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Y01

Chemotactic Drug Targeting (CDT) Synthesis and *in vitro* application of chemotactic drug delivery systems

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In the field of targeted drug delivery, numerous bioconjugates have been developed to enhance the efficiency and specificity of novel antitumor therapeutics. These kind of drug delivery systems (DDSs) usually consist of a carrier, a drug and targeting moieties. During the past decade, several carrier systems have been envolved depended on the target organ. Forasmuch the receptor mediated endocytosis may provide the appropriate pathway for cellular uptake, targeting moieties have amended the structure of DDSs. Our aim was to develop targetable oligopeptide-based chemotactic drug delivery systems for the treatment of cancer. These biodegradable conjugates consist of a drug (DFMO or Daunomycin), a spacer, an oligopeptide carrier (Tp20) and chemotactic targeting peptide moieties, which can bind to their specific cell-surface receptors and might be internalized by receptor mediated endocytosis. Carriers (H-[TKPPR]4-NH2) with tuftsin-like targeting moieties (H-TKPR, For-TKPR, H-TKPPR, For-TKPPR) in branches were synthesized by solid phase synthesis using mixed Boc and Fmoc strategies. Drug molecules with or without enzyme labile spacer were attached to the carrier system in solution. The bioconjugates were characterized by analytical RP-HPLC and ESI-MS. *In vitro* biological assays (chemotaxis, cellular uptake, apoptosis assay) were investigated with the prepared conjugates and their components on MonoMac6 and HL-60 human tumor cell lines. Most of the conjugates had advatageous chemotactic properties, they can be internalized rapidly and could trigger toxic effect on the cells. Our results suggest that these novel oligopeptide-based chemotactic drug delivery systems might be potential candidate for targeted cancer chemotherapy.

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Y02

Understanding the mechanism of cell penetrating peptides (CPPs) by a new approach based on advanced model membranes

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Understanding the internalization mechanism of carrier peptides is fundamental to their use as delivery vectors in pharmaceutics. Although a wide literature has been reported on CPPs interaction with model membranes no data show to take in account the heterogeneity and domain segregation of living cells. A new approach to study peptide/lipid interaction is recently considering innovative models where the membrane is viewed as phase coexistence of immiscible liquid ordered (Lo) and liquid disordered (Ld) domains. These models can be reproduced in giant unilamellar vesicles (GUVs), self-assembled from synthetic or natural purified lipids, and giant plasma membrane vesicles (GPMVs), obtained from chemically induced vesiculation -"blebbing"- of living cell membranes. Fluorescence microscopy enables the observation of coexisting fluid domains containing Lo-like and Ld-like phases, detectable by fluorescent probes. Moreover, it is a powerful tool for the characterization and quantification of specific biophysical parameters, such as membrane line tension. On these bases, we present here a fluorescence microscopy study aimed at investigating the still unveiled import mechanism of penetratin, penetratin conjugated peptide -A42- and pep-1. The biological activity of A42 was recently demonstrated by us. Our data show that the internalization mechanism of the peptides under scrutiny is crucially dependent on the choice of the experimental conditions - peptide concentration, model systems-. Moreover we proved that GPMVs represent a suitable model of plasma

membranes, resembling the real plasma membrane composition. Using these systems we demonstrated that more than one internalization mechanism may be hypothesized for the CPPs, including endocytotic and/or non endocytotic pathways.

Y03

Neoglycopeptides as glycoprobes for lectin-carbohydrate interaction studies by SPR

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Glycosylation, the most abundant post-translational modification in proteins, plays a key role in multiple biological processes, such as protein folding, lysosomal enzyme traffic and several cell communication events, such as fertilization, immune response, pathogen-cell anchoring, or metastasis. For this reason, there is an increasing interest in finding powerful analytical tools to study these molecular recognition events in details.

Surface plasmon resonance (SPR) is one of the most poweful tools for studying sugar-protein interactions, due to its sensitivity, low sample consumption and real time monitoring. In SPR, one of the two interacting entities (sugar or protein) must be immobilized onto the sensor surface, while the other is flown across. The resulting read-out enables kinetic and thermodynamic analysis of the interaction. In SPR-based sugar-protein interaction studies, the sugar-on-chip (*vs.* lectin-on-chip) alternative has demonstrable advantages, including novel lectin capture/identification and study of its carbohydrate specificity.

Our group has developed an SPR approach to monitor carbohydrate-protein interactions in which the sugar is immobilized via a tailor-made peptide module on the sensor surface (1, 2). The glycopeptide module is prepared by chemospecific oxime ligation between the reducing-end of the sugar and an aminooxyacetic acid (Aoa) residue at the *N*-terminus of the peptide module.

In this presentation we will describe the preparation of neoglycoprobes to correctly display different, small, sugar epitopes (di- and trisaccharides) as well as their application in SPR interaction studies.

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Y04

Synthesis and conformational analysis of a series of cyclic i-to-i+4 side chain-to-side chain 1,4-disubstituted [1,2,3]triazolyl-bridged PTHrP(11-19) derivatives

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Side chain-to-side chain cyclization is used to stabilize bioactive conformations and to reduce proteolytic degradation. Among the numerous modes of cyclization, bioisosteric modifications and cyclizations that do not require orthogonal protection schemes, are of great interest. The recently introduced Cu^{I}-catalyzed azide–alkyne 1,3-dipolar Huisgen's cycloaddition[1-3] as click chemistry reaction[4] presents a promising opportunity to develop a new paradigm for intramolecular cyclization. In fact, the proteolytic stable 1,4-disubstituted [1,2,3]triazolyl represents a constrained isosteric surrogate of the peptide bond. We report the preparation of N^{α}-Fmoc-ω-azido-α-amino acids by either diazo-transfer of the N^{α}-protected ω-amino-α-amino acid or a multistep strategy from the N^{α}-protected ω-hydroxy-α-amino acids. The N^{α}-Fmoc-ω-ynoic-α-amino acids were prepared by alkylation of Ni^{II} complexes of the Schiff bases derived from glycine and a chiral inducer with alk-ω-ynyl bromides. These building blocks were used in solid phase synthesis of a series of 8 linear nonapeptides derived from the sequence of PTHrP(11-19) where ω-azido- and ω-ethynyl-α-amino acids replaced Lys^{13} and Asp^{17}. Cleavage from the resin and side chain deprotection, followed by intramolecular Cu^{I}-catalyzed click reaction, generated a series of i-to-i+4 1,4-disubstituted [1,2,3]triazolyl-bridged cyclopeptides.[5] The CD and NMR conformational analysis allowed to identify the permutations that closely mimic cyclo[Lys13,Asp14]PTH(11-19). This study lays the ground work to design novel bioactive cyclopeptidomimetics using this novel intramolecular side chain-to-side chain rigidification mode.

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Y05

Nanostructure formation through incorporation of acyl chains enhances the activity of anti-LPS peptides

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Sepsis and its acute state, septic shock, are the first cause of mortality in intensive care units and a leading cause of mortality worldwide. In 2003, septicemia was the 10th foremost cause of death in the U.S. Sepsis and other infectious diseases are produced by a bacterial endotoxin, the lipopolysaccharide (LPS), a major component of the cell wall in Gram-negative bacteria. Therefore, peptides that interact with LPS can provide the basis for the development of new antisepsis agents. In this regard, the inhibition of LPS at the early beginning of the process (i.e. extracellularly neutralization of LPS) is considered a promising approach. In the present study, we have focused on three LPS-binding proteins: i) the Limulus anti-LPS factor (LALF), ii) the bactericidal permeability-increasing protein (BPI), and iii) the serum amyloid P (SAP). Several N-acylated peptides derived from LALF-14c were synthesized, cyclized and evaluated for anti-LPS activity. An increase in activity of at least 10-fold was observed for C16-LALF-14c over the parent peptide, C2-LALF-14c. On the basis of TEM images, these enhanced activities could be associated with the peptide's capacity to form nanostructures. TEM studies revealed that long fatty acyl chains promote the formation of micellar and fibrilar superstructures. This biological activity/nanostructure formation correlation has been corroborated with other anti-LPS peptides, BPI and SAP, which also displayed improved activities. Furthermore, applications of these peptides at cell-tolerated concentrations as cell-penetrating micellar-based peptides will be discussed.

Y06

Chemoenzymatic synthesis of C-terminal peptide thioesters for chemoselective ligations

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Over the last 15 years, several techniques for the chemoselective ligation of peptides have been introduced. Most interesting are those that produce

a genuine amide bond in the ligation product. Today, native chemical ligation,[1] which features a cysteine-thioester ligation is the most powerful. Alternatively, the so called Staudinger ligation[2,3] between phosphine thioesters and azides has potential to enable couplings independent of the amino acid side chain at the site of ligation. Since both methods rely on C-terminal thioester building blocks, the preparation of peptide thioesters is a key step in both methods. However, general Fmoc-based SPPS methods are not compatible with thioester synthesis. We,[4] and independently others,[5] have addressed this issue by the development of a novel chemo-enzymatic strategy. In this strategy, the application of a protease under optimized conditions allowed for the regio- and stereoselective C-terminal conversion of side chain unprotected peptide C-terminal methyl esters and carboxylic acids to obtain peptide C-terminal thioesters. The described strategy encompasses a new route, not limited by the method for peptide synthesis, to peptide thioesters as building blocks for chemoselective ligation reactions.

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Y07

The structure and interactions of the proline rich domain of ASPP2

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Peptides are valuable tools to study the structure and interactions of proteins. Here we used peptides in combination with biophysical and biochemical methods to study the proline-rich domain of ASPP2, a pro-apoptotic protein that stimulates the p53-mediated apoptotic response. The C-terminus of ASPP2 contains Ankyrin repeats and SH3 domains, which mediate its interactions with proteins such as p53 and Bcl-2, and a proline-rich domain (ASPP2Pro), whose structure and function are unclear. Our results show that the ASPP2Pro domain is natively unfolded. We found that ASPP2Pro domain makes an interaction with the ASPP2Ank-SH3 domains, and mapped the interaction sites in both domains. Using a combination of peptide array screening and biophysical and biochemical techniques, we found that ASPP2Pro does not mediate interactions of ASPP2 with peptides derived from its partner proteins. ASPP2Pro-Ank-SH3 bound a peptide derived from its partner protein NFkB weaker than ASPP2Ank-SH3 bound this peptide. This suggested that the presence of the proline-rich domain inhibited the interactions mediated by the Ank-SH3 domain. This was confirmed by our observation that a peptide from ASPP2Pro competed with a peptide derived from NFkB on binding ASPP2Ank-SH3. Based on our results, we propose a model in which the interaction between the ASPP2 domains regulates the intermolecular interactions of ASPP2 with its partner proteins.

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Y08

Polarity versus hydrophobicity: The impact of fluorinated amino acids on the self-assembly of coiled coil peptides

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The substitution of hydrogen by fluorine is one of the most auspicious structural modifications of organic compounds with improved pharmaceutical activity. Fluorinated amino acids are therefore prominent building blocks for modulating the structure and interactions of peptides and proteins. However, because fluoroalkyl groups uniquely combine two contrary properties - polarity *and* hydrophobicty - their effects as side chain substituents in native protein environments are not easily predicted. To test this, a new model system has been designed on the basis of a heterodimeric α-helical coiled coil peptide. A native monomer was used to screen ten complementary peptides that carry various fluorinated amino acids at either of two positions within the hydrophobic interface of the dimer. The heteromers were thermodynamically characterized by temperature induced unfolding monitored by CD-spectroscopy as well as by theoretical methods (MM-PBSA energy calculations). In all cases heterodimer formation was retained albeit with less stability. With the support of MD-simulations, we find that, depending on the position, the coiled coil structure imposes different conformations upon the fluorinated side chains. Consequently, the impact of fluorine induced polarity depends on the immediate environment of the specific amino acid.

Applying surface plasmon resonance we are currently investigating the impact of fluorine substations on the kinetics of coiled coil formation. These studies will help to gain a better understanding of the way fluorinated amino acids direct peptide/protein interactions and pave the way towards a directed application of fluorinated peptides in medicinal chemistry.

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Surface-bound cationic antimicrobial peptides: The effect of immobilization upon the activity spectrum and the mode of action Bagheri, Mojtaba	Y09
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β- turn peptide mimics as tool for understanding allosteric antagonism of GPCR activity Bourguet, Carine	Y11
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Peptide Nanobioscience: Heterobifunctional Linkers for Chemoselective Coupling of Peptides Thygesen, Mikkel B.	Y16

Y09

Surface-bound cationic antimicrobial peptides: The effect of immobilization upon the activity spectrum and the mode of action

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Due to the formation of biofilms and their intrinsic resistance to conventional antibiotic therapy, the development of materials which directly inhibit the growth of pathogens at surfaces is a promising approach to combat surface-associated infections. One strategy to develop such biomedical surfaces is the covalent attachment of antimicrobial agents. Among the various antimicrobial compounds, cationic antimicrobial peptides (CAPs) represents a promising alternative to classical antibiotics. However, there are few studies addressing the activity and mechanism of action of surface-bound CAPs. How the immobilization of antimicrobial peptides at different chain positions and the length of spacer between the active sequences and the solid matrix influence the activity against bacteria, and the mode of action has to be investigated. In this report, we evaluated the antimicrobial activities of two surface-fixed CAPs with different activity profiles: an amphipathic 18-residues model peptide (KLAL) and a magainin peptide (MK5E). The peptides were immobilized on TentaGel S NH2 and HypoGel 400 NH2 resins characterized by different spacer lengths and loading capacities as model surfaces. The peptides were attached covalently at the C-terminus, N-terminus and side chains, and the activities of the solid matrix-bound peptides were investigated against B. subtilis and E. coli. The peptides retained their activity but, TentaGel S NH2-bound peptides with a long spacer are in general more active than HypoGel 400 NH2-fixed compounds with a spacer shorter than the thickness of the bacteria outer membranes. Furthermore, whereas there was no difference between the activities of KLAL immobilized at different chain positions against both strains, the C-terminally immobilized MK5E showed distinctly higher activity than MK5E fixed at other chain positions. The results suggest a carpet mode of action and toroidal-pore formation as mechanisms of surface-loaded KLAL and MK5E, respectively.

Y10

Quantitative evaluation cellular uptake of 22 CPPs in 4 different cell lines

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Most molecules that are not actively imported by living cells are impermeable to cell membranes, including practically all macromolecules and even many small molecules whose physicochemical properties prevent passive membrane diffusion. A more and more popular class of membrane translocating agents are the cell penetrating peptides (CPPs). To our best knowledge no single systematic study comparing a majority of existing CPPs has been carried out since nowadays. Differences in the numerous variables of the experimental procedures employed by various groups make it harder to compare studies and arrive at a conclusion especially on the mechanism of internalization. However, since there has been no systematic attempt to determine which CPP sequence show the optimal cellular uptake, we undertook studies to compare the delivery of 22 different CPPs in the four cell lines Cos-7, HEK293, HeLa and MDCK. In a first step we define standard conditions allowing the direct comparison of the measured cellular uptake. Thereafter, we started to analyze systematically the properties of the CPPs by changing one of the conditions such as trypsinization, endocytose inhibitors or temperature. Base on our results, we could clearly demonstrate the differences in cell penetration between the various CPPs. Furthermore, we could conclude that the endocytose consists in multiple mechanism which can be influenced only using several CPP:cell line couples. Taking altogether, our quantitative evaluation of cellular uptake ins an important contribution to obtain the best CPP:cell line couple for a targeted biological assay. In view of these considerations, CPPs offer a promising new tool for targeting protein network within the cell and furthermore, for noninvasive and specific delivery of pharmacological agents.

Y11

β-turn peptide mimics as tool for understanding allosteric antagonism of GPCR activity

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Preterm labor, for which medical and social costs are estimated at $ 9 billion per year in the USA (the highest per patient cost of any disorder), has steadily increased over the last thirty years. We have targeted the prostaglandin F2α receptor, a member of the G-protein coupled receptor family, for the development of a method to prevent preterm labor, because this receptor is directly involved in uterine contractions and over-expressed at onset and during labor. Our reseach has led to allosteric modulators of the PGF2α receptor, that feature a turn geometry in the peptide and small molecule mimics [1,2] . To explore the importance of the turn conformation for biological activity, the central residue in the peptide mimics has been modified using different types of beta-turn mimics. For example, the enantiomeric forms of indolizidin-2-one (m=1, n=1), indolizidin-9-one (m=0, n=1), and quinolizidinone (m=1, n=2) amino acids were introduced into the peptide mimic to induce type II and II' geometry. Our presentation will discuss the synthesis, conformational analysis and biological activity of these novel allosteric modulators of prostaglandin PGF2α receptor activity.

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Y12

Design and synthesis of cell-penetrating nucleopeptides

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In recent years peptide nucleic acids have gained much attention as oligonucleotide mimics with very good affinity and selectivity. On the other side such molecules have poor water solubility and do not penetrate efficiently through biological membranes.

We therefore focused our attention on nucleopeptides, a different class of nucleotide analogues containing nucleo-amino acids, i.e. amino acids carrying nucleobases in their side chains. In nucleopeptides, backbone conformation controls the relative orientation and distance of the nucleobase side chains, thus modifying the nucleobase ability to interact with complementary sequences.

Our project aims at investigating the effects of backbone structures in sequential nucleopeptides on peptide-peptide and peptide-oligonucleotide base pairing properties. To do so, we designed sequential nucleopeptides with a polyalanine backbone in which nucleo-amino acids are placed every third residue and two lysine residues are set at the N- and C- terminus in order to increase water solubility and allow peptide functionalization with a fluorescent moiety or with biotine.

The solution-phase synthesis of β-alanyl nucleo amino acids carrying the four DNA bases (thymine, adenine, cytosine, guanine) will be described, as well as the solid-phase synthesis and functionalization of nucleopeptides.

The nucleopeptide pairing properties have been investigated through surface plasmon resonance and pairing of a thymine-containing and a surface bound adenine-containing nucleopeptide has been observed. The affinity of the same adenine containing nucleopeptide towards complementary DNA and RNA oligos has also been studied.

Fluorescent and biotinylated nucleopeptide derivatives have been detected in cytosolic and nuclear compartments of tumor RENCA cells; cell penetration is most probably due to endocytosis.

Cytotoxicity tests have shown that such nucleopeptides do not affect cell viability.

Y13

Conversion of non-contiguous active regions in proteins into intestinally permeable macrocyclic scaffolds: the HIV-1 CD4:gp120 model.

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Cycloscan is a method in which libraries of backbone cyclic peptides are synthesized and screened for biological activity. The diversity of the Cycloscan libraries is a spatial diversity (also called conformational diversity) namely; all the members in the library maintain the original side chain sequence of the parent linear peptide. Members of the cycloscan libraries differ from each other in the following diversity parameters (1) mode of cyclization (2) ring position (3) ring size (4) ring chemistry. The diversity of cycloscan allows the target to select the best peptide that undergoes conformational complementarity. In this study we have used Cycloscan to convert a non-contiguous active region in proteins into an intestinally permeable macrocycle. We have used the CD4:gp120 interaction as a model. Based on the x-ray structure of the HIV-1 CD4:gp120, we synthesized backbone cyclic peptide libraries that include two non-contiguous active regions of CD4. From this library we selected a potent inhibitor of HIV-1 replication in cells. We managed to minimize the size of the inhibitor to a small macrocyclic scaffold that preserved the two pharmacophores Arg59 and Phe43 of the CD4 extra cellular domain and maintained the inhibitory activity. We have further synthesized a focused stereo isomeric macrocyclic library that contains the Arg59 and Phe43 side chains and maintains the same ring size and ring chemistry as the potent HIV inhibitor obtained from the cycloscan library. Only the S,S isomer has demonstrated an extremely high intestinal permeability and intestinal metabolic stability. This study demonstrates the use of Cycloscan to convert non-contiguous active regions in proteins into backbone macrocycles with extremely high intestinal permeability and metabolic stability.

Y14

Involvement of alpha-2 domain in prion protein conformationally-induced diseases

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The alpha-helix 2 of the prion protein (PrP) possesses chameleon conformational behaviour, gathers several disease-associated point mutations, can be toxic to neuronal cells and strongly fibrillogenic. Therefore it is a suitable model to investigate both structural determinants of PrP misfolding and rational structure based drug design of compounds able to block or prevent prion diseases (1). The intriguing structural properties of this protein domain prompted us to investigate the conformational landscape of the alpha-helix 2. The alpha-helix 2 of hPrP was used as a template for designing alpha2-helix-derived peptides, which were synthesized by SPPS and characterized by CD and NMR in aqueous buffer at different pH, in structuring media and in presence of anions (2) and bivalent metal cations (3). The neurotoxicity of these peptides was also assayed on B104 neuroblastoma cells. In addition, the affinity of the alpha-helix 2-derived peptides for potential PrP-binding molecules was investigated by integrated spectroscopical and computational studies (4). All our data highlight the importance of the alpha-helix 2 as nucleation point of prion misfolding and oligomerization and also as target for therapeutic and diagnostic approaches in prion diseases.

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Y15

Development of Miniature b(-HLH-)ZIP Peptidosteroid Models

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Dimerization is often crucial in selective biomolecular recognition, both governing numerous protein/protein and protein/DNA interactions.(1) Contemporary research at the interface of chemistry and biology focusses on the understanding of these interactions and the design of synthetic receptors based on naturally occurring systems can be of help in this respect. Consequently, the design of minimized proteins, containing the minimum requirements necessary for recognition of biomolecules is actively pursued. Besides the simple desire to mimic Nature, this research provides insight in the complex interplay between various biochemical systems and has potential therapeutic applications.
We are currently investigating the design of new peptidic receptors, based on the attachment of (relatively) short minimal recognition peptides to a steroid core with defined geometrical properties. Dimerization being a prerequisite, the correct geometrical positioning of the peptides relative to the target is another necessity. An additional feature of the steroid scaffold is the orthogonal reactivity of the attachment points, permitting the design of not only homo-, but also heterodimers.(2)
The DNA major groove targeting leucine zipper (bZIP) and basic/helix-loop-helix/zipper (b/HLH/ZIP) protein dimers, involved in the transcription of DNA to mRNA,[1] are studied in the context of the current project. Peptide dimers consisting of the basic DNA contact regions of naturally occurring GCN4, cMyc and Max proteins were developed. The solid-phase-synthesis and electrophoretic evaluation of DNA recognition will be discussed.

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Peptide Nanobioscience: Heterobifunctional Linkers for Chemoselective Coupling of Peptides

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Quantum Dot (QD) and Gold (Au) nanoparticles are at the heart of the nanobiotechnology revolution. Methods for the regio- and chemoselective covalent anchoring of biomolecules to nanoparticles to form functional nano-scale objects will become central tools for biochemistry, biomedicine, and nanobioscience. It is our aim to develop such efficient and robust methods for the preparation of oligo(ethylene glycol) (OEG) linkers with built-in chemistry for highly chemoselective reactions. These linkers allow covalent attachment of biomolecules, especially peptides, proteins and carbohydrates, to nanoparticles and other surfaces in a biocompatible manner, *i.e.* the linker prevents proteins from becoming denatured and enables interaction of the nanobio-probe with other biomolecules. Furthermore, the OEG linkers provide aqueous solubility to the intrinsically hydrophobic nanoparticle core.
We have developed a two-step chemoselective approach, whereby peptides may be attached via oxime ligation and OEG conjugates are immobilized on nanoparticles via self-assembly. For synthesis of heterobifunctional linkers, Mitsunobu chemistry was used to introduce *N*-hydroxyphthalimide and tritylmercaptan on OEGs. Polymer-supported triphenylphosphine was used to ease purification. Protected OEG linkers were investigated in various strategies for the attachment of peptide aldehydes to nanoparticles. For example, chemoselective oxime ligation and liberation of thiols allowed attachment to nanoparticles. Using these systems, we studied the self-assembly and ligand density of peptide ligands and chemoselective ligation chemistry on nanoparticles.
These new bifunctional linkers enable sequential chemoselective reactions for efficient immobilization of biomolecules to nanoparticles, thus enabling a wide range of applications, including bio-imaging.