin has been shown to play an essential role in regulating gene expression in *Escherichia coli* and in mammalian cells [24]. Moreover, in mammalians > 2000 biotin-dependent genes have been identified and biotinylation of histones plays an essential role in cell proliferation, gene silencing and cellular response to DNA damage [25].

Biotin is bound to cellular carboxylases and decarboxylases, which catalyze the transfer of CO_2 to and between metabolites in gluconeogenesis, lipogenesis, amino acid degradation and energy transduction [26]. The attachment of biotin to the ε-amino group of a specific lysine moiety in carboxylases is catalyzed by biotin protein ligase in an ATP-dependent reaction [24]. The number of biotinylated carboxylases varies from species to species: in *E. coli*, the bacterial biotin ligase BirA biotinylates only one protein called acetyl-CoA carboxylase, whereas in mammalian cells acetyl-CoA carboxylase, methyl crotonyl-CoA carboxylase, propionyl-CoA carboxylase and puryvate carboxylase are biotinylated [24].

Biotinylation of proteins is an attractive alternative to epitope tagging owing to the strong (strept)avidin–biotin interaction. Although a wide range of chemical biotinylation techniques exist, they are limited by the fact that chemical biotinylation is not site-specific, requires prior purification of the substrate and can lead to inactivation of the target protein. Given the difficulties in chemical biotinylation, many efforts are now devoted to developing systems exploiting nature's own biotinylation machinery. Early studies using this approach took advantage of the 1.3S subunit of *Propioni bacterium shermanii* transcarboxylase (PSTCD), which is naturally biotinylated at lysine 89 [24]. The expression of PSTCD fusion protein led to its enzymatic biotinylation in *E. coli* and *Saccharomyces cerevisiae*. Later on, this approach was expanded to mammalian cells and animals [27,28]. Isolation of new shorter biotin acceptor peptide (BAP) substrates

(~ 13 – 20 residues) for BirA have increased further the potential of this system for biotechnolgical applications [29].

3. Avidin and biotin in targeting

The specific characteristics of avidin have been shown to be of great advantage in drug targeting: the high positive charge of avidin augments the efficiency of cellular uptake of biotin-coated particles [30-32], whereas incubation of bioconjugated avidin with biotinylated cell lines results in rapid surface attachment and endocytosis, with efficiencies approaching 100% [33]. In addition, (strept)avidin has been demonstrated to accumulate in specific tissues [17,18], especially in tumors *in vivo* [34]. Consequently, avidin alone can enable some targeting of gene therapy vectors to specific tissues, while tissue specificity can be altered by biochemical modification of the protein. The strong avidin–biotin interaction can be used to develop targeted therapies by the biotinylation of ligands or tissues *in vivo* [35-37]. Several studies have shown promising results using monoclonal antibodies in targeting of biotinylated therapeutic or diagnostic compounds by means of avidin in experimental animals [38-40]. The body can be protected from the drug by incorporating it inside the carriers and targeting the complex then to correct location. For example, paclitaxel was incorporated into biotinylated PLA-PEG nanoparticles and targeted to tumor cell *in vitro* using transferrin ligand. The release of drug from nanoparticles was rapid (24 h) and results indicated that nanoparticles showed increased antitumoral activity [41].

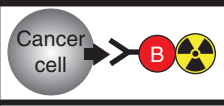
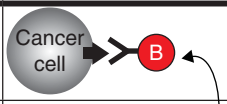
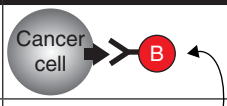
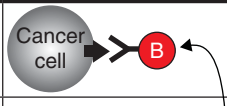
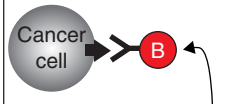
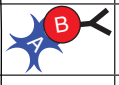
The avidin-biotin system has also been utilized in drug pretargeting applications, which involves the separation of the targeting antibody from the subsequent delivery of a therapeutic agent, which then finds its way to the tumor-localized antibody. This reduces the level of free radiolabeled agent and thus improves the exposure ratio between healthy and tumor tissue. Pretargeting methods can be divided into several 'step' approaches (**Table 1**). The two-step pretargeting methods based on avidin-biotin make use of antibodies conjugated to streptavidin or avidin to enable the binding of biotin [42,43] or biotinylated antibodies capable of complexing with avidin or streptavidin (**Table 1**) [5,44]. In both cases the second step of the treatment consists of injection of a radiolabeled effector molecule. The principle of a three-step protocol is first to target the tumor with a biotinylated antibody, followed by the injection of a clearing agent and finally by the biotinylated therapeutic compound, which binds to the (strept)avidinylated tumor cells (**Table 1**) [5,45]. The first three-step approach uses a streptavidin-conjugated antibody followed by the injection of a clearing agent and a third injection of radiolabeled biotin [46]. The second approach consists of the first injection of biotin-conjugated antibody followed by an injection of avidin and streptavidin, acting as clearing and bridging agents [5]. Avidin undergoes quite a rapid clearance from the circulation, which has been preferentially utilized as a second step for the clearance of non-bound antibody from the blood flow. Streptavidin can be injected on the following day because it has a longer half-life and it has more time to bind to the biotinylated antibody localized at the tumor site [47]. Historically, the injection of avidin followed by streptavidin has been considered as one step. For clarity, this approach will from now on be called a four-step approach. The avidin-biotin system can also be exploited in the reverse way. For example, in a clinical trial Shen *et al*. targeted a streptavidinylated antibody to gastrointestinal cancer cells, followed by clearing of this compound from the circulation by treatment with a galactosylated-biotinylated clearing agent, and finally by the administration of the radiolabeled biotin [48].

3.1 Application of avidin-biotin-based pretargeting in therapy

The use of avidin-biotin technology in pretargeting was first envisioned at the end of 1980s [10,49]. Following these initial preclinical studies, two preliminary clinical studies using opposite two-step pretargeting strategies were reported. First, Kalofonos *et al*. reported improved tumor-to-normal tissue radioactivity ratios after the delivery of streptavidin-anti-MUCl IgG (HMFG1) with a radioactive ^{111}In-biotin to patients with squamous cell carcinoma of the lung [43]. In a second study, Paganelli *et al*. demonstrated a new method to target ovarian tumors using biotinylated antifolate receptor monoclonal antibody and ^{111}In-labeled streptavidin [50]. Patients showed no signs of toxicity against the treatment and the average tumor-to-tissue ratios were 9:1, superior to the conventional approach, suggesting the feasibility of the method. Paganelli *et al*. also developed a three-step pretargeting configuration that started with the injection of a biotinylated anti-carcinoembryonic antigen (CEA) IgG, followed by an injection of unlabeled streptavidin, and 2 h later radiolabeled biotin [5]. The proof-of-principle of this approach after systemic delivery was demonstrated in 20 patients with CEA expressing tumors [51]. Biotinylated anti-CEA monoclonal antibody (mAb) injections were followed by unlabeled avidin as a clearing agent 3 days later, followed by a third step of ^{111}In-labeled biotin. No signs of toxicity or adverse effects were observed in any of the patients. Tumors and metastases were detected in 18 out of 19 patients, demonstrating improvement in the tumor localization. As the circulatory half-life of avidin is much shorter than that of streptavidin, making it better fitted to the role of clearing agent, the group in Milan further optimized their pretargeting protocol to include four steps. A clinical imaging study with 40 cancer patients consisted of biotinylated monoclonal antibody injection, avidin as a clearing agent, streptavidin and ^{111}In-labeled biotin [47]. All the lesions were detected in the patients with single photon emission computed tomography imaging (SPECT), proving the potential of this system for diagnosis of recurrent ovarian carcinoma. Later on, biotinylated albumin was introduced as a fifth step to clear excess streptavidin from circulation.

In 1999 the first Phase I/II study results were published [45]. In this study, 48 patients with histologically confirmed

Table 1. Principle of pretargeting approaches.

Method	Steps	Schematic diagram	Explanation
1-step	Biotinylated mAb (or bsmAb) labeled with therapeutic agent		Accumulation into the tumor
2-step	Biotinylated mAb or bsmAb (or avidinylated mAb)		Accumulation into the tumor
	Labeled (strept)avidin (or labeled biotin)		(Strept)avidin binds to biotinylated mAb
3-step	Biotinylated mAb		Accumulation into the tumor
	(Strept)avidin		Binds to biotinylated mAb
	Labeled biotin		Binds to (strept)avidin
4-step	Biotinylated mAb		Accumulation into the tumor
	Avidin		Clears the nonbound, freely circulating biotinylated mAb
	Streptavidin		Binds to biotinylated mAb
	Labeled biotin		Binds to streptavidin
5-step	Biotinylated mAb		Accumulation into the tumor
	Avidin		Clears the nonbound, freely circulating biotinylated mAb
	Streptavidin		Binds to biotinylated mAb
	Biotinylated albumin		Clears the nonbound, freely circulating streptavidin
	Labeled biotin		Binds to streptavidin
Explanation of the symbols	Therapeutic agent (radiolabel, drug, etc) Biotin Biotinylated antibody		Avidin Streptavidin Biotinylated albumin

grade III (n = 17) or IV (n = 31) gliomas were treated using a five-step protocol with biotinylated anti-tenascin monoclonal antibody, avidin, followed by streptavidin, biotinylated albumin, and ^{90}Y-tetra-azacyclododecane tetra-acetic acid (DOTA). Tumor mass reduction of > 25% was observed in 12 patients of whom 8 sustained the state at least for 12 months. Treatment protocol that used 2.2 – 2.96 GBq/m^2 of radiation showed the lowest dose to be well tolerated, allowing ^{90}Y-DOTA treatment without bone marrow transplantation. The maximum tolerated dose was set to 2.96 GBq/m^2, which is ~ 5 times higher when compared with non-targeted injection of ^{90}Y-DOTA. In the follow-up study, 37 high-grade patients in a controlled, open non-randomized study were treated in a similar manner to the Phase I/II study [52]. The aim was to evaluate the overall survival of the patients as well as the time to relapse. All patients underwent surgery and radiotherapy and 19 also received adjuvant radioimmunotherapy. The overall median survival of the study group was 33.5 months, at which point one patient was still without evidence of the disease relapse. Disease-free interval of the study group ranged from 9 to 59 months, having a median of 28 months. The control group perished with a median survival of 8 months.

Other similar studies are being conducted for the treatment of colon cancer, B-cell lymphoma and gastrointestinal malignancies [48,53-55]. For example, promising results were achieved when 15 patients suffering B-cell non-Hodgin's lymphoma were treated with a single-chain variable region of B9E9 monoclonal antibody (anti-CD20) fused to a monomer of streptavidin and in two patients the remission was complete. In this study, no significant toxicities were observed [54]. In another B-cell lymphoma study the side effects of biotinylated and radiolabeled monoclonal antibody were minimized as unbound molecules were removed from the circulation by filtering whole bold through an avidin-coated filter [55]. Important future promises include treatments combining pretargeted radioimmunotherapy to intratumoral or systemic application of a chemotherapeutic agent, which in preliminary studies have shown significant survival benefit in glioblastoma patients [56,57].

4. Pretargeting by expression of avidin or biotin on cell surface

Another pretargeting approach consists of delivering an avidin gene into the target tissue [58]. In the authors' lab, a gene consisting of avidin fused to a macrophage scavenger receptor (Scavidin) was delivered to tumor cells using recombinant viral vectors, leading to its expression on the surface of target cells (**Figure 2A**) [59]. Scavidin showed efficient binding and endocytosis of biotinylated ligands *in vitro*. Furthermore, the expression of Scavidin was demonstrated *in vivo* in implanted immunocompetent malignant rat glioma BT4C cells after retrovirus-mediated gene delivery and allowing accumulation of biotinylated horseradish peroxidase (HRP) at the tumor site [59]. Scavidin has also been used in imaging applications, taking advantage of its ability to internalize biotinylated magnetic resonance imaging contrast agents [60]. Another similar fusion protein, called Lodavin, consists of a fusion between avidin and the endocytotic low-density lipoprotein (LDL) receptor [61]. Similarly to Scavidin, the expression of avidin fusion protein has been shown near the injection site in a rat malignant glioma model after the transduction, and this fusion protein was also capable of binding biotinylated HRP [61]. However, higher cell surface expression and biotin-binding capacity was achieved using Lodavin™ (Ark Therapeutics Oyj, Kuopio, Finland), compared with Scavidin, which was therefore chosen for subsequent studies. The early Lodavin studies were performed with Semliki Forest virus (SFV). However, potential toxicity and a short expression time hindered further studies [62], therefore lentivirus was chosen as a new delivery vector [63]. Long-term expression of the avidin fusion protein on the cell surface was confirmed and the ligand binding studies indicated that a second ligand administration is already feasible 1 h after the first administration owing to the endocytic nature of low-density lipoprotein receptor (LDLR). Moreover, the applicability of this approach for drug targeting was confirmed using biotinylated nanoparticles carrying paclitaxel *in vitro*, and *in vivo* studies are underway. As discussed earlier, avidin has proved to be immunogenic [20,21], and in line with this Lodavin also evoked an immune response. Importantly, the authors' study showed that anti-avidin antibodies were unable to block the ligand binding capacity of the avidin fusion protein [63].

An opposite approach to avidin display consists of biotinylation of the target tissue and subsequent delivery of avidinylated therapeutic compounds (**Figure 2B**). This approach has been limited mostly to direct chemical biotinylation of surface proteins (**Figure 2C**) [35,36] or synthetic sugars [64]. Nevertheless, proof-of-principle of this method in the delivery of biotinylated molecules to biotinylated rabbit renal arteries has been demonstrated [36]. In 2006, however, a study by Tannous *et al.* showed for the first time expression of a metabolically biotinylated cell surface receptor on the cell surface by fusing BAP with the transmembrane domain of platelet-derived growth factor receptor [65]. Biotin display was used for *in vivo* imaging of labeled streptavidin moieties bound by transduced cells, but one can envisage future application of this approach also in drug targeting [65].

5. (Strept)avidin-biotin technology in vector targeting

(Strept)avidin-biotin technology can be adapted to improve the targeting of gene therapy vectors [12]. It provides substantial advantage over other adaptor systems by assuring sufficient stability of the vector-adaptor complex even under physiological conditions [66]. Early studies with

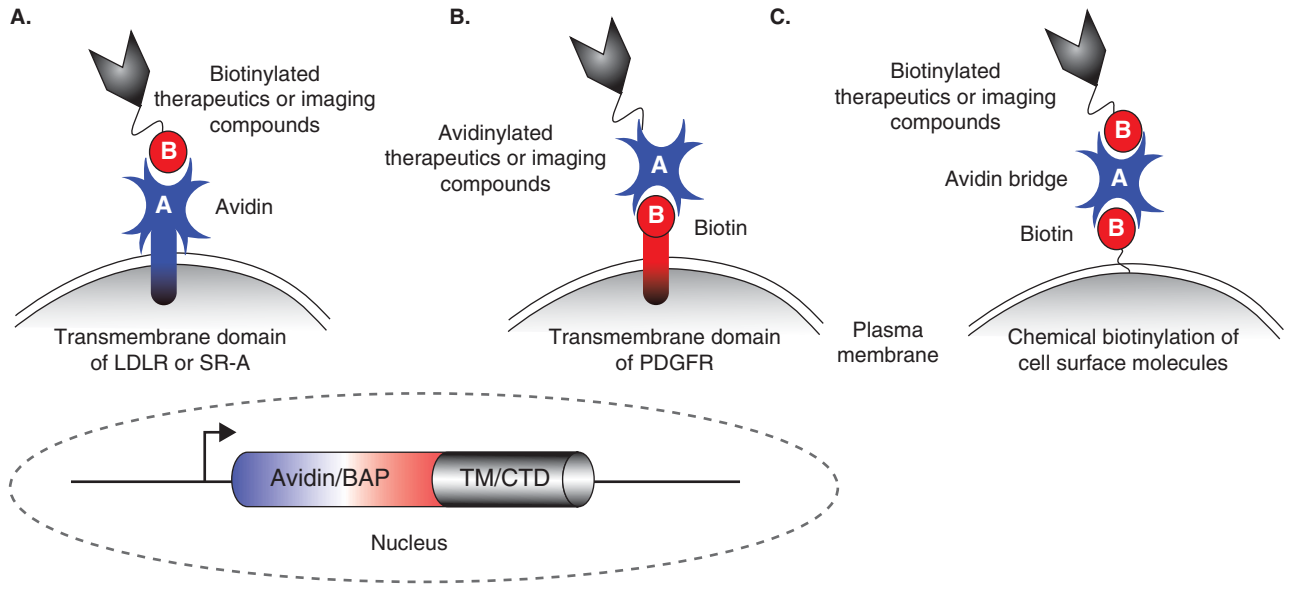


Figure 2. The principle of pretargeting based on display of avidin (A) or biotin (B, C) on the target cell surface. A. Expression of avidin or streptavidin on the cell surface allows local delivery of biotinylated drugs to the site of interest. This can also be achieved by (**B**) the delivery of a gene encoding for protein containing biotin acceptor peptide (BAP) or (**C**) chemical biotinylation of the tissue even though the functionality of these approaches for drug pretargeting remains to be demonstrated. In both cases of biotin display the therapeutics or imaging compounds can be (strept)avidinylated or biotinylated, the latter approach including an extra step of avidin administration that functions as a bridge between the target cell and effector molecule.
TM/CTD: Transmembrane/cytoplasmic tail domain of VSV-G.

retroviruses and streptavidin-bound antibodies specific for both viral and cell membrane epitopes provided the proof-of-principle even though only low transduction efficiencies were attained [67,68]. Since then, the (strept)avidin-biotin-based targeting has followed two different approaches (**Figure 3**). First, gene therapy vectors can be biotinylated either chemically or metabolically (**Figure 4**), while bringing the biotinylated targeting molecules in conjunction to avidin [11]. First studies by Smith and colleagues were performed with a chemically biotinylated adenovirus vector. They demonstrated successful vector targeting to hematopoetic cells through an avidin bridge carrying biotinylated c-Kit receptor ligand, resulting in up to 2400-fold increase in reporter gene expression [69]. Later on, chemically biotinylated retrovirus, adeno-associated virus (AAV) and vaccinia virus were created, all of which showed significantly increased transduction of target cells [70-72]. Not only the viral vectors, but also biotinylated non-viral DNA vectors have been used for targeting [73].

Chemical biotinylation, however, often leads to nonspecific labeling, inactivation of target proteins and requires prior purification of the virus (**Figure 4**). Therefore, metabolic biotinylation, achieved directly in living cells, is considered a more promising approach [24]. The concept of metabolically biotinylated gene therapy vectors was first introduced in the context of adenovirus showing up to 300-fold increase in reporter gene expression *in vitro* [74-78]. Since then, several studies have shown that metabolic biotinylation provides an efficient means to target other vectors, such as AAV [79] and lentivirus [80]. The authors have recently constructed a metabolically biotinylated baculovirus that enhanced the transduction of tumor cell lines by 15 – 60% when targeted to transferrin receptor, EGFR and CD46 after only 15 min exposure of the cells to the virus [81]. Owing to the large availability of (strept)avidin-biotin-based purification methods, metabolical biotinylation of gene therapy vectors has also been applied for virus concentration [75,79,81-84].

Many adaptor-based targeting systems face the problem of suboptimal stability under physiological conditions. For example, in targeting strategies that are based on the antibody binding to the virus surface, the weak interaction under physiological conditions may lead to the removal of the antibody from the viral surface [12]. The strong binding between avidin and biotin is, however, less likely to be affected by this. Pereboeva and co-workers provided evidence to support this by demonstrating efficient retargeting of metabolically biotinylated adenovirus to ovarian carcinomas and lung endothelium of EGFR transient transgenic mice [66]. Interestingly, a recent study by Stachler *et al.* took advantage of a metabolical addition of a ketone analogue of biotin on AAV surface, which allowed subsequent chemical linkage of targeting molecules on vector surface, with no need for avidin conjugation [85]. This new method resulted in effective gene

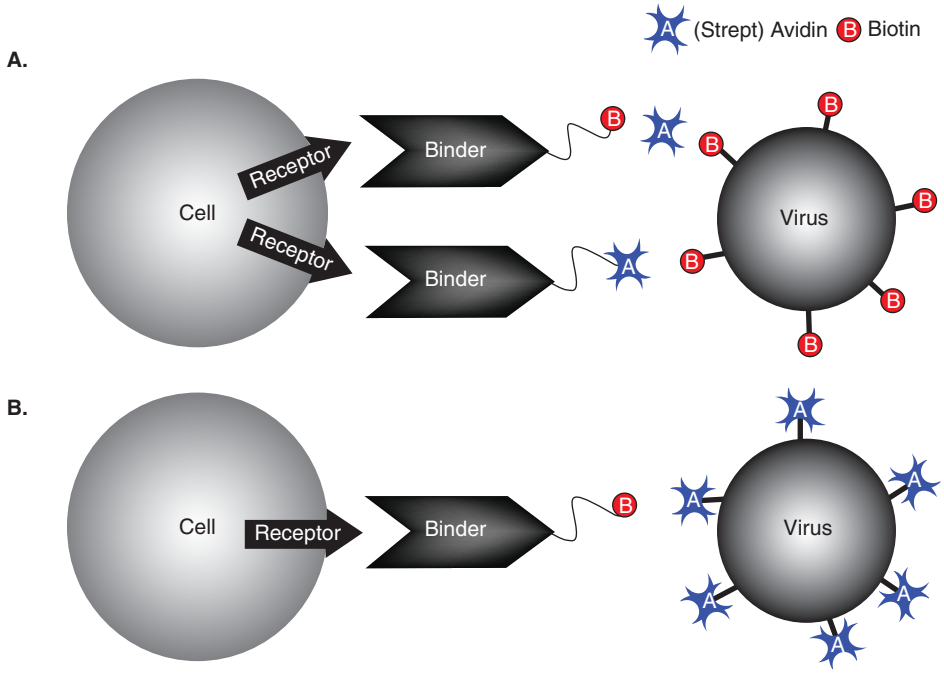


Figure 3. The principle of (strept)avidin-biotin technology in vector targeting. The vector can display either biotin (**A**) or avidin (**B**), allowing attachment of biotinylated or avidinylated targeting molecules on the virus surface. This will guide the virus to recognize specific receptors on target cells (binders, e.g., ligands, antibodies).

transfer to tumor vasculature by RGD-mediated targeting in a murine model of ovarian carcinoma [85].

An opposite targeting approach consists of avidin displaying vectors (**Figure 3A**). To this end, chemically avidinylated PEI vectors [86] and adenoviruses [87], avidin-polymer-coated adenoviruses [88] together with genetically engineered avidin displaying baculoviruses have been described [89]. In the latter study, Räty and colleagues expressed an avidin-gp64 fusion protein on the baculovirus surface thus providing binding sites to biotinylated targeting ligands [89]. This two-step system is easier to control than the three-step process using the avidin bridge between desired biotinylated molecules and offers better valence for covering because all four biotin binding sites are available for biotinylated ligands. Avidin displaying baculovirus showed a fivefold increase in transduction efficiency of rat glioma cells and a 26-fold increase in rabbit aortic smooth muscle cells compared with wild-type virus without major cytotoxicity. Enhanced transduction was also observed with biotinylated cells and biotinylated EGF enabled targeting to EGFR expressing cells. In addition, the use of biotinylated paramagnetic particles allowed magnetic targeting.

To develop lentivirus vectors with targetable gene delivery, the authors recently designed new gp64-pseudotyped lentivirus vectors expressing avidin or streptavidin fused to the transmembrane anchor of VSV-G on the virus envelope [90]. Separation of the targeting moiety and the envelope protein hoped to leave the fusion protein intact for endosomal escape thus avoiding the common problem of decreased infectivity caused by the modified envelope proteins [91,92]. To redirect the specificity of infection of streptavidin or avidin displaying vectors, biotinylated ligands and antibodies selectively targeting transferrin, EGF and CD46 receptors expressed at high levels on certain tumor cells were used. The results demonstrate that target cell-specific transduction of these vectors can be increased 10 – 300% by the use of biotinylated ligands and antibodies *in vitro*. However, the transduction of (strept)avidin displaying lentivirus was dependent on gp64, which has shown quite broad tropism to different tissue types, such as liver, lung, skin, glia, endothelium or thymus [93]. To overcome this problem, removing or greatly reducing the natural binding activity of gp64, without disturbing the fusion, by mutagenesis, could be attempted. Alternatively, pseudotyping with other binding-defective mutants, such as hemagglutinin (HA) of influenza A (Lin *et al.*, 2001) [99] and Sindbis virus [94] envelope proteins, could be considered.

In addition to targeting, avidin-biotin technology provides the possibility of imaging. The pretargeted radioimmunotherapy for cancer treatment described above has been widely applied for simultaneous radioimmunoimaging [1]. Furthermore, conjugation of baculovirus vector to biotinylated iron oxide particles has enabled imaging of the viral particle biodistribution by magnetic resonance imaging [95], or for conjugation of baculovirus or lentivirus by radionuclides, the biodistribution has been imaged by SPECT *in vivo* [90,96].

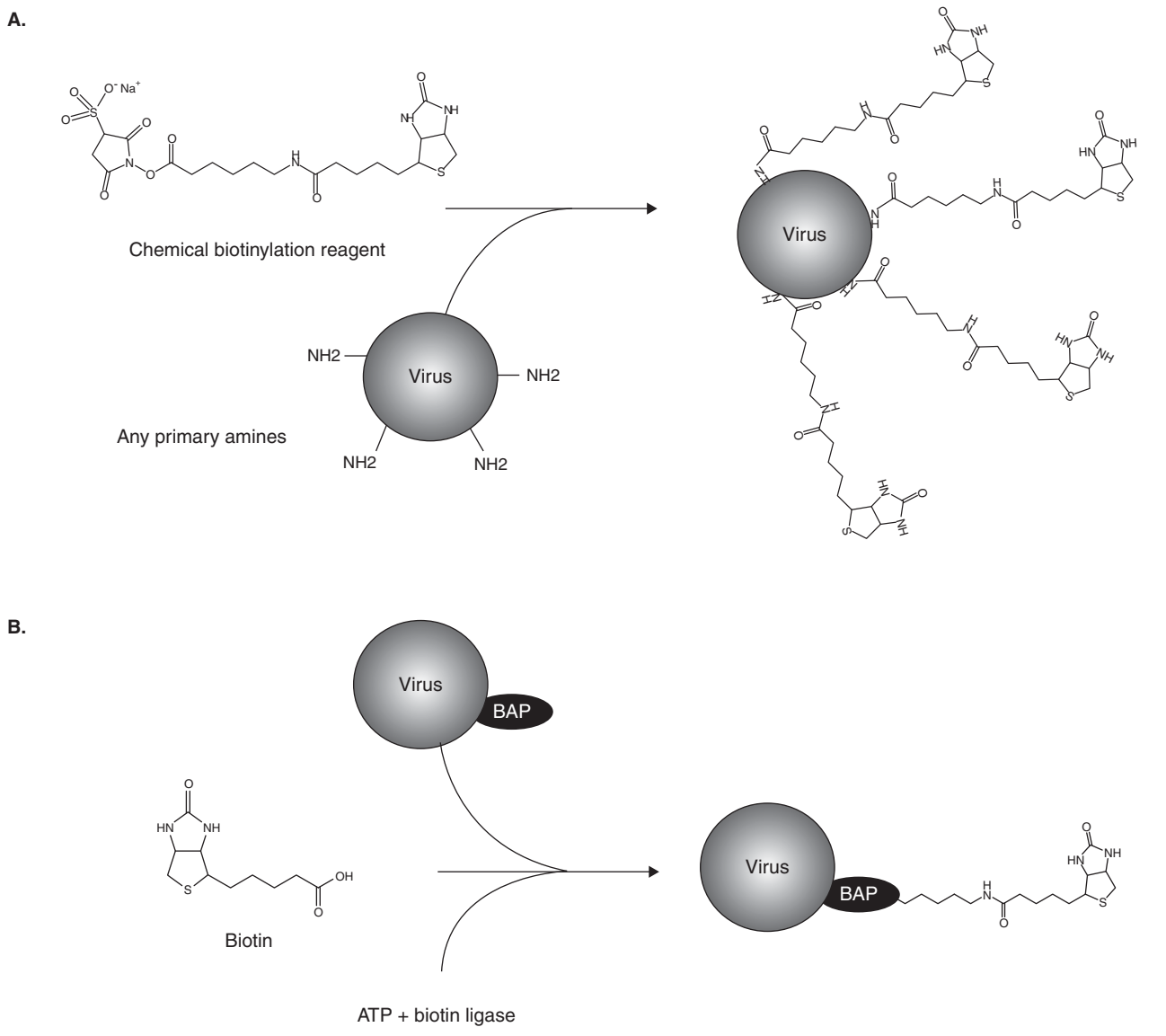


Figure 4. Comparison of chemical and metabolic biotinylation methods. A. Biotin labeling of viruses by chemical methods consists of conjugation of an activated biotin derivative to the protein surface residues (commonly primary amines) *in vitro.* This may result in heterogeneous modification leading to the virus inactivation or aggregation after addition of (strept) avidin. **B.** Metabolic biotinylation can be achieved by fusing a biotin acceptor peptide (BAP) domain on virus surface proteins. Biotin protein ligase (endogenous or coexpressed exogenous such as bacterial BirA) then catalyses a formation of an amide bond between biotin and ε-amino group of the lysine on BAP in a reaction involving ATP. Metabolic biotinylation can thus be achieved during the production of the virus and site-specifically.

6. Conclusion

The avidin-biotin system offers a versatile tool for targeting purposes. One application has been pretargeting where the administration of monoclonal antibodies has been separated from that of a low-molecular-mass radionuclide ligand. This multistep (two to five steps) approach has been shown to improve tumor-to-normal tissue radiation dose ratios because the targeting molecule administered first is not radiolabeled. Subsequent administration of (strept)avidin removes the excess circulating antibodies or binds to tumor cells, preparing them to receive radiolabeled biotin. This strategy has already been followed in clinical trials for the treatment of malignant glioma, demonstrating efficacy on cancer progression without significant toxicity [45,52,97].

The second application is to deliver the gene-encoding avidin to the target cells using a viral vector. Scavidin and Lodavin are examples of such a pretargeting approach. After the local viral transduction, cells were able to express avidin fusion protein on the surface and were capable of internalizing

biotinylated ligands [59,61,63]. Also, display of biotin on the target cells, achieved by genetic or chemical means, could allow drug targeting, but the potential of this approach for drug targeting needs to be studied further [59,61,63].

The third application is to utilize the avidin-biotin system for targeting of viral vectors. After systemic administration, viral vectors are present in the blood circulation. The affinity of the vector to the target cell is based on the interaction between the viral receptor and the viral surface proteins. By modifying the surface, viruses can be targeted to specific cells or tissues. This transductional targeting can be achieved by displaying avidin or biotin on the viral surface and coupled to suitable biotinylated or avidinylated targeting ligands [12]. In most of the studies, an enhanced transduction was achieved *in vitro* [69-71,89,90], but also evidence of the suitability of this approach for *in vivo* targeting has recently been demonstrated [66].

7. Expert opinion

Avidin-biotin-based radiommunotherapy has already entered clinical trials owing to its improved selective targeting of cancer cells that can benefit both imaging and therapeutic applications. However, the radioimmunotherapy of solid tumors remains a challenge because of their resistance to radiation and requirement for high doses of radiation [1]. The most promising approach for the future consists of combining immunotherapy with other treatment modalities. As new entries to the pretargeting field, one could envisage the delivery of gene encoding for avidin-fusion protein to tumor cavity following standard surgery. Subsequent administration of a radio- or chemotherapeutic agent to the bloodstream could thus destroy the newly growing cancer cells. This strategy has recently been proven superior to standard care for the treatment of operable primary glioblastoma using adenovirus-delivered thymidine kinase gene together with ganciclovir (Cerepro®; Ark Therapeutics group PLC, London, UK, Phase II results) [98].

Use of the avidin-biotin system for vector targeting provides a means to avoid the necessity to engineer a new vector for each new ligand while helping to ensure the stability of the conjugation between targeting molecules in the presence of serum. Moreover, it allows attachment of large ligands (e.g., antibodies) and non-protein ligands (e.g., carbohydrates, lipids, etc.) to the virus surface while enabling direct comparison of these different targeting modalities [77].

Although the use of the avidin-biotin system for vector targeting has been used successfully to generate vectors that recognize specific cells, there is still little evidence of whether this can eliminate the problem of nonspecific distribution after *in vivo* administration. The concern of unspecific binding is largely caused by viral capsid proteins (e.g., adenovirus fiber) or envelope molecules interacting with blood and tissue components. The development of binding-defective mutants of virus surface proteins without compromising viral titers is underway to surmount this problem. Such future enhancements are hoped to result eventually in specific targeting to desired tissues for which local or *ex vivo* gene delivery is not possible.

Avidin-biotin technology has been used for 30 years for different kinds of biotechnological application. A lot of effort has been made to improve the system further. These mutants have shown, for example, reduction of nonspecific binding of avidins, while retaining their thermal stability and the ability to bind biotin tightly. Also, the production of dual and single chain avidins, avidins with dual affinities and streptavidin binding biotin in a monovalent fashion has been demonstrated, and reviewed in [22]. Apart from these, the next-generation (strept)avidins with multiple ligand binding sites or ability to bind new ligands with high affinity hold great promise for the field of targeted therapies.

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Declaration of interest

HP Lesch, MU Kaikkonen and JT Pikkarainen are employees of Ark Therapeutics Oyj.

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