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A HIGHLY EFFICIENT PREPARATION OF NEOGLYCOCONJUGATE VACCINES USING SUBCARRIERS THAT BEAR CLUSTERED CARBOHYDRATE ANTIGENS

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Abstract: A limited amount of spacer-equipped carbohydrate haptens was linked by reductive amination to a subcarrier, an oligopeptide containing 16 amino groups, to give a hapten-carrying subcarrier (HCS). It was then linked, via the remaining free amino groups, to chicken serum albumin (CSA) to give a cross-linked neoglycoconjugate bearing the haptens in the form of clusters. Alternatively, the same type of a glycoconjugate, but with higher conjugation efficiency, was obtained when HCS was treated successively with squaric acid diethyl ester and CSA.

Classical work by Goebel and Avery has shown[1,2] that immunogenicity of carbohydrate antigens can be increased by linking them to suitable protein carriers. Use of a vaccine against *Hemophilus influenzae* b,[3] based on this principle, has had an unprecedented impact on public health in countries where the vaccine is routinely used. When designing conjugate vaccines we try to mimic the situation on the surface of disease-causing bacteria, namely the density and distribution of antigens on the carrier. These molecules often occur on the surface of pathogens as multiple, not single molecules. It is, therefore, not surprising that the arrangement of antigens on neoglycoconjugates in the form of clusters results in their enhanced immunogenicity.[4–6] This phenomenon has been referred to as the cluster effect.[7]

We are interested in using synthetic fragments of the O-polysaccharides (O-PS) of bacterial pathogens as the antigenic components of conjugate vaccines. Currently, we are working towards a conjugate vaccine against cholera and have already reported[8,9] on the preparation of some neoglycoconjugates from functionalized fragments of the O-PS of *Vibrio cholerae* O:1, which were directly linked to chicken serum albumin (CSA) by single-point attachment.[10] To compare the immunogenicity of such conjugates with their counterparts bearing antigens in the form of clusters, we have now prepared clusters from the same haptens and linked them to CSA. Conventional cluster type conjugates, such as those described by Toyokuni et al.[6] or Lee et al.,[7,11] are prepared by linking small clusters of haptens to a carrier protein by single point attachment. Cluster neoglycoconjugates described here are conceptually different in that they are formed by chemical attachment of a limited number of carbohydrate antigens to a subcarrier containing many reactive groups, to give a hapten-carrying subcarrier (HCS, Figure 1, where a-c are spacer-equipped haptens suitable for linking to amino group(s) containing molecules by reductive amination, squaric acid diester chemistry[12,13] and carbodiimide reagents, respectively). This is a multivalent neoglycoconjugate (later referred to as a neoglycocluster) of relatively low molecular mass, still containing some of its reactive groups unchanged (in the present case, amino groups). The neoglycocluster is

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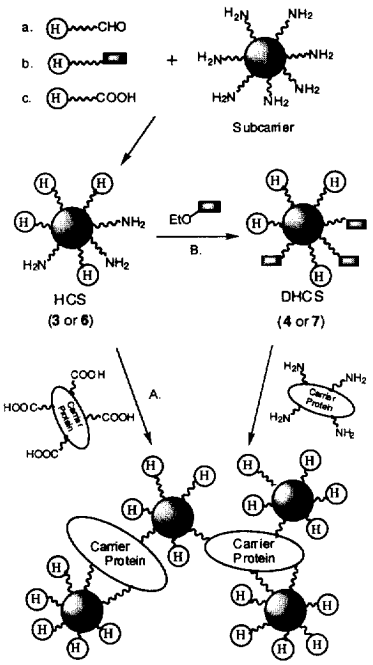
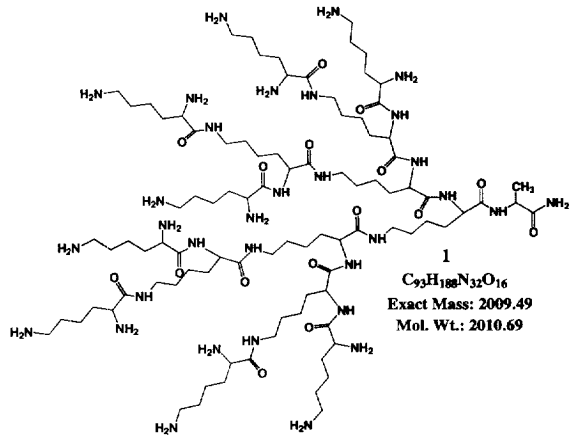


Figure 1

Squarate end
Linker-Equipped Hapten
HCS Hapten-Carrying Subcarrier
DHCS Derivatized HCS

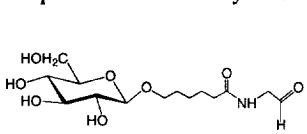


then linked chemically (employing, for example, a carbodiimide type coupling reagent), through these remaining reactive groups, to a protein carrier (Figure 1, Pathway A), yielding the target, high molecular mass HCS—protein neoglycocluster conjugate. Alternatively, the HCS can be further derivatized (Figure 1, Pathway B), if this is advantageous in the next conjugation step, to yield a *derivatized* hapten-carrying subcarrier (DHCS). DHCS is then conjugated to a protein carrier. This new concept is applicable not only to carbohydrates but to other areas where conjugation of haptens in the form of clusters to macromolecules is sought. Depending on the nature of immunogens, means other than those shown here (Figure 1) can be involved, and other types of subcarriers can be employed.

The subcarrier we used is the branched oligopeptide **1**.[14] Similar materials fully saturated with carbohydrate antigens have been described,[15,16] evaluated, and found as inhibitors with remarkably increased potency in various protein–carbohydrate interactions. To our knowledge, use of dendridic lysine or other cores as *sub*carriers, *incompletely* saturated with carbohydrate antigens, and their subsequent linking to suitable protein carriers through their remaining reactive groups has hitherto not been described. Compound **1** contains 16 primary amino groups available for chemical transformation. For example, coupling *n* haptens to such a subcarrier, will leave (16 – n) amino groups on the resulting HCS available for subsequent coupling to a carrier protein. Use of such a subcarrier substantially increases the efficiency of conjugation of neoglycoclusters to carrier proteins. This is because only one of the many remaining free reactive groups in the HCS (amino groups in the present case, Figure 1) has to react with the carrier protein to form an HCS—carrier–protein conjugate. By comparison, in the conventional single point attachment model[7] there is only *one* reactive group per antigenic molecule (a hapten or a cluster) available for conjugation. That one has a much-diminished chance to react subsequently with the carrier protein and form a neoglycoconjugate. Consequently, conjugation of an HCS containing 8 free amino groups, for example, should be 8 times more efficient than that of a conventional hapten.

Achieving the highest possible conjugation efficiency is particularly important when labor-intensive, synthetic haptens are to be conjugated. The presence of free reactive groups in HCS (or DHCS) also increases the likelihood of cross-linking with multiple carrier molecules (Figure 1) during their subsequent linking to a protein carrier. This increased possibility of cross-linking should prove to be advantageous in the making of neoglycoconjugate vaccines according to the concept presented here, since others indicated[17,18] that the cross-linked neoglycoconjugates do show enhanced immunogenicity.

The feasibility of making the neoglycoconjugates as just discussed was tested first using a readily available spacer-equipped derivative of D-glucose, **28** (mol. mass, 335.16 Da). It was coupled by reductive amination with the subcarrier **1** at a molar ratio of 10:1. The MALDI-TOF analysis of the product showed that, on average, the D-glucose-bearing HCS (**3**, average mass 5019 Da) formed contained 9.4 D-glucose residues/mol. To follow Pathway B (Figure 1), the foregoing material was treated with an excess of squaric acid diethyl ester to produce the D-glucose-containing DHCS (**4**, average mass 5553 Da). MALDI-TOF analysis showed **4** to contain, on average, 4.3 squaric acid monoethylester residues/mol.



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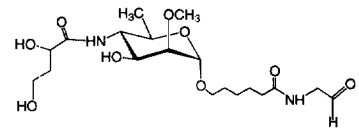

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To study the coupling efficiency and incorporation of neoglycoclusters in carrier proteins, direct conjugation of HCS **3** was carried out at various HCS/CSA ratios, using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetrametyluronium hexafluoro-phosphate (HATU).[19,20] The product was analyzed by quantitative, carbohydrate phenol-sulfuric acid assay. The result showed (Table 1) that some HCS remained uncoupled when HCS/CSA ratios of 8:1 and 16:1 were used. In addition, data in Table 1 show that coupling efficiency decreases when higher HCS/CSA ratios are used. Nevertheless, the efficiency of coupling using HATU is reasonably high. On the other hand, analysis of the neoglycoconjugates resulting from coupling of the DHCS **4** to CSA showed (Table 1) that even at the highest HCS/CSA ratio virtually all DHCS used was incorporated in the DHCS—CSA neoglycocluster conjugate. Thus, the concept of using a multivalent subcarrier, such as **1**, greatly increases the conjugation efficiency. Considering that each HCS **4** contained 9.4 haptens/mol, on average, some 90 and 150 haptens could be easily incorporated in CSA (mol. mass 67000 Da, itself containing only 46[21] amino groups), when HCS and DHCS were employed, respectively. This clearly demonstrates the potential the present approach has in synthetic vaccine development.

Synthesis of the neoglycocluster conjugate from the immunologically dominant determinant of the O-PS of *Vibrio cholerae* O:1, serotype Ogawa[22] was carried out according to the above protocol using the spacer-equipped hapten **5** (mol. mass 434.23)[8] as hapten. The synthesis involved preparation of the hapten **5**-containing HCS (**6**, MALDI-TOF *m/z* 5516), using compounds **5** and **1** in a ratio of 9.3:1. MALDI-TOF analysis showed **6** to contain 8.4 residues of **5**. The HCS cluster **6** was then treated with an excess of squaric acid diethyl ester to give the **6**-containing DHCS (**7**). MALDI-TOF analysis of **7** showed it to contain 4.4 of squaric acid monoester residues. Subsequent coupling of **7** with CSA at various DHCS ratios yielded the cross-linked neoglycoconjugates containing monosaccharide **5** in the form of clusters. The phenol-sulfuric acid carbohydrate assay showed that virtually all DHCS **7** used was linked to CSA (Table 1).

Table 1. Carbohydrate Assay for the (D)HCS—CSA Neoglycoconjugate[a]

Conjugation (Neoglycocluster)	(D)HCS:CSA	Carbohydrate incorporated		Conjugation Efficiency [%]
		Theoretical[b] [wt.%]	Found [wt.%]	
Carbodiimide (HCS, **3**)	2:1	5.14	4.96	96.5
	4:1	9.02	7.90	87.5
	8:1	14.45	10.49	72.6
	16:1	21.05	12.54	59.6
Squarate (DHCS, **4**)	2:1	5.08	5.01	98.6
	4:1	8.80	8.73	99.2
	8:1	13.90	13.04	93.8
	16:1	19.58	19.41	99.1
Squarate (DHCS, **7**)	1.6:1	5.36	5.98	~100
	3.2:1	9.52	10.69	~100
	6.4:1	15.57	15.03	96.5
	12.8:1	22.82	20.05	87.9

[a]Carbohydrate amount expressed as methyl glycoside analogs of **2** or **5**, as required. [b]Based on the amount of hapten in (D)HCS used.

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