ISSN 1070-3632, Russian Journal of General Chemistry, 2014, Vol. 84, No. 2, pp. 357–363. © Pleiades Publishing, Ltd., 2014.
Original Russian Text © A.V. Maksimenko, 2012, published in Rossiiskii Khimicheskii Zhurnal, 2012, Vol. 56, Nos. 3–4, pp. 89–96.

Development and Application of Targeted Therapeutic Protein Conjugates

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Received September 1, 2012

Abstract—The present review considers therapeutic protein conjugates (bioconjugates) in terms of the concept of drug targeting delivery in the body.

DOI: 10.1134/S1070363214020376

INTRODUCTION

Intensive research in 1970–80s of immobilized enzymes and protein–protein interactions [1, 2] resulted in that much a broader range of forms of conjugated proteins fell under the scrutiny of science. Researches focused not only on naturally conjugated proteins classed, in terms of the chemical nature of their prosthetic groups, into hemoproteins, phytochromes, lipoproteins, glycoproteins, and so on, but also on synthetic protein conjugates. The interest in them was further stimulated by the advent of antibodies [3, 4], which had served as the driving force for the development of the concept of drug targeting delivery [5]. This concept implies the development of systems for targeted delivery of drugs [6] to an injured organ or tissue, which ensures their specific binding and a high therapeutic effect.

The most common carriers used in drug targeting delivery in the body are bioconjugates, dendrimers, liposomes, polymerosomes, polymer micelles, nanogels, nanoparticles, and other nano objects [6].

Types of Protein Conjugates

Protein conjugates are one of the carriers for drug targeting delivery. Their common model structures obtained by chemical synthesis includes a polymer biodegradable matrix bound with a therapeutic agent and with a vector compound which is responsible for recognition and specific binding of the bioconjugate with target. Over the past few decades the class of synthetically conjugated protein has appreciably extended and altered. It is considered that the first bioconjugate products were conjugate vaccines [7]. They were prepared by covalent binding of weakly antigenic bacterial polysaccharides with stronger antigenic protein carriers which impart immune properties to the attached molecules.

Another abundant group of protein conjugates includes poly(ethylene glycol)-conjugated proteins (PEG-proteins). The covalent "wrapping" of proteins by PEG molecules of different molecular weights makes the proteins more stable and bioavailable in the body.

Antibodies to foreign surface antigens of damaged and cancer cells are being introduced to clinical practice for efficient recognition of the latter and delivery to then of therapeutic agents. Clearly, these possibilities are first of all demanded in oncology, where to reduce the systemic toxicity of a drug and increase it accumulation in the injury site (see table) are the key problems. Most commercially available antibodies used in cancer therapy activate the immune system of the body to affect cancer cells. However, such agents rarely lead to stable remission as a result of total destruction of a tumor. To approach the problem of cancer relapses, antibodies conjugates with cytotoxic drugs (antibody–drug conjugates) are being developed. The vigorous progress in the synthesis of such conjugates gave grounds to declare a change from Paul Ehrlich's "magic bullets" to a "guided missile" concept [7]. Furthermore, genetic engineering made possible efficient biological synthesis of hybrid (chimeric) protein forms. Such forms favorably

Advantages and disadvantages of antibody conjugates for cancer therapy [8]

Advantages	Disadvantages
Specific binding with target antigen	Need for tumor testing for the target antigen
Targeted delivery of highly toxic agents to tumor cells	Possible presence of the target antigen in healthy tissues, threat of toxic effects
Wide therapeutic index	Possible toxic effects at the initial stage of conjugate injection
Stability of conjugates in the bloodstream, prolonged half-life	Insufficient local concentration for a lethal effect on tumor cells
Reduction of harmful effects	Heterogenous expression of the target antigen, especially in solid tumors

combine antibodies to markers of injury site and therapeutic proteins. Depending on the composition and method of production, bioconjugates are divided into the following groups: conjugate vaccines, PEG proteins, antibody–small molecule drug/or radionuclide conjugates, protein and protein–peptide conjugates obtained by chemical synthesis, and fusion protein conjugates obtained by genetic engineering.

Anticancer Bioconjugates

There are four types of anticancer bioconjugates which presently attract researcher's attention [8]: antibody–enzyme; antibody–protein toxin or antibody–toxin fusion protein; antibody–radionuclide; and antibody–small molecule drug (antibody–drug conjugate, ADC). The first two types of conjugates have not yet reached the stage of approved clinical trials. Antibody–enzyme conjugates are expected to be introduced in clinical practice along with the introduction of a small molecule prodrug for the latter to convert into an active substance (drug) in an injury site. This approach is called the antibody–direct enzyme prodrug therapy (ADEPT technology). A successful realization of this approach is encumbered by the immunogenic of the bacterial enzyme component and a short half-life of the conjugate in the body [8]. According to other sources, the approach is redeemable, since it proved possible to selectively activate an anthracycline prodrug by means of the ADEPT technology [9]. A reliable answer is still to be obtained.

As to antibody–protein toxin bioconjugates, their clinical potential is seriously restricted by the low probability that a therapeutic concentration of such an immunotoxin can be delivered to solid tumors [8].

Radioimmunotherapy with antibody–radionuclide conjugates offers the following advantages over conventional radiotherapy: more exact targeting of a radioactive agent to tumor and metastasis sites and effect on tumors with heterogeneous antigen. Antibody–radionuclide conjugates are already used in the therapy of hematological malignancies (lymphoma) with CD 20-targeted agents: ^{131}I-tositumomab (Bexxar) and ^{90}Y-ibritumomab tiuxetan (Zelavin) [10]. Testing antibody–radionuclide conjugates as potential therapeutic agents against solid tumors have not still brought encouraging results, primarily because of a low radiosensitivity of cells of this type [8].

Clinical trials of antibody–small molecule drug conjugates (doxorubicin, vinblastin, methotrexate) revealed their limited applicability in view of their immunogenicity, insufficient efficiency, and tumor/normal tissue selectivity [8]. These disadvantages could be overcome by the replacement of the ADC components. To reduce the immunotoxicity, mouse antibodies were replaced by humanized or human antibodies, and the efficiency was enhanced by increasing the cytotoxicity of the formulation and more accurately selecting the target and antibodies. As a result, trastuzumab emtansine (T-DM1) and brentuximab vedotin (SGN-35) showed promising activity. They both are presently at an advanced stage of clinical assessment, and another about twenty ADC conjugates are at an early stage of clinical trials [8].

Important safety and efficiency requirements to antibody conjugates include a water-soluble formulation, prolonged stability in aqueous media and blood plasma, availability for conjugation (presence of corresponding functional groups and a sufficiently stable linker), optimal conjugation degree of the components (for ADCs: 3–4 drug molecules per one antibody molecule), and homogeneity of dimensions and their reduced level (on a comparative scale) in the resulting bioconjugates [8, 9]. Because of the

impossibility to meet or ignoring some the above requirements, positive clinical results could be obtained only with antibody–radionuclide and antibody–small molecule drug conjugates.

Bioconjugates in the Therapy of Other Pathologies

Evidence for the universality of experimental targeted conjugate therapy approach was obtained *in vivo* for other pathologies. Thus, for the therapy of mice with liver fibrosis (the model of CCl_4-induced acute liver injury), mannose-6-phosphate-modified human serum albumin was used as a vector carrier, and this ensured binding of the carrier with the insulin-like growth factor type 2/mannose-6-phosphate (IGF-2/M6P) receptor [11]. This receptor assisted hepatic stellate cell activation by Rho kinase in liver fibrosis. The Rho kinase inhibitor was bound to the carrier, and the effect of the resulting bioconjugate was compared *in vivo* with the effect of a free inhibitor. It was found that the specific targeting of Rho kinase in the form of a conjugate provides increased accumulation of the drug in hepatic stellate cells and slows down liver fibrosis progression [11]. The bioconjugate reduced local cell activation, whereas the free inhibitor in the equimolar quantities did not produce such effect.

Haverdings et al. [12] compared the efficiencies of the bioconjugate of the angiotensine-converting enzyme (ACE) inhibitor (captopril) with the protein lysozyme and free captopril in the therapy of renal disease in rats [12]. It was found that 6-h intravenous infusion of the conjugate led to a more expressed increment of renal blood flow and about a 5-fold increase of natriuresis compared to equimolar amounts of free captopril; at the same time, systemic effects (blood pressure, response to intravenously administered angiotensin I, and strong attenuation of plasma ACE inhibition) were essentially lower. In the case of nephrotic syndrome, the conjugate again proved to be more efficient than free captopril in terms of the antiproteinuric response and blood pressure reduction. The obtained data show that the captopril–lysozyme bioconjugate leads to more selective renal ACE inhibition, as well as enhanced local and attenuated systemic effects compared to captopril itself [12].

The bioconjugate of interferon-β (IFNβ) with dextran was used to treat experimental choroidal neovascularization (CNV) in the eye cornea of rabbits [13]. The bioconjugate was prepared by mixing IFNβ with dextran and diethylenetriaminoacetic acid (DTPA, metal-chelating agent) in aqueous solution containing Zn^{2+} cations. The resulting IFNβ–DTPA–dextran conjugate was compared with free IFNβ with respect to inhibition of the progression of the model CNV of rabbit's eye (the antiangiogenic effect). This disease was induced by subretinal injection of gelatin microspheres containing basic fibroblast growth factor. The residual activity of IFNβ in the conjugate was roughly estimated at 44% of the initial activity. Conjugation allowed prolongation of the plasma half-life of IFNβ and ensured its accumulation (according to the immune-enzyme analysis) in CNV lesions. Hystology revealed an expressed inhibitory effect of the IFNβ–DTPA–dextran bioconjugate on CNV progression, especially upon long-term (4 weeks) injection in small doses (0.75 MIU/kg/day). Thus, the bioconjugate ensured INFβ targeting to the injury site and enhanced its therapeutic effect [13].

The presented data provide evidence showing that targeted bioconjugates can be prepared not only with antibodies, but also with other proteins and polysaccharides chosen with account for the pathology [12] or preliminary chemically modified [11, 13]. Practical feasibility of the research results can be confirmed by immunological and toxicological assessment of the safety of clinical application of bioconjugates, as well as by clinical trials.

Bioconjugates in Cardiology Research and Practice

Much effort is presently focused on R&D of bioconjugates for cardiology [14, 15]. Chemically synthesized bioconjugates of urokinase-type plasminogen activator (u-PA) conjugated by 4-succinimidyl-oxycarbonyl-alpha-methyl-alpha(2-pyridyldithio) toluene (SMPT) with monoclonal antibodies (RE8F5) against the surface pulmonary vascular endothelial membrane protein in rats [16], as well as recombinant fusion proteins of low-molecular-weight urokinase-type plasminogen activator (lmw-scu-PA) fused with a single-chain variable fragment (scFv) of a platelet-endothelial-cell adhesion molecule (PECAM-1) antibody [17].

The model of pulmonary embolism in rats was used to show that the u-PA–SMPT–RE8F5 bioconjugate, which preserves 85% of the initial urokinase activity, exhibits a 12–16-stronger thrombolytic activity compared to urokinase and retavase (a truncated mutant of tissue-type plasminogen activator, r-PA) not causing systemic activation of plasminogen and decrease of the fibrinogen level [16]. The fused bioconjugate in the

form of a lmw-scu-PA-scFv prodrug specifically bound PECAM-1-expressing cells, and, after the cleavage by plasmin of the Lys-158–Ile-159 bond in lmw-scu-PA generated fibrinolytically active lmw-uPA form [17]. This favored bioconjugate accumulation in the lungs of wild-type mice (but not PECAM-1 knockout mice) after intravenous injection, as well as more efficient lysis of pulmonary emboli compared to lmw-scu-PA. The obtained results provide support for the concept of thromboprophylaxis using recombinant fusion proteins that target the endothelial surface [18].

It should be noted that the methods of synthesis of bioconjugates, applied in the research practice, produce plasminogen activators which have a larger molecular size than the parent forms. At the same time, the molecular size of the plasminogen activators applied in the thrombolytic practice, especially of new-generation ones (retavase, metalize) are smaller compared to the parent molecules [15]. Metalize and retavase intravenously injected by the single- and double-bolus scheme, respectively, are recombinant truncated forms of tissue plasminogen activator. The clinical use of thrombolytic biopreparations, too, gave evidence for the therapeutic efficiency of antibodies of reduced molecular size (abciximab and monafram, respectively, the Fab and F(ab′)2 fragments of monoclonal antibodies to glycoprotein IIb/IIIa which inhibits platelet aggregation [15]. The above-mentioned feature of the research approaches to and clinical practice with cardiological bioconjugates raised the question about an optimal range of the molecular size of such bioconjugates and importance of its control (endothelium internalizes conjugates 100–300 nm in diameter, but not monomolecular antibodies or micron-size conjugates [19]). On the other hand, a concrete way to practical implementation of research projects was pointed out, specifically, combining domains/fragments of the protein components of targeted bioconjugates in the form of protein molecules or as bioconjugated nanoparticles [15].

Antioxidant Bioconjugates

As known, cardiovascular disorders are associated with oxidative stress. This circumstance raised interest in antioxidant bioconjugates [20]. It was established that superoxide dismutase (SOD) and catalase (CAT) conjugates with antibodies to PECAM-1 bind to endothelium (but not to PECAM-1-negative cells), accumulate in lung vessels, and block reactive oxygen species [21]. Moreover, it was sound that the dependence of the efficiency and specificity of targeting (after intravenous injection in mice) of bioconjugates (enzyme–antiPECAM-1) on their molecular size follow looks like a bell-shaped curve peaking at the conjugate diameter of about 300 nm. Thus, the molecular size of bioconjugates affects the efficiency and specificity of their targeting, and their optimal diameter can be determined in *in vivo* experiments [21].

Han et al. [22] established in their experiments with endothelial culture that the conjugate CAT–antiPECAM-1 (but not SOD–antiPECAM-1) decreases the hyperpermeability of the endothelial monolayer, induced by hydrogen peroxide [22]. The conjugate SOD–antiPECAM-1 (but not CAT–antiPECAM-1) was found to efficiently inhibit the vascular endothelial growth factor-induced increase of endothelial permeability, thus predetermining the limiting role of superoxide radical in the development of pathological lesions under these conditions. The established difference [22] in the protective effects of antioxidant enzymes underlines the urgent need in that a universal activity SOD–CHS–CAT bienzyme conjugate (hereinafter, SOD–CHS–CAT) is present in the developing lesion site (especially extracellularly) in vivo [20]. The use of separate SOD and CAT conjugates allows one to vary the efficiency of intracellular delivery. The SOD–antiPECAM-1 conjugate (but not CAT–antiPECAM-1 or untargeted enzymes), injected intravenously, accumulated in vascular endothelium, localized in endothelial endosomes, and produced 70% inhibition of the lipopolysaccharide-induced expression of vascular cell adhesion molecules (VCAM-1) [23]. The resulting data provide direct evidence for the involvement of the endosomal supeoxide radical in the endothelial inflammatory response and its blocking due to the targeted delivery of the above conjugate to endothelial endosomes, which may be of importance for an exogenous anti-inflammatory effect.

The activities of the two antioxidant enzymes (SOD and CAT) were combined by their conjugation via CHS (endothelial glycocalyx glycosaminoglycan) [20]. Such conjugation not only helped to target the bienzyme complex to affected sites of the luminal surface of vessels (in the case of atherosclerosis, they contain increased CHS levels, and oxidative stress is provoked), enhanced the biocatalytic effect of enzymes (conversion of reactive oxygen species into water and molecular oxygen), but, which is important, the

bioconjugate acquired nanoparticle properties: size dependence of properties. Actually, the SOD–CHS–CAT conjugate inhibited the ADP-induced platelet aggregation due to the enzymatic activity and acquired supramolecular structure, which is not inherent in the native enzymes and free CHS.

The antithrombotic effect of the bienzyme conjugatea *in vivo* was reliably higher that the respective effects of other combinations of native and modified enzymes, as well as their combinations with CHS [24]. The model of artherial thrombosis in rats, induced by treatment of a vessel by a saturated iron chloride solution, was used to show that the bienzyme conjugate is more active (by the antithrombotic effects: occlusion time and thrombus weight) at doses lower by an order-of-magnitude than with the component enzymes individually modified by CHS (or their mixture) and lower by two orders of magnitude than with native enzymes. The efficient mitigation by the bienzyme conjugate of the hydrogen peroxide-induced hemodynamic perturbations in rats (arterial pressure, heart rate), possible use for prophylaxis, and low acute toxicity allow the SOD–CHS–CAT conjugate to be identified as a drug candidate [15, 20, 24].

The use of glycocalyx components for enhancing the therapeutic efficiency of bioconjugates deserves interest in terms of drug targeting and stabilization. A biotinylated tissue plasminogen activator (t-PA) was associated via streptavidin with biotinylated erythrocytes. The resulting adduct ensures fast and prolonged reperfusion in rats with cerebral thrombosis, by contrast to the t-PA itself even if it is injected in order-of-magnitude higher doses [25]. The association of t-PA with erythrocytes decreased its susceptibility to the plasminogen activator inhibitor-1 (PAI-1), on account of the fact that the erythrocyte glycocalyx protects t-PA from interaction with PAI-1, and this protection is no longer effective after treatment of erythrocytes by a mixture of neuramidase, hyaluronidase, and heparinase enzymes [26]. The protective effect of glycocalyx is explained by that it shields t-PA reactive centers and alters the electrostatic interaction network. To make further use of the potential of glycocalyx glycosaminoglycanes, Soto et al. [27] synthesized chimeric monoclonal antibodies to sulfated molecules. These antibodies (chP3R99) react with sulfated glycosaminoglycanes, mainly CHS, thereby inhibiting association and oxidation of low-density lipoproteins in the artery wall.

Thus, the obtained antibodies were found to show the antiatherosclerosis effect, and, therewith, endothelial glycocalix components act as a therapeutic target [27, 28].

Large Bioconjugates and Nanoobjects

To preserve the oxygen-binding properties of hemoglobin in an extracellular environment, stabilize its active form (to prevent its oxidation to the inactive methemoglobin form), and protect it from reactive oxygen species, hemoglobin was covalently "cross-linked" with SOD and CAT via dicarboxymethylated poly(ethylene glycol) (MW 2 kDa) [29]. The resulting bioconjugate had the molecular weight of 1000 kDa and retained 70 and 90% of the initial activity of SOD and CAT. The adduct showed reduced tendency to transform to methemoglobin both on conjugation and on storage (4°C, one month) and enhanced resistance to superoxide radical and hydrogen peroxide [29]. Under conditions of acute hypoxia, this bioconjugate exhibited an appreciable protective effect: The viability of pancreatic β-cells was 80% higher versus control [30]. These results provide evidence showing the large bioconjugates have a good perspective to be introduced in clinical practice.

The introduction of bioconjugates in clinical practice is favored by medical nanotechnologies [31], based on the use of nanomaterials (liposomes, dendrinmers, gold nanoparticles, quantum dots, fullerenes, nanotubes). Having a developed surface, such nanoobjects can be loaded with a lot of drug molecules, which is quite essential for oncology.

Tumors have a poor lymph drainage, their vessels are highly porous, and this facilitates diffusion and accumulation of nanoparticles in the tumor tissue. Nanoparticles loaded with chemotherapeutic agents can deliver the latter to cancer cells. The specific drug loading of nanoparticles is determined by the amounts of components in the lesion site, required for an appreciable therapeutic effect [6]. To estimate the drug delivery density, different models are being developed, which allow, in particular, determination of the efficiency of the reaction of functionalized nanocarriers with endothelial cells, mediated by the cell glycicalyx [32]. It was found that nanocarrier adhesion can be controlled by the resistance of glycocalyx, flexibility of cell receptors, and strength of the receptor–ligand bond.

Among the diverse applications of nanomaterials, targeted delivery of antibodies, peptides, and oligo-

nucleotides folded into complex structures (so-called aptamers) occupy an important place. The properties of such nanoobjects are being studied along different lines. In a noncovalent complex of SOD with cyclodextrin (a polysaccharide nanotube with a hydrophobic inner and a hydrophilic outer surfaces), the activity of the enzyme increases by 52% [33]. Analysis of the molecular docking of these agents revealed reaction sites on the protein and cyclodextrin rings, which ensures specific conformational transformations of SOD. Correlations using these data may prove a useful guidance for further research.

The share of combined therapeutic agents (not only in the form of covalent bioconjugates) is being constantly growing. The preparation of combined agents is based on the results of research on drug cocktails and combinations, such, in particular, as drug–biological agent combinations [34].

Recombinant human/mouse chimeric antibodies to the epidermal growth factor receptor (Erbitux, ImClone Systems) were found to be efficient against colorectal cancer. It should be noted that this receptor is expressed (>35%) in all solid malignant tumors. Such antibodies as a biological agent were approved by the FDA for monotherapy and for combined therapy together with iriniotecan as the first course of head and neck cancer therapy in patients above 45 years of age (in combination with radiotherapy) [32].

The wide use of combined cancer therapy is probably associated with the fact that cancer lesions are multifactor in nature. Thus, it was noted that the therapeutic effect of extracellular oxidoreductases may not necessarily affect the total extracellular redox status but may disturb redox signaling or its control by changing the behavior of cancer cells [35]. This may lead to progress in treatment but not to complete recovery. Therefore, antioxidant additives only are not sufficient for anticancer therapy: Combined therapy together with proteins controlling the redox potential. Naturally, such tentative conclusions do not argue against the importance of extracellular reductases as potential therapeutic targets for cancer therapy. The relatively new concept of the modulation of intra- and extracellular redox statuses in oncogenesis assigns to oxidoreductases the role of potential targeted therapeutic agents [33]. The most therapeutically efficient agents in the research series of covalently and noncovalently bound large bioconjugates and nanoobjects, which is presently under development, are still to be identified.

CONCLUSIONS

The described picture of R&D work on targeted bioconjugates provides evidence for violent effort for the sake of higher efficiency and safer treatment in different fields of medicine: oncology, cardiology, ophathalmology, hepatology, etc. Some of the developed drugs are already used in clinic. The common feature of such bioconjugates is their reduced molecular size. In oncology they include antibody–small molecule drug conjugates, and in cardiology these are antibodies (antithrombotics) and recombinant plasminogen activators discriminated by certain domains (thrombolytics).

Extensive research revealed a series of new targets for effort to be directed. The important factors favoring clinical promotion of bioconjugates include the importance of their molecular size and reliable control, density of population by therapeutic agents of the lesion site, use as vector carriers of not only antibodies (including cellular adhesion molecules and sulfated glycosaminoglycanes), but also modifies proteins and polysaccharides, various nanomaterials, use of bioantioxidants and combined agents (with covalently and noncovalently bound drugs), etc. In view of the great diversity of lines of research and well-substantiated goals of research, a potential breakthrough point is quite difficult to predict. It is quite evident that this will to a great extent be determined by swift-flowing time and sufficient funding.

ACKNOWLEDGMENTS

The work was financially supported by the Russian Foundation for Basic Research (project nos. 12-04-00015 and 12-08-00010), as well as by the Ministry of Health of the Russian Federation.

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