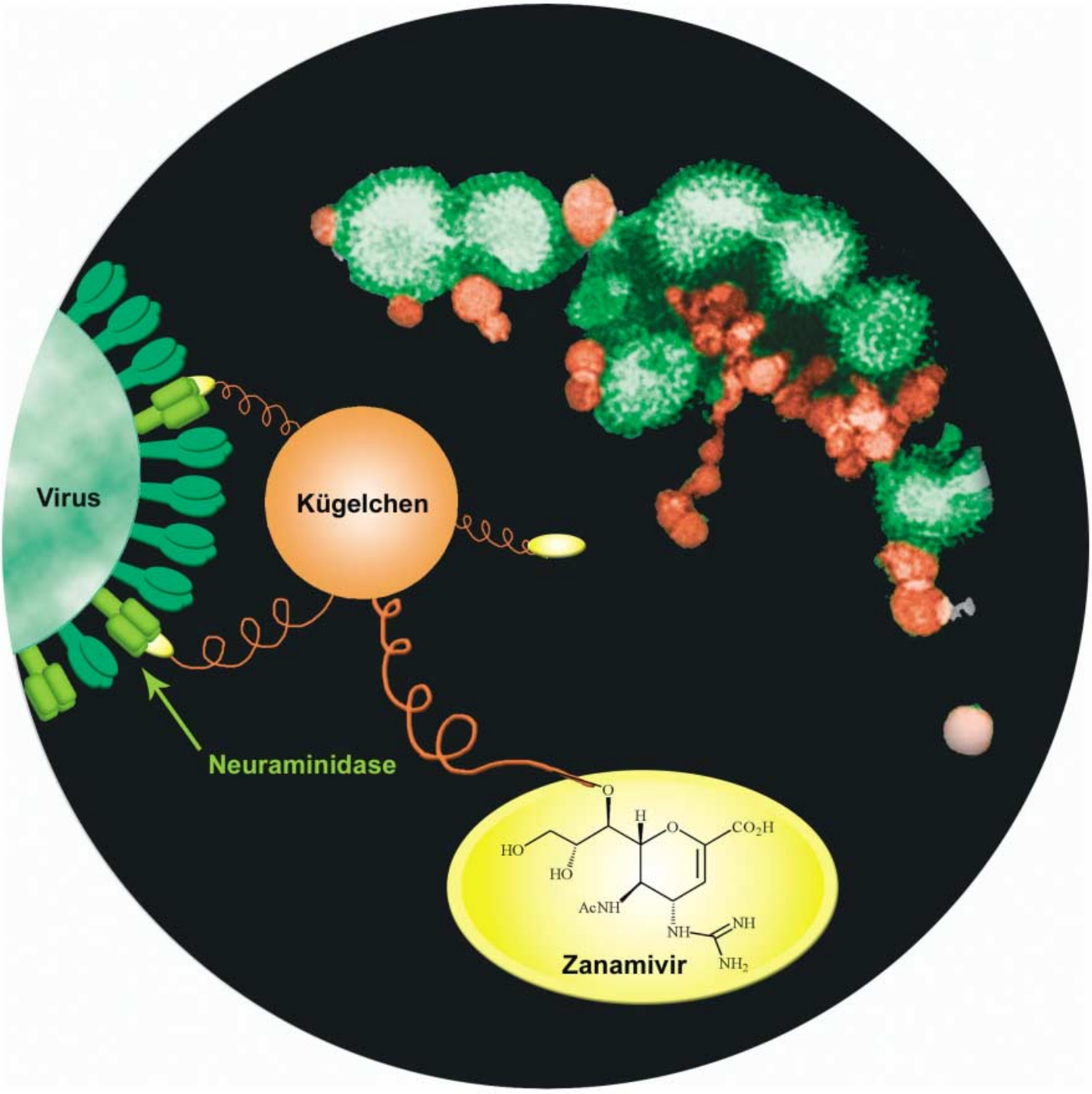
Zuschriften



Das TEM-Bild zeigt, wie Influenza-Virionen an Mikrokügelchen gebunden werden und aggregieren. Die Bindung wird durch Moleküle des wirksamen Neuraminidase-Inhibitors Zanamivir vermittelt, die sich an der Oberfläche der Kügelchen befinden. Mehr Informationen hierzu finden Sie in der Zuschrift von J. L. McKimm-Breschkin, K. G. Watson et al. auf den folgenden Seiten.

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Towards Influenza Detection

Tethered Neuraminidase Inhibitors That Bind an Influenza Virus: A First Step Towards a Diagnostic Method for Influenza

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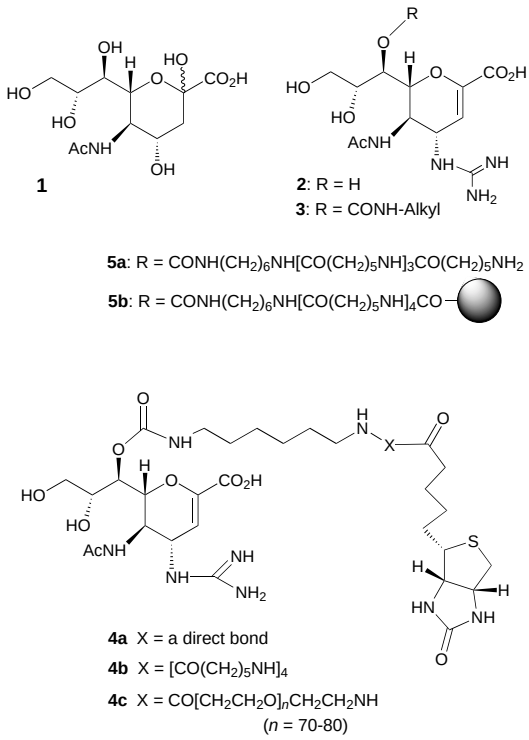
Influenza viruses are isolated as small (80 to 120 nm diameter), generally spherical particles.[1] The lipid bilayer envelope of the virus is derived from the host cell and protruding from this membrane on the surface of the virus are two virus-encoded glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA is the major surface antigen of the virus and is responsible for the binding of virions to the receptors of the host cell and for fusion between the virion envelope and the host cell.[2] NA, the minor surface antigen, is a glycosidase that cleaves sialic acid, **1** (*N*-acetylneuraminic acid) from the terminal position of glycoproteins and it is thought to be important for the release of virions from the surface of the host cell, and possibly also enhances infectivity by allowing movement of the virus through the mucins lining the respiratory tract.[3] Herein we describe the synthesis of tethered NA inhibitors, which potentially have a high affinity for all strains of influenza A and B, and can be used to capture influenza virions.

The structure of influenza NA has been extensively studied and found to consist of a tetrameric head that is anchored to the viral membrane by a long stalk.[4] Studies of the X-ray crystal structure of influenza NA bound to sialic acid revealed the location of the enzyme active site[5] and showed that it is lined with about 20 amino acids that are invariant in the NA of all known strains of influenza A and B. On the basis of the X-ray crystallographic structural information for influenza NA, several novel inhibitors were designed and synthesised[6] including 2,3-dehydro-2,4-dideoxy-4-guanidino-*N*-acetyl-neuraminic acid, **2** (4-guanidino-Neu5Ac2en, zanamivir), which has been approved for the treatment of influenza.[7]

The X-ray crystal structure of zanamivir bound to influenza NA indicates that the 7-hydroxy group has no close interactions with the protein, and in fact points directly out and away from the active site.[8] Based on this information, Andrews et al. prepared a series of 7-carbamate derivatives **3** of zanamivir **2** and found that the compounds retain high affinity for influenza NA and many also have good antiviral properties.[9] Importantly, compounds **3** showed a similar affinity for both influenza A and B, in contrast to other derivatives of **2** with altered glycerol side chains.[10]

Seeking an influenza-detection method that is independent of viral strain, we explored the feasibility of using functionalized 7-carbamate derivatives of zanamivir to selectively capture influenza virions. Thus zanamivir-biotin conjugates **4a–c** with different length linkers were prepared (Scheme 1) as outlined in Scheme 2 and their inhibition



Scheme 1. Structure of sialic acid **1**, zanamivir **2** and 7-carbamate derivatives **3**, **4a–c** and **5a–b.** In the case of microspheres **5b**, there are many **5a** units bound to each bead.

constants for influenza NA were measured in an enzyme assay. The data (Table 1) shows that all three conjugates, although not as active as **2**, are good inhibitors of influenza NA and bind to three representative influenza strains with approximately equal affinity. There is a gradual reduction in binding affinity of zanamivir derivatives **4a–c** as the size of the appended group increases, but even compound **4c**, which has a 3400 dalton polyethylene glycol (PEG) spacer group

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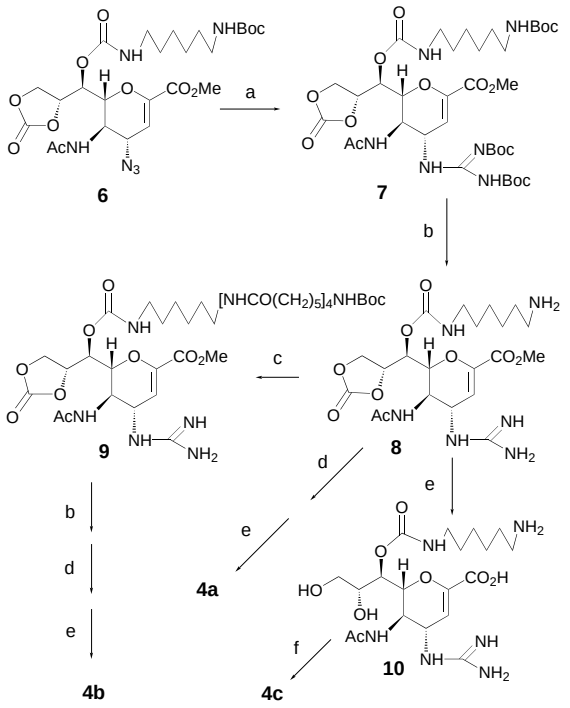

Scheme 2. Synthesis of zanamivir–biotin conjugates **4a–c**. a) H_2, Pd–C, MeOH/Toluene, room temperature, 1 h, then bis-Boc-pyrazole-1-carboxamidine, MeOH, 30 °C, 5 days, 49%; b) TFA, room temperature, 1 h; c) $HO_2C(CH_2)_5[NHCO(CH_2)_5]_3NHBoc$, KO*t*Bu, *i*BuOCOCl, MeOH, 0→20 °C, 32%; d) NHS-biotin, K_2CO_3, H_2O/acetone, room temperature, 3 h; e) Et_3N, H_2O/MeOH, room temperature, 16 h; f) NHS-$COCH_2CH_2$-3400PEG-NH-biotin, pyridine, room temperature, 3 days, 36%.

Table 1: Inhibition of influenza virus NA by **2** and biotin conjugates **4a–c**.

Compound	IC_{50} [nM] on neuraminidase from three influenza types		
	A/NWS/Tokyo/3/67(H1N2)	A/NWS/G70C/75/(H1N9)	B/Victoria/02/87
2	1.3	1.0	2.5
4a	10	4.8	22
4b	30	20	50
4c	180	200	220

(approximately 75 PEG units or 225 atoms in length), still shows submicromolar inhibition (IC_{50} 2×10^{-7} M) of the enzyme.

The ability of the biotinylated compounds **4a–c** to capture influenza particles was tested in an ELISA-based assay. Avidin coated plates were saturated with the conjugates **4a–c**, the plates were washed to remove unbound conjugate, and serial dilutions of influenza virus were added starting with 100 Hemagglutinating units (HAU, 100 HAU is approximately 5×10^6 virus particles). The plates were washed again to remove unbound virus, and the captured influenza was detected by using an anti-HA antiserum–horseradish peroxidase (HRP) conjugate and chromogenic substrate. Initial experiments with compound **4a**, which has only a short 1,6-hexanediamine spacer group between the drug and biotin molecules, failed to show significant capture of the virus (data not shown), whereas experiments with a biotinylated anti-neuraminidase antibody control resulted in a strong signal. In contrast to **4a**, compounds **4b** and **4c** gave rise to strong positive signals, thus indicating the successful capture of the influenza virus, with the longer PEG3400 conjugate **4c** giving rise to a signal that was similar to that from the 1 μg mL^{-1} NA antibody–biotin control NC-10 (Figure 1).

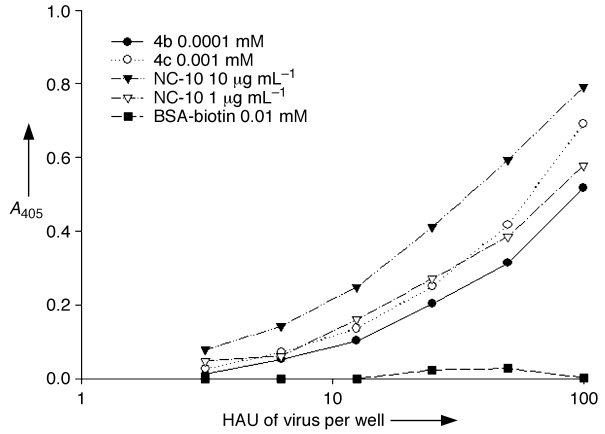

Figure 1. ELISA based detection of influenza A (NWS/G70C) captured with biotinylated compounds **4b–c**, or biotinylated NA–antibody control NC-10, bound to avidin coated microplate wells and detected with a HA–Ab/Ab–HRP enzyme conjugate.

Previous reports have demonstrated the importance of a long spacer group in the application of biotin[11] and antibody[12] conjugates for the capture macromolecules or binding to microspheres. The ability of tethered NA inhibitors **4a–c** to capture influenza may be determined by several steric factors including the numerous HA spikes on the influenza surface, the presence of oligosaccharides on the NA heads,[4] and the fact that the enzyme active site is about 10 Å below the surface of the protein. Based on the calculated root mean square length (end-to-end distance) for PEG3400 in water, the tethered zanamivir in surface-bound conjugate **4c** would be an average distance of about 45 Å from the avidin surface (Figure 2).[13] The slightly superior capture of virus by the

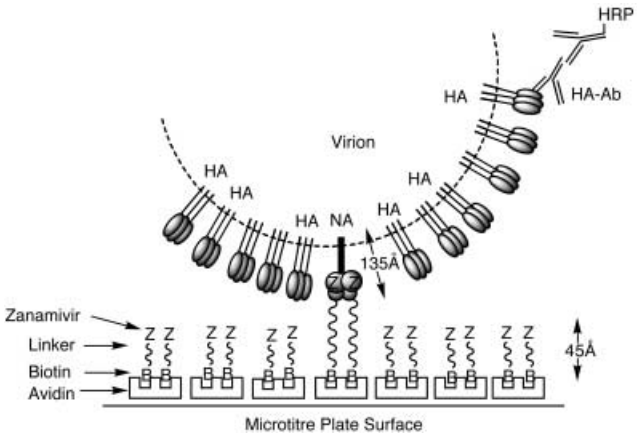

Figure 2. Schematic representation of a captured influenza virion showing the NA, HA, and ELISA detection

biotinylated NA-antibody control NC-10 (Figure 1) may be due to a combination of factors including the greater affinity ($K_D = 1.3 \times 10^{-8}$ M) of the NA/antibody interaction,[14] and a reduction of surface steric effects resulting from the antibody binding site being held further from the surface by the larger (> 100 Å) framework of the antibody molecule.

To visualize the interaction between influenza virions and tethered zanamivir we prepared microspheres coated with covalently bound zanamivir for examination under transmission electron microscopy (TEM). Thus the zanamivir tetracaproamide derivative **5a** was coupled with carboxylated polystyrene microspheres of average diameter 32 nm to give functionalized beads represented by formula **5b** (Scheme 1). When mixed with influenza and examined by TEM, microspheres **5b** were observed to interact closely with the surface of the virions (Figure 3A). In addition, self-aggregation of the microspheres was commonly seen, along with spheres, which were either decorated with free NA molecules or cross-linked to other spheres by NA molecules or viral membrane fragments containing NA. To confirm the involvement of NA binding in the cross-linking role played by **5b**, excess zanamivir **2** was added, whereupon the previously observed aggregates were almost completely eliminated (Figure 3B). To further exclude the possibility that the association of microspheres **5b** with influenza virus was nonspecific, a second experiment was performed in which Respiratory Syncytial Virus (RSV), a virus that has no NA, was mixed with the influenza virus suspension, and the sample was treated as described above. It was found that while the great majority of influenza virions were closely associated with microspheres, RSV virions were almost always isolated, even when **5b** was present in considerable excess (TEM not shown).

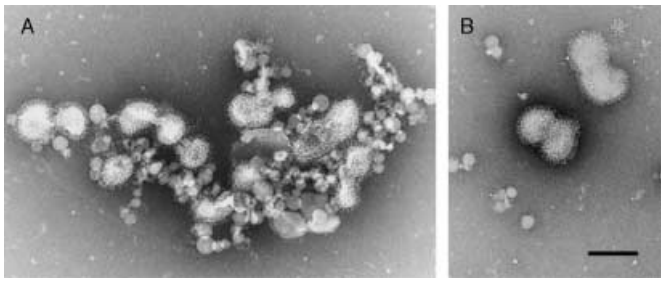

Figure 3. A) TEM images of influenza virus showing 32(±10 nm) microspheres **5b** binding to virions and causing aggregation. Some spheres appear to be decorated or cross-linked by free NA or small fragments of cell membrane containing NA. B) Addition of excess zanamivir **2** prevents virus aggregation by **5b**. Magnification bar 100 nm.

Antibodies to the influenza surface proteins HA or NA, being strain-specific, are not useful for the general detection of influenza and thus the limitation in our present results is the use of a strain-specific anti-HA antibody/enzyme conjugate as the detection reagent. One possible extension of our results would be to employ a nonspecific influenza capture surface and then use a neuraminidase-binding reagent, such as compound **4c**, together with an avidin/enzyme conjugate, for the detection stage. For example, there are various reported methods for the general, nonspecific capture of influenza virus onto a surface including the use of the fetuin (a sialylated glycoprotein),[15] nitrocellulose[16] and nylon membrane.[17]

There is a clear need for more rapid, specific and clinically relevant detection methods for influenza.[18] The results of our experiments indicate that the use of tethered NA inhibitor **4c** or the microspheres **5b** could form the basis for a new detection method for all strains of influenza A and B. We believe this to be the first demonstration that it is possible to take an inhibitor of a viral enzyme, tether it to a surface, and then capture that virus.

Experimental Section

Details of the synthetic methods used for the preparation of compounds **4a–c** are provided in the Supporting Information. Briefly, compounds **4a–c** and **5a** were prepared by starting from the known 4-azido sialic acid derivative **6**[9] as outlined in Scheme 2. Zanamivir coated microspheres **5b** were prepared by coupling 32 nm carboxylated polystyrene beads (Bangs Laboratories) with the tetracaproamide derivative **5a** of zanamivir.

The NA inhibition assay was carried out following the method of Potier et al.[19] by using methylumbelliferone-*N*-acetyl neuraminic acid as substrate. All samples were tested in duplicate and for comparison zanamivir **2** and 2,3-dehydro-2-deoxy *N*-acetyl neuraminic acid (DANA) were included in each assay.

ELISA experiments: PVC 96-well ELISA plates (Dynatech, USA) were coated overnight with avidin, 2 μg mL^{-1} in PBS. Plates were blocked with PBS/Tween 20 (0.1%) and washed with PBS prior to the addition of the biotinylated compounds **4a–c.** A biotinylated anti-NA antibody NC-10[20] (specific to the test strain of influenza) was used as a positive control and a BSA-biotin conjugate provided a negative control. After a 1 hour incubation, the plates were washed to remove unbound compound and then, starting with 100 Hemagglutinating units (HAU) of virus, serial twofold dilutions of the reassortant NWS/G70C (H1N9, obtained from R. Webster[20]) were added and incubated for 1 hour at room temperature. After another wash, captured virus were detected by treatment with a polyclonal anti-HA antiserum, followed by a sheep antirabbit-horseradish peroxidase conjugate and treatment with a chromogenic (ABTS) substrate. Very similar results were obtained with plates coated with 0.1, 0.01 or 0.001 mM of the biotinylated compounds, thus indicating that all concentrations were saturating the available avidin sites.

TEM experiments: Influenza virus was diluted in phosphate-buffered saline (PBS) to a concentration of approximately 10^7 particles mL^{-1}. Mixtures of viral suspension and microspheres **5b** in a variety of ratios were incubated at 4°C for 15 mins. Droplets of the mixture (3 μL) were then applied to glow-discharged, carbon-coated 400-mesh grids for 30 s and were stained with 2% potassium phosphotungstate at a pH of 6.5. The grids were air-dried and examined at 60 kV accelerating voltage in a JEOL 100B transmission electron microscope at magnifications between 40000 and 100000×. Electron micrographs were recorded on Kodak SO-163 film and were developed in Kodak D19 developer. In some experiments excess zanamivir **2** (100 μM) was added to the mixture of virus and microspheres **5b** prior to incubation.

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