Synthetic Vaccines

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Synthetic Vaccines Consisting of Tumor-Associated MUC1 Glycopeptide Antigens and a T-Cell Epitope for the Induction of a Highly Specific Humoral Immune Response**

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Dedicated to Professor Gunter Fischer on the occasion of his 65th birthday

The mucin MUC1 is an attractive target for the development of an immunotherapy against cancer.[1,2] Its extracellular domain contains numerous tandem repeats of the sequence HGVTSAPDTRPAPGSTAPPA, which includes five potential glycosylation sites.[3–6] On epithelial tumor cells, MUC1 is extensively overexpressed, and owing to down-regulation of certain glycosyltransferases and concomitant overexpression of sialyl transferases, the tumor-associated MUC1 bears short saccharides with premature sialylation.[7–10] As a result of the incomplete glycosylation of MUC1, which on normal cells is covered by large saccharides, the peptide backbone is accessible to the immune system in tumor-associated MUC1. Therefore, the saccharide as well as the peptide structure contribute to the tumor-associated epitopes.[2,11,12] As a consequence, antibodies that selectively bind to the surface of tumor cells should be inducible using glycopeptides from the tandem repeat region of MUC1. However, MUC1 glycopeptides are not sufficiently immunogenic and additional stimulation is necessary to elicit a strong humoral immune response.[13–17] This stimulation should be achieved by activation of T_H cells through binding of their T-cell receptor (TCR) to a T-cell peptide antigen presented by the major histocompatibility complex MHCII on the surface of an antigen-presenting cell (APC).[18]

Recently, we showed[14] that specific antibodies against MUC1 glycopeptides can be induced by a synthetic vaccine consisting of a MUC1-glycododecapeptide and a T_H-cell peptide epitope from ovalbumin ($OVA_{323–339}$).[19] Here, we describe synthetic vaccine constructs **1**, **2**, and **3** containing mono-, di-, and triglycosylated complete tandem repeat peptides linked through a nonimmunogenic spacer amino acid to the OVA T-cell epitope (Figure 1). The tumor-associated saccharide sialyl T_N, which has been identified in mammary, stomach, and colon carcinomas,[20–22] was incorporated in all three vaccines through linkage to threonine 6. The di- and triglycosylated peptides in addition contain T_N antigen glycans at the other threonines. Thus, the triglycosylated MUC1 vaccine **3** is glycosylated in the immunodominant PDTRP motif.[23,24] This region of MUC1 is masked on normal cells with long-chain glycans but becomes accessible in tumor cells. There is currently discussion whether this region becomes more or less immunogenic by glycosylation of this threonine.[5–12] The results reported herein suggest that glycosylation in this position decreases the immunogenicity.

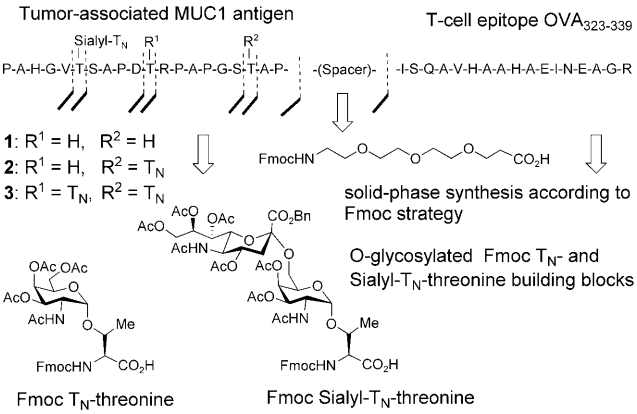

Figure 1. Strategy for the synthesis of MUC1–$OVA_{323—339}$ vaccine construct consisting of a MUC1 glycopeptide antigen, a nonimmunogenic spacer amino acid, and an immunostimulating OVA T-cell epitope.

The mono-, di-, and triglycosylated MUC1 tandem repeat peptides and OVA T-cell epitopes were synthesized on solid phase using a Wang resin[25] loaded with Fmoc arginine (Scheme 1). The glycosylated threonine building blocks[14,26–28] were applied in two equivalents and coupled manually with HATU/HOAt,[29] whereas other Fmoc amino acids were applied in 20 equivalents and automatically coupled with HBTU/HOBt.[30]

The 38-amino-acid vaccine constructs were detached from resins **4**–**6**, and all acid-sensitive protecting groups were simultaneously removed using trifluoroacetic acid (TFA)/triisopropylsilane (TIPS)/H_2O (15:0.9:0.9). Isolation was achieved by precipitation with diethyl ether and purification by preparative HPLC. After hydrogenolysis of the NeuNAc benzyl ester, the O-acetyl groups were removed by transes-

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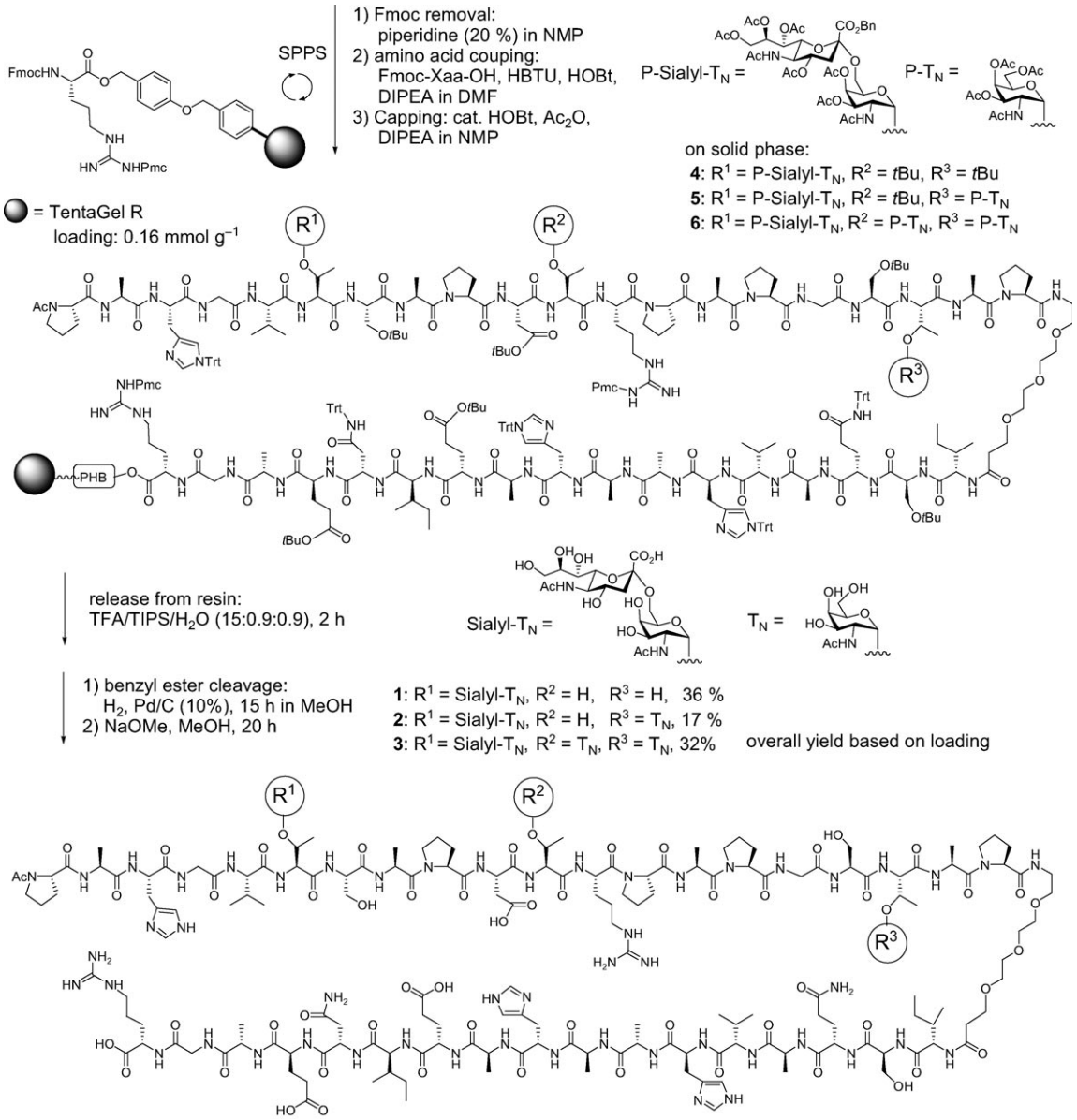
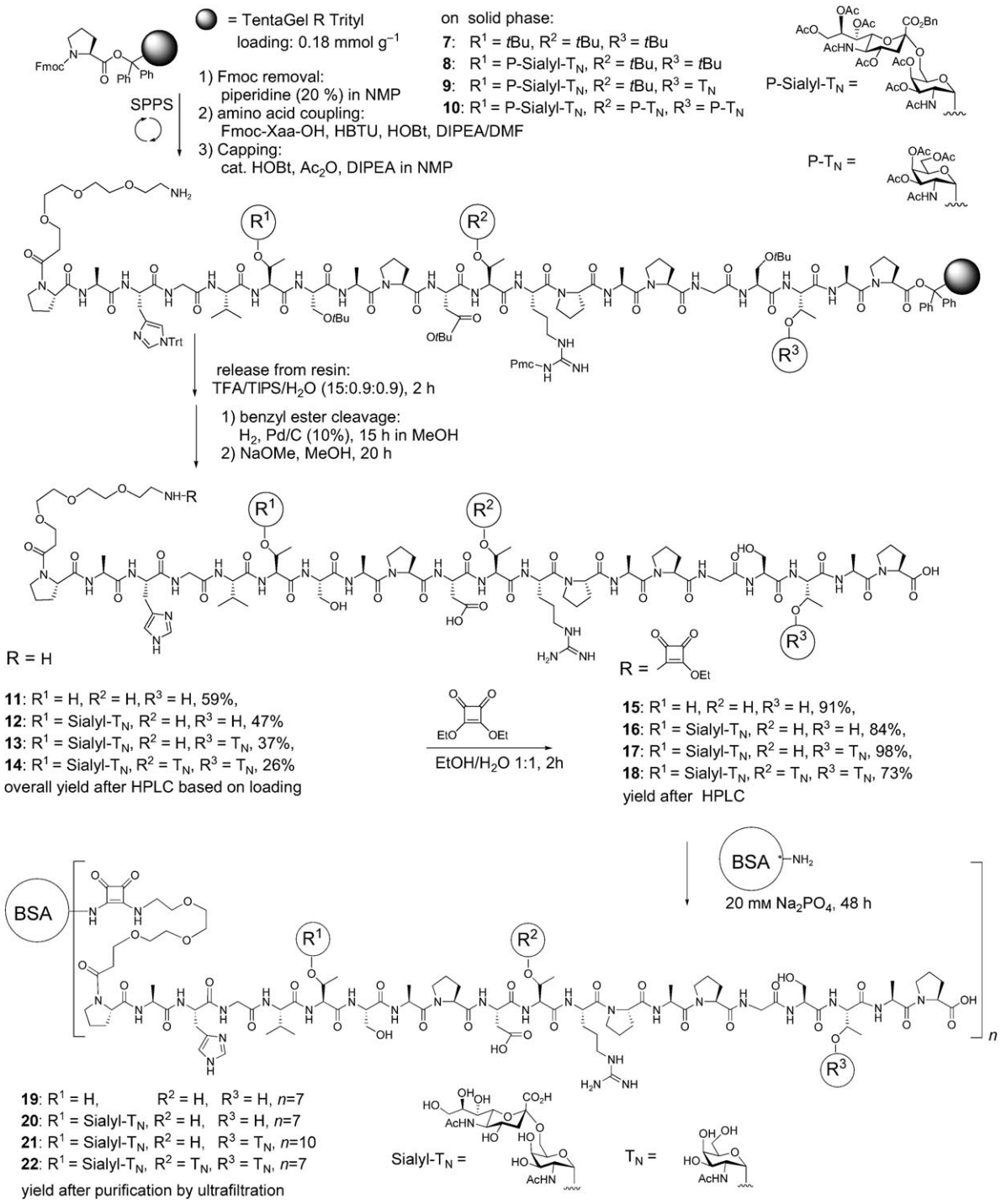


Scheme 1. Synthesis of mono-, di-, and triglycosylated MUC1–$OVA_{323–33}$-vaccine constructs; HBTU: *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt: 1-hydroxybenzotriazole, DIPEA: diisopropylethylamine, HATU: *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt: 7-aza-1-hydroxybenzotriazole, PHB: *p*-hydroxybenzyl, Pmc: pentamethylchromanesulfonyl, Trt: trityl.

terification in methanol with catalytic NaOMe at pH 9.5. Purification by preparative HPLC gave the glycopeptide–$OVA_{323—339}$ vaccines in overall yields of 36 % (**1**), 17 % (**2**), and 32 % (**3**). These compounds have been used for vaccination of mice transgenic in a CD_4 receptor for $OVA_{323–339}$.

In order to detect the antibodies, BSA conjugates of the non-, mono-, di-, and triglycosylated MUC1 peptides were synthesized. The antigens were assembled in protected form (**7**–**10**) on a resin equipped with a trityl linker in a fashion analogous to that for the vaccines (Scheme 2). Release from the resins using TFA/TIPS/H_2O, hydrogenolysis, deacetylation by transesterification, and purification by HPLC gave the (glyco)peptides in overall yields of 59 % (**11**), 47 % (**12**), 37 % (**13**), and 26 % (**14**). In order to conjugate these glycopeptides to BSA, they were reacted with diethyl squarate at the N-terminal amino group in EtOH/H_2O (1:1) at pH 8 to give the squaric ester monoamides, which were isolated after preparative HPLC in yields of 91 % (**15**), 84 % (**16**), 98 % (**17**), and 73 % (**18**). The coupling to BSA was performed in aqueous Na_2HPO_4 buffer at pH 9.5. After ultrafiltration, the BSA conjugates displayed an average loading of $n = 7$ (**19**), $n = 7$ (**20**), $n = 10$ (**21**), and $n = 7$ (**22**).

For induction of a humoral immune response against the MUC1–glycopeptide antigens, transgenic mice (DO11.10), whose T cells express a receptor specific for the $OVA_{323–339}$ T-cell epitope, were immunized with 10 μg of the synthetic vaccines **1**–**3** together with complete Freund's adjuvants (CFA). Mice 4–6 were immunized with the monoglycosylated vaccine **1**, mice 7–9 with the diglycosylated vaccine **2**, and mice 10–12 with the triglycosylated vaccine **3**. As a control,



Scheme 2. MUC1–(glyco)peptide–BSA conjugates (**19**–**22**) for detection of the induced anti-MUC1 glycopeptide antibodies by ELISA; BSA: bovine serum albumin.

mice 1–3 (PBS group) were treated with buffer solution instead of the vaccine. After 21 days booster immunizations were performed with 10 μg vaccine and incomplete Freund's adjuvants (IFA). Five days after the third immunization blood was drawn from each mouse and the supernatant of the centrifuged blood was subjected to antibody analysis. For the detection of MUC1-specific antibodies, ELISA was performed on microtiter plates coated with the MUC1–(glyco)-peptide–BSA conjugates. The sera, increasingly diluted, were added and the antibodies photometrically detected by binding of biotin-labeled antimouse antibodies followed by binding of streptavidin linked to horseradish peroxidase (HPO).[32] Mouse 4, immunized with the monoglycosylated vaccine **1**, and mice 7 and 9, immunized with the diglycosylated vaccine **2**, displayed high antibody titers all specific to the MUC1–glycopeptide antigens (Figure 2 a–c). In contrast, none of the

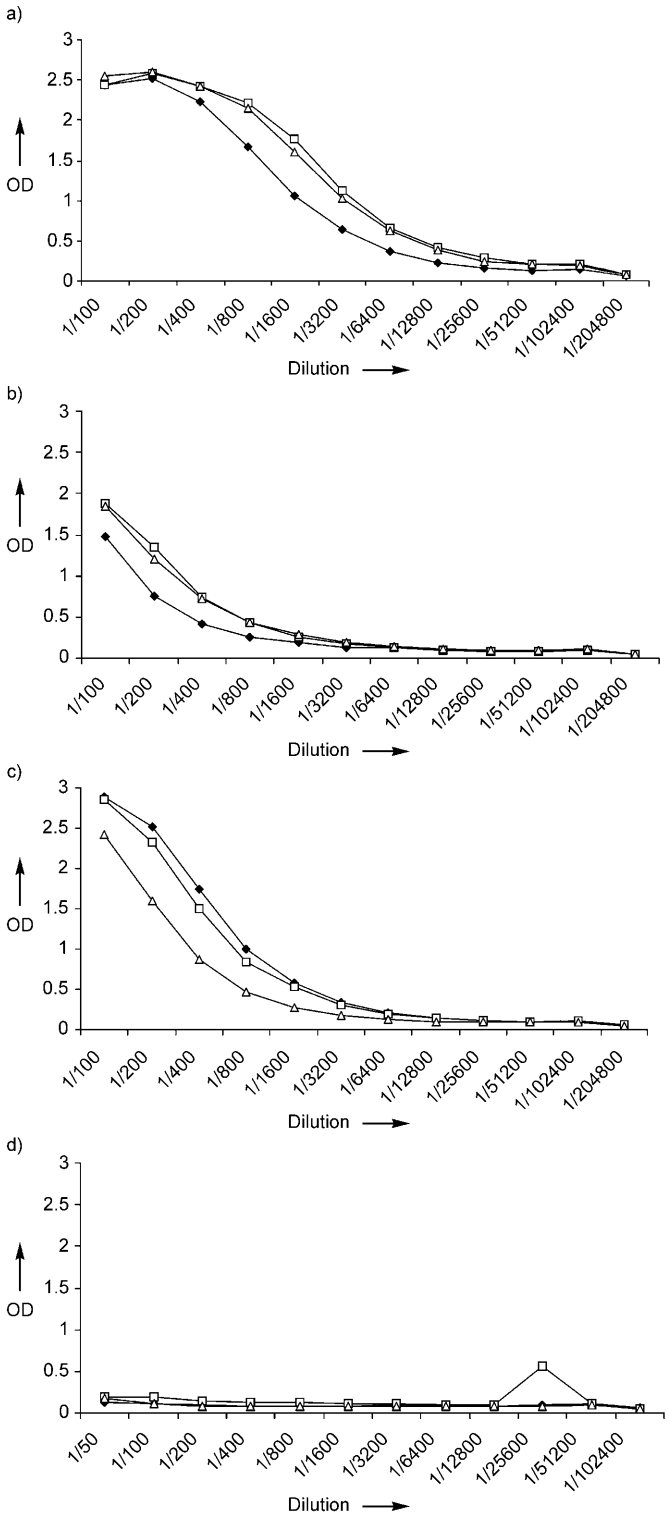


Figure 2. Detection of the MUC1-specific antibodies by HPO-catalyzed oxidation of the dye ABTS[32] (optical density (OD) at $\lambda = 410$ nm); ◆ third immunization; □ fourth immunization; △ fifth immunization. a) mouse 4, immunization with monoglyco-MUC1-OVA vaccine **1**, binding on **20**; b) mouse 7 and c) mouse 9, both immunized with diglyco-MUC1-OVA vaccine **2**, binding to **21**. d) MUC1-antibody titer ($\lambda = 410$ nm) after 3. immunization with triglyco-MUC1-OVA vaccine **3** in ◆ mouse 10, □ mouse 11, △ mouse 12 (here coating of wells with **22**).

mice 10–12 immunized with the triglycosylated vaccine **3** showed any immune response (Figure 2d). Further immunizations carried out with mice who showed a response and with the control group did not increase the antibody titers (Figure 2a–c).

All antibodies induced in mice 4, 7, and 9 showed specific binding to the mono- (**20**), di- (**21**), and triglycosylated (**22**) MUC1–BSA conjugates (Figure 3b–d) but no or low binding to the non-glycosylated MUC1–BSA conjugate **19** (Figure 3a). From these observations, it is concluded that the antibodies induced with the mono- (**1**) and diglycosylated (**2**) MUC1–tandem repeat–OVA vaccines are highly specific. Not only the saccharide but also the peptide backbone is important for the epitope recognition. The latter obviously is so important that the triglycosylated MUC1–BSA conjugate (Figure 3d) also is recognized by the antibodies induced by the vaccines **1** and **2** although it carries a T_N antigen saccharide in the immunodominant domain PDTRP and is not immunogenic in the form of its OVA construct **3**.

When the fully synthetic vaccines **1** and **2** containing the complete tandem repeat domain of the tumor-associated mucin MUC1 and a single sialyl T_N antigen (**1**) or this together with a T_N antigen (**2**) linked by a nonimmunogenic spacer to a T-cell epitope of ovalbumin were applied in mice, highly specific humoral immune responses were induced. Both antibodies induced against **1** and **2** recognize the glycopeptide antigen structures (**12, 13**) typical for epithelial tumor cells in their BSA conjugates **20**–**21** (Figure 3b,c) and, in addition, the triglycosylated MUC1–glycopeptide antigen **14** in the BSA conjugate **22**, but not the BSA conjugate **19** of the non-glycosylated MUC1 tandem repeat peptide. This selectivity is also confirmed by corresponding neutralization of the antibody induced by **1** through glycopeptide antigens **12**–**14**. The results show that the MUC1–glycopeptide, glycosylated in the PDTRP epitope, and not immunogenic itself, is recognized by the antibodies induced with the synthetic vaccines. This may explain why tumor-selective antibodies induced with biological material also bind to this structure.[6,23]

The antibody induced with the monoglycosylated sialyl T_N-MUC1 antigen vaccine **1** (Figure 3) exhibits the highest binding affinity to all three tumor-associated MUC1–glycopeptide antigen BSA conjugates (Figure 3b–d). This gives evidence that a specific immune response can be induced with a synthetic vaccine (**1**) that is directed to several aberrant glycopeptide structures present on a tumor cell. On this basis it should be possible to develop antitumor vaccines which at the same time are specific and have a useful scope of recognition.

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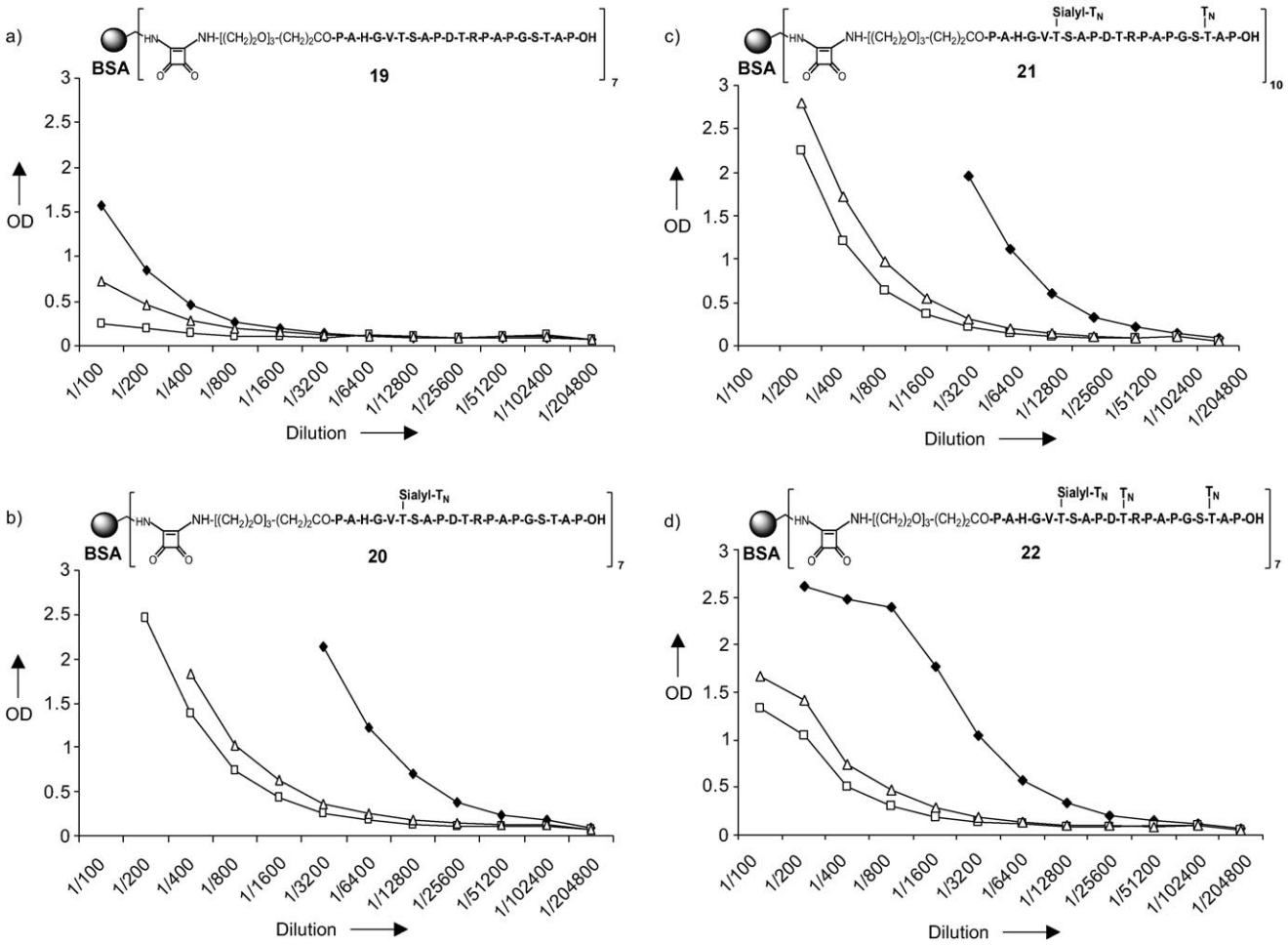

Figure 3. Specific binding of the induced anti-MUC1-glycopeptide antibodies (after the fifth immunization): ◆ mouse 4, □ mouse 7, △ mouse 9; detection (optical density at $\lambda = 410$ nm); ELISA binding assay on plates coated with glycopeptide antigen-BSA conjugates: a) non-glycoMUC1-BSA **19**; b) mono-glyco-MUC1-BSA **20**; c) diglyco-MUC1-BSA **21**; d) triglyco-MUC1-BSA **22**.

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