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Ambient Ion–Surface Reactions

Peptide Cross-Linking at Ambient Surfaces by Reactions of Nanosprayed Molecular Cations**

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The ability to control the chemical composition of surfaces is of fundamental interest and growing technological importance due to developments in nanotechnology, biotechnology, organic electronics, and functionalized materials.[1] The motivation behind this study is to generate new materials at surfaces, including new covalently bound surface coatings, by using molecular ions in the ambient environment as chemical reagents. In particular, we are interested in generating two- and three-dimensional peptidic macromolecular structures by cross-linking the linear peptide chains generated by conventional solid-phase synthesis[2] using suitable ambient ionic reagents. There is already a substantial literature on peptide cross-linking[3] but our approach is unique in that we use ionic reagents generated by mass spectrometry (MS) to effect rapid, small-scale derivatization. In pursuit of this larger aim, the present study focuses on cross-linking of oligolysines (K_n, $n = 3, 5$) and on the model derivatization reaction of aromatic aldehydes with Girard T reagent. The results indicate that exposure of a nominally dry derivatizing agent (e.g., Girard T or an *N*-hydroxysuccinimide (NHS) ester reagent) present at a surface to a stream of charged microdroplets containing the analyte (e.g. an aldehyde or a peptide) is a rapid and efficient method of small-scale chemical synthesis.

There is a growing interest in the study of the reactions of organic ions outside of the mass spectrometer, both at surfaces and in the gas phase. For example,[4] dry pyrylium ions react in air at surfaces bearing amines to give the pyridinium derivatives. This reactive scattering experiment at atmospheric pressure is the analog of typical vacuum-based ion/surface reactions performed with mass-selected ions at surfaces inside a mass spectrometer.[5] As examples of gas-phase reactions, organic ions generated by electrospray ionization (ESI) and dried in a heated metal tube[6] undergo atmospheric pressure ionic reactions like the Fischer indole synthesis, the Borsche–Drechsel cyclization, and the pinacol rearrangement outside the mass spectrometer. Charged microdroplets are used to interrogate surfaces in desorption electrospray ionization (DESI), a method of ambient surface analysis.[7,8] If a reagent is included in the primary spray solvent, then chemical reactions occur at greatly accelerated rates in the secondary droplets splashed[9] from the surface. For example, the rate of derivatization of ketones by the Girard T reagent is accelerated by two to three orders of magnitude in the droplet/surface process compared to conventional bulk solution.[10,11] The reactive DESI experiment also facilitates identification of reaction intermediates,[12] something also achieved with ESI.[13]

Ambient peptide cross-linking was examined in two ways, in one experiment the cross-linking reagent was ionized and delivered in charged microdroplets to the peptide present on an ambient glass surface. In the other experiment, the reagents were interchanged and the peptide was sprayed onto the cross-linker. We deal with the latter case first: droplets of oligolysine cations to be cross-linked were generated using an array of ten nanoelectrospray (nanospray) emitters arranged in two concentric circles (see Experimental section for details). This array provided a reagent ion current of about 0.60 μA in a small area ($\approx 0.8\ cm^2$) causing chemical reactions at the ambient surface pre-coated with cross-linker. This current corresponds to a singly-charged ion deposition rate of $3.6 \times 10^{12}\ s^{-1}$ and in a typical 1 min reaction, to the deposition of 3.6×10^{-10} mol of material. For the predominantly doubly-charged tripeptide KKK, molecular weight 402, this corresponds to 0.15 μg of peptide and to 43% of a monolayer. The total amount of cross-linking reagent was 2 μg, although the total area on which this amount of reagent is coated ($0.8\ cm^2$) was somewhat larger than the total area sprayed. Under these circumstances all the electrosprayed peptide is exposed to the derivatizing reagent and, as will be shown below, conversion of peptide to cross-linked product is close to 100% efficient.

The nanospray array was used to deliver the cross-linking reagent suberic acid bis(3-sulfo-*N*-hydroxysuccinimide ester) sodium salt (SBS) for in situ derivatization of peptides at ambient surfaces. The protonated free-acid form of SBS (acidic SBS, **1**, M_w 528 $g\,mol^{-1}$) was generated in situ by exposing the dry sodium salt to an electrosprayed acidified solution of peptide. It can react (Scheme 1) through one or both of its activated ester groups to form either a simple covalently modified peptide (**2**) or a cross-lined peptide (**3**) with one or two successive eliminations of 1-hydroxy-2,5-dioxopyrrolidine-3-sulfonic acid (M_w 195 $g\,mol^{-1}$). Formation of simple amides (**2**) occurs by nucleophilic attack of a peptide primary amine at a carbonyl carbon of the ester and is characterized by the gain of 333 Da in mass. Similarly, formation of a cross-linked peptide involves two such reactions and the net addition of 138 Da in molecular weight.

After brief exposure of dry SBS to charged microdroplets containing micro-solvated peptide cations ($[M+H]^+$ and $[M+2H]^{2+}$) derived from KKK, MS analysis of the material removed from the surface showed the formation of cross-

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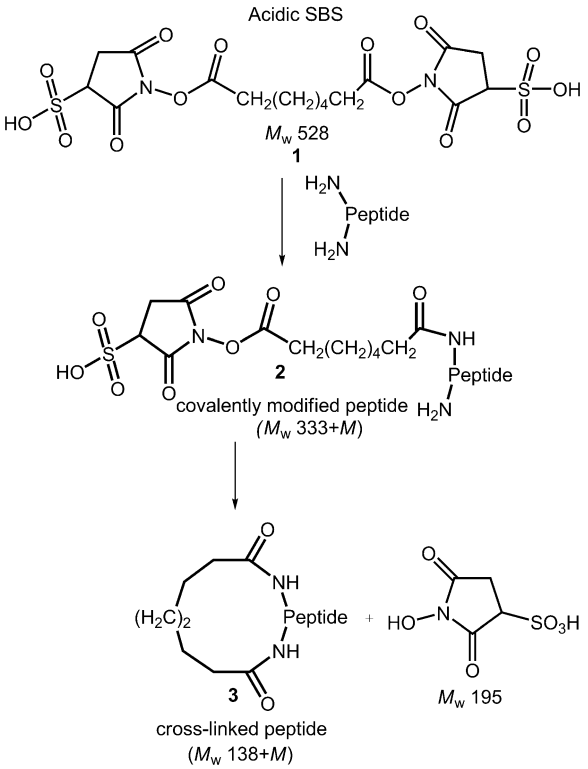
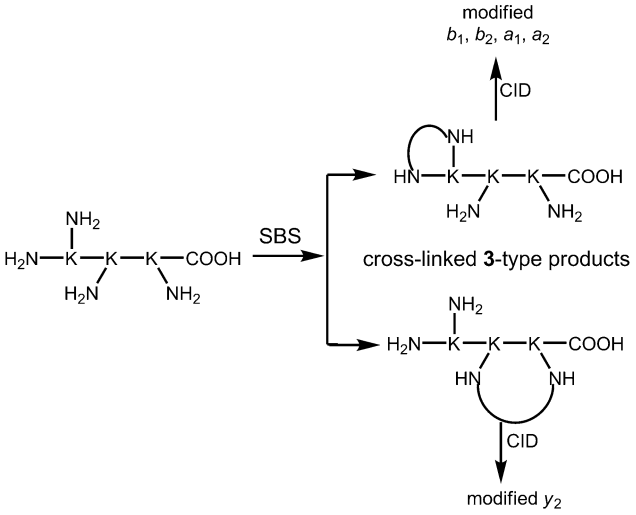


Scheme 1. Sequence of reactions leading to cross-linking of a peptide (M) by SBS.

linked products in high abundance (Figure 1b). Characterization of the cross-linked product (m/z 541) by collision-induced dissociation (CID) gave modified b_1 and b_2 type ions together with the corresponding a ions (Figure 1c). This result suggests that the cross-linked product was formed from the N-amino terminus and the ε-amino of the first lysine in the peptide, as shown in Scheme 2. However, the presence of modified y_2 ions suggests that isomeric structures also



Scheme 2. Two different ways in which tripeptide KKK is cross-linked during the surface reaction as elucidated by the CID MS/MS spectrum.

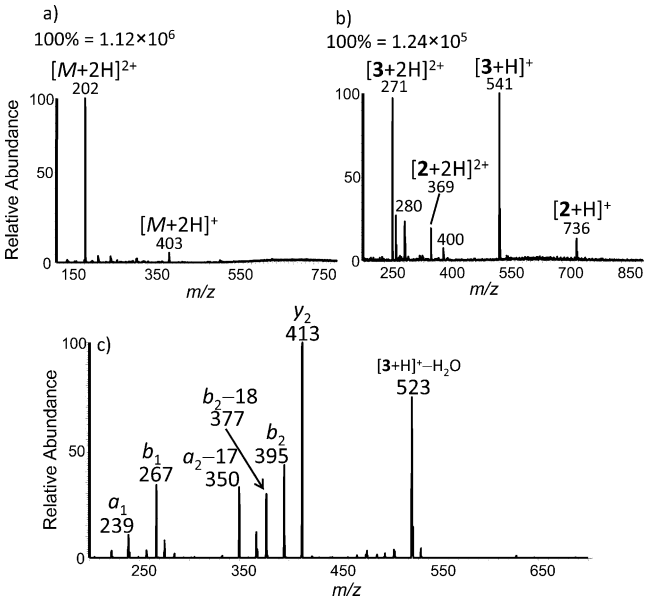


Figure 1. Nanospray-MS of a) pure KKK (1000 ppm peptide solution (1000 ppm in methanol/water/acetic acid, 49.5:49.5:1, vol/vol/vol) peptide solution, b) surface reaction mixture after spraying peptide solution onto surface bearing dried SBS ester cross linker on a glass slide (2 μg absolute amount, on total area of 0.8 cm^2) for 1 min, c) MS/MS product ion scan of m/z 541, $[M+138+H]^+$ indicating modified a-, b- and y-type fragment ions.

contribute to the cross-linked peptide (e.g., that involving second and third ε-amino groups of lysine). The cross-linking reaction yield was determined by measuring the relative responses of the peptide and the product in mixtures of various compositions. This calibration data (see Figure S1 and S2 in the Supporting Information) shows that the ionization efficiencies of peptide KKK and its cross-linked derivatitives are very similar (actually 1:1) so from the peak height measurements in (Figure 1b) a lower limit of 98% can be placed on the reaction yield. By contrast with the ambient surface reaction, practically no cross-linked product **3** [and very little (<5%) product **2**] was observed in the bulk solution-phase reaction after reaction for 1 h (Figure S3).

Participation of the side chain primary amines in the derivatization reaction at the ambient surface was investigated further using the model KK dipeptide. Nanospray-MS analysis of KK solution (1000 ppm) in methanol/water/acetic acid (49.5:49.5:1, vol/vol/vol) showed singly- and doubly-charged (major contributor) peptide cations (Figure S4) but very little reaction product of either type **2** or **3**. Similar results were obtained from other small model peptides such as MRFA and AEKAA. The low reaction efficiency of SBS towards these singly- and doubly-charged peptides is ascribed to the simple fact that modification at the primary amine site of a peptide requires the amine to act as a nucleophile. Protonation of the amine reduces its basicity.[14,15] Hence, when all potential reactive sites are protonated (e.g., as is the case for $(MRFA)^{2+}$ and $(AEKAA)^{2+}$), the peptide ion shows poor reactivity toward the SBS reagent.

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Cross-linking was further investigated using another model peptide, KKKKK. Nanospray-MS analysis of pure KKKKK solution shows singly-, doubly- and triply-charged peptide cations (Figure 2a). Note that even for the $[M+3H]^{3+}$ cation, two ε-amino groups on lysine residues remain unprotonated and can react with SBS. After surface reaction, a nanospray mass spectrum of the removed surface reaction product was recorded plus, for purposes of comparison, a nanospray-MS of the bulk solution-phase product of reaction of peptide and SBS (Figure 2b and c). From this data, 60 s of peptide cation exposure to the SBS reagent resulted in cross-linking of the peptide by reaction with the two free ε-amino groups on lysine (see Figure S5 for the CID data). The cross-linked peptide was detected in the +3, +2 and +1 charge states at *m*/*z* 267, 399 and 797, respectively (Figure 2b). However, under bulk solution-phase conditions (acidic medium using the methanol/water/acetic acid, 49.5:49.5:1, vol/vol/vol solvent, 30 min reaction and of equal aliquots of 1000 ppm peptide and SBS solutions) gave no observable *m*/*z* 267 cross-linked product (Figure 2c) and only traces of the other charge states. The dominant product under the bulk-phase conditions is simply the amide derivative (product **2**) which occurs at *m*/*z* 497 as a doubly-charged ion and was characterized by CID (Figure S6). Practically no free peptide cations were collected from the reaction surface after 60 s of electrospray, attesting to the speed of cross-linking in the charged microdroplet environment.

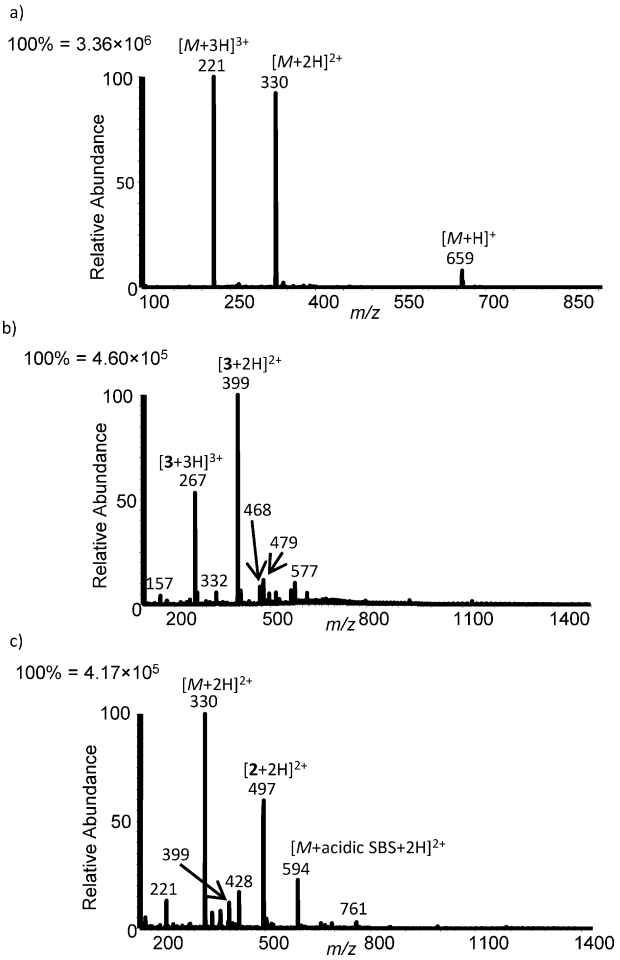

Figure 2. Nanospray-MS of a) pure KKKKK (1000 ppm) peptide solution without added SBS reagent. The next panels show peptide and SBS reaction mixture after b) 1 min surface reaction and c) 30 min of bulk solution-phase reaction. Peptide solution (1000 ppm) in methanol/water/acetic acid (49.5:49.5:1, vol/vol/vol) solvent was sprayed using the array apparatus with 3.5 kV applied voltage. Bulk solution-phase reaction mixture was allowed to react at ambient temperature for 30 min before analysis by nanospray-MS. Surface reaction product was washed using 10 μL of methanol/water (1:1, v/v) after microdroplets containing peptide ions had impinged on the dried SBS (2 μg absolute amount) for 1 min.

The cross-linking reaction was also investigated by reversing the reagents and electrospraying the cross-linker (i.e. SBS) onto a peptide covered ambient surface. The experiment was done after evaporating the peptide from an acidified methanol/water solution. Reaction occurred readily and yields were high although the fact that the spray area was smaller than the surface area of the peptide spot meant that some peptide was not exposed to the reagent. The experiment was done by spraying the tripeptide KKK with positively- and negatively-charged droplets of the cross-linking reagent (Figure S7). The former gave the cross-linked product in good yield. The latter gave mostly the singly derivatized product.

In a different experiment, the charged microdroplet environment was also applied to the conversion of benzaldehyde into the corresponding charge-labeled hydrazone by reaction with Girard T reagent.[16] This reaction is useful in analytical mass spectrometry since aldehydes often exhibit low ionization efficiency in ESI-MS analysis. Surface reaction involving benzaldehyde and Girard T was achieved simply by spraying positively charged microdroplets containing benzaldehyde in acetonitrile onto a surface coated with nominally dry Girard T using the nanospray array (Figure S8). The total ion currents (ca. 0.6 μA) derived from this nanospray array (ca. 0.2 $\mu L\,min^{-1}$, total) were approximately equal to those generated by conventional ESI (flow rate 5 $\mu L\,min^{-1}$) to spray benzaldehyde yet the array gave much more product (by a factor >10). This is due to the differences in the size of the droplets generated by the two different ionization techniques—the smaller droplets derived from nanospray allow effective solvent evaporation and thus provide reagents that are present as concentrated films at the surface compared to the bulky ESI droplets. Removal of the surface reaction product and analysis by nESI-MS show that 95% of the Girard T reagent present at the surface was converted into product (ca. 2 μg quantity product, *m*/*z* 220) in less than 10 min of spray time. This was confirmed by in situ on-surface SIMS (secondary ion MS) analysis of product using Cs^+ primary ions under vacuum, in which no unreacted reagents were detected from the reaction surface (Figure S9). By contrast, the corresponding bulk solution-phase reaction condition provided only 10% product yield in 60 min of reaction time using the same quantity of starting materials.

In conclusion, peptide cross-linking is readily achieved by directly delivering solvated peptide cations generated by nanospray onto a nominally dry SBS reagent. Multiplexing of the nanospray emitters allowed relatively large molecular ion

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currents to be used for the surface reaction, thus substantially reducing reaction time. The charged microdroplet environment should be widely applicable for chemical synthesis at ambient surfaces under mild reaction conditions. This advantage is expected to impact analytical MS by generating species with high ionization efficiency by on-line chemical derivatization, and synthetic chemistry at large through waste reduction and accelerated reaction rates. In general, almost 100% of all the molecular cations delivered onto the reaction surface were converted into the desired reaction product in a short exposure time (< 60 s). Since the ions are generated and reacted in the open laboratory environment at atmospheric pressure, analysis of the ensuing products by other analytical techniques is straightforward.

Ionic reactions performed in the open laboratory environment extend the capabilities of mass spectrometers and allow easy access to reaction products for subsequent use. The fact that these reactions occur quickly is consistent with previous observations on ionic reactions in confined volumes,[17] including reactions in droplets and in thin surface films.[18] Cross-linking of oligolysines yielded only intramolecular products for the model peptides tested. The absence of intermolecular cross-linking suggests that 1) solution-phase complexes[19–22] can be studied at ambient surfaces by stabilizing the complex through rapid cross-linking in the ambient environment, and 2) studies of the three-dimensional structures and configurations of proteins should be facilitated through intra-molecular cross-linking using charged droplet/surface reactions.

Experimental Section

Nanospray emitter array: Individual glass capillaries were pulled using Flaming/Brown micropipette puller (Sutter Instrument Co., Novato, CA) to tip sizes of about 10 μm (Figure 3b). To create the nanospray array (Figure 3a), a 1.2 cm-thick, 2.5 cm-diameter Teflon disk (Figure 3c) was drilled with 1/16 inch holes arranged in two concentric circles, all pointing towards a common area 1 cm in diameter. Electrical contact with the analyte solution contained in the glass capillary was achieved using Nichrome wire (≈250 μm in diameter) inserted into each capillary.

Nanospray emitter array characterization: The nanospray array was characterized by connecting the ten emitters in parallel to a single DC HV power supply and measuring the total ion current at atmospheric pressure as a function of the applied voltage. The total ion current increased with an increasing number of emitters in the array. However, the current increase was not linear and was low when applying the typical nanospray voltage of 2.0 kV to the array. A three-fold increase in total current was achieved by increasing the applied voltage from 2.0 to 3.5 kV with no observable discharge, yielding about 15 times more ion current than a single nanospray emitter operated at 2.0 kV. The relatively higher voltage requirement for the array configuration (compared with ca. 2 kV for a single emitter) and the non-linear behavior with number of emitters (Figure S10), are well-characterized phenomena attributable to electrical shielding of emitters located in the central part of an array.[23,24] This shielding introduces some inefficiency into the surface reaction method in terms of surface coverage and reagent consumption.

Nanospray ionization mass spectrometry for product analysis: All MS experiments were performed using a Thermo Fisher Scientific LTQ mass spectrometer (San Jose, CA, USA). Capillary voltage: 15 V, capillary temperature: 150 °C, and tube lens voltage: 65 V. An isolation window of 1.5 Th (mass/charge units) and a normalized collision energy of 15–35% (manufacturer's unit) were selected for the CID experiments.

Chemicals and reagents: Peptide solutions were typically prepared in 49.5:49.5:1 (vol/vol/vol) water/methanol/acetic acid mixture at a concentration of 1000 ppm. All aldehyde solutions including Girard T reagent were prepared at a concentration of 1000 ppm in acetonitrile. SBS ester solution was prepared in water/acetonitrile (1:2, vol/vol) at concentration of 1000 ppm.

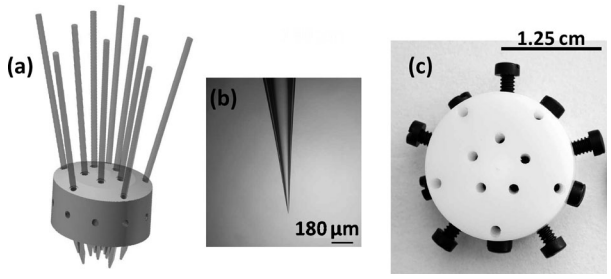

Figure 3. a) Nanospray emitter array of ten glass capillaries held in a Teflon holder, b) typical 10 μm-tip glass capillary used in the array, c) 1.2 cm thick, 2.5 cm diameter Teflon disk capillary holder with 1/16 inch drilled holes arranged in two concentric circles. The 10 emitters in the two concentric circles all point towards a target of ca. 1 cm diameter.

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