Characterization of the Biocide Polyhexamethylene Biguanide by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

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ABSTRACT: The biocide polyhexamethylene biguanide (PHMB) has been characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Previously, no method has been able to provide a detailed structural characterization of PHMB. MALDI-TOF MS was able to detect PHMB oligomers with $n \leq 6$. Six different PHMB product types were identified, which possess combinations of amine, cyanoamine, guanidine, or cyanoguanidine end-groups. Postsource decay (PSD) fragmentation was used to confirm the correct assignment of PHMB structure for the dominant PHMB molecular ion. MALDI-TOF MS analysis of a ^{15}N-labeled PHMB confirmed the correct assignment of PHMB molecular ions, and also indicated the existence of a polymerization–depolymerization equilibrium during melt polymerization of the polymer. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 4928–4936, 2006

Key words: polyhexamethylene biguanide; MALDI-MS; PSD fragmentation

INTRODUCTION

Rose and Swain[1] first described the synthesis of polyhexamethylene biguanide (PHMB) in 1954. PHMB is utilized in a variety of antibacterial applications, such as industrial disinfection, preservation of cosmetics, and as an antibacterial treatment for textiles. The antimicrobial activity of PHMB is due to the basicity of the biguanide group, which has been shown to be around eleven.[2] PHMB is a highly adsorptive molecule whose mechanism of biocidal action is considered to be (i) damage to the bacterial cytoplasmic membrane and (ii) irreversible loss and precipitation of cellular constituents.[3]

In contrast with most other widely used biocides, PHMB is a polymeric material, with a repeating unit consisting of a biguanide group linked to a hexamethylene chain (Fig. 1). Therefore, the biocide consists of a mixture of different polymer lengths, with an average repeating unit number of 12. The route of PHMB synthesis (Fig. 1) is based on the reaction of cyanoguanidine groups with amine hydrochloride moieties, to form the biguanide group.[2] Theoretically, each PHMB oligomer should be terminated with amine and/or cyanoguanide end-groupings.[4]

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Because of the highly heterogeneous nature of PHMB, analysis of the material is complex and results in data of limited utility. Gel-permeation chromatography (GPC) analysis will provide an estimation of the average molecular weight for PHMB. Structural characterization of PHMB has been performed by NMR spectroscopy, to reveal the structure of (i) the PHMB repeating unit, and (ii) PHMB end-groups.[2,5] In addition, IR spectroscopy can be used to determine the presence of expected functional groups.[2,5] However, neither NMR nor IR spectroscopy can elucidate the structure and polymer chain length of individual polybiguanide chains within the PHMB mixture. Therefore, the soft ionization technique MALDI-TOF mass spectroscopy would appear to provide the best opportunity to achieve a thorough characterization of individual polymeric chains within the mixture. Indeed, as a precedent, structural analysis and M_w estimation of the structurally related biocide PHMG and PHMG structures based on ethoxylated linker groups has been reported using MALDI-TOF.[6,7]

MALDI-TOF is suited to the analysis of synthetic polymers.[8] MALDI-TOF results in relatively simple mass spectra, which reveal mainly singly charged quasimolecules with little fragmentation, thereby facilitating ion assignment. The technique will reveal information on repeating group structure, end-group identity, and sometimes molecular weight distribution.[9] Here, we report development of a MALDI-TOF method for the structural characterization of PHMB. Assignment of resultant molecular ions on generated

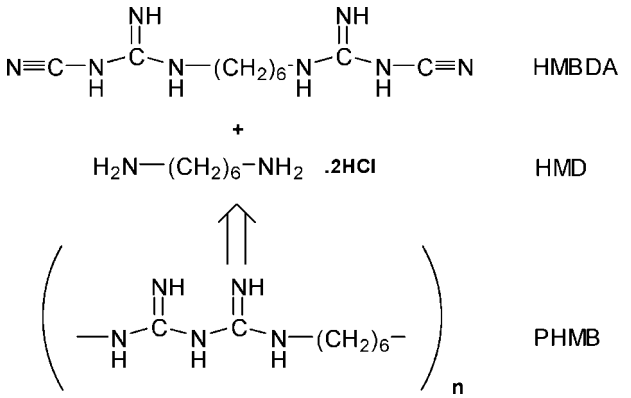


Figure 1 PHMB synthesis, by melt polymerization of hexamethylene diamine (HMD) and hexamethylene bisdicyandiamide (HMBDA).

MALDI-TOF mass spectra is reported, with confirmation of correct assignment provided by postsource decay (PSD) fragmentation of a prominent precursor ion. Interpretation of the obtained MALDI-TOF MS spectra with respect to the mechanism of PHMB polymerization is also reported.

EXPERIMENTAL

Materials

Standard PHMB solution (20% (w/v) Cosmocil CQ) was sourced from Avecia (Grangemouth, UK). Unless stated otherwise, all other laboratory reagents were sourced from Fisher Scientific (Loughborough, UK) and were of the highest purity possible.

Preparation of ^{15}N-labeled PHMB

^{15}N-labeled PHMB (labeled at the *N*-1 position of the biguanide group) was synthesized using doubly ^{15}N-labeled hexamethylene diamine (HMD) [$H_2{}^{15}N(CH_2)_6{}^{15}NH_2{\cdot}2HCl$], ammonium chloride (both obtained from Sigma-Aldrich, Gillingham, UK) and hexamethylene bisdicyandiamide (HMBDA, obtained from Avecia, Grangemouth, UK) as reactants. HMBDA (0.83 g), ^{15}N-labeled HMD (0.59 g), ammonium chloride and distilled water (0.2 mL) were mixed and heated for 2 h at 160°C. After this time, the temperature was reduced to 100°C and distilled water (6 mL) was added with stirring until a mobile liquid was obtained. The obtained liquid was allowed to cool, then made up to a 15 mL volume with distilled water. Analysis of the resultant polymer (10% w/v, in water) was conducted by GPC to observe that a satisfactory value of average molecular weight and expected Gaussian distribution had been achieved.

Preparation of polydisperse PHMB molecular weight fractions

Polydisperse PHMB samples with both higher and lower average molecular weights than standard PHMB were prepared by a two-step dialysis method. Two 100-mL volumes of standard PHMB were placed in lengths of 2000 Da molecular weight cut-off (MWCO) dialysis membrane (Spectra/Por 6, regenerated cellulose, 45 mm width, Spectrum Europe B.V., Breda, The Netherlands). Dialysis was conducted by placing the sealed membrane in 3 L of distilled water at room temperature for 24 h, without stirring of the outer liquid. On completion of dialysis, the solution inside the membranes (Samples 1 and 2) was retained. The solutions present outside were evaporated to dryness by rotary evaporation, followed by reconstitution in 100 mL of water. Subsequently, the reconstituted samples were subjected to a second dialysis step in either 1000 Da (Spectra/Por 6, 45 mm width, regenerated cellulose) or 500 Da (Spectra/Por Biotech, 31 mm width, cellulose ester) MWCO dialysis membranes. On completion of the second dialysis step, the solutions inside from the 1000 Da (Sample 3) and 500 Da (Sample 4) were retained. The solutions outside were again evaporated to dryness and reconstituted in 50 mL of water, to result in samples 5 and 6 from dialysis in the 1000 and 500 Da membranes, respectively.

Each PHMB molecular weight fraction was analyzed by GPC, using a Waters 2690 Separations Module (Waters, Elstree, UK). A Waters 2410 refractive index detector was used to monitor the GPC effluent. PHMB (20 μL injection volume, 200 ppm) was separated on two Zorbax PSM60 analytical columns (6.2 $\times$ 250 mm^2, 5 μm pore size) placed in series. Eluent consisted of (per liter) methanol (430 mL), distilled water (550 mL), ammonium chloride (4.3 g), and 1*M* HCl (20 mL), used at a flow rate of 0.5 mL min^{-1}. Sample weight–average molecular weight (M_w) was determined by integration of resultant GPC profiles to a calibration curve constructed from the known mass (log molecular weight) and retention time of PHMB monomer, dimer, trimer, and tetramer. Millennium software was used to perform all data manipulations.

Matrix assisted laser desorption ionization mass spectrometry

Before MALDI-MS analysis, PHMB samples were concentrated and purified by solid phase extraction (SPE), using Hysphere C2 8 μm SPE columns (Spark, Holland). Columns were solvated first with 2 mL of methanol and then 2 mL of distilled water. PHMB samples (50 mg L^{-1}) were adjusted to pH 2.3 by adding 2*M* HCl. Subsequently, the acidified PHMB solution (1 mL) was added to the column, followed by a wash step with distilled water (2 mL). Finally, PHMB was eluted in 1 mL of methanol.

MALDI-TOF mass spectra were obtained using a Voyager DE-STR time-of-flight mass spectrometer (Applied Biosystems, Framingham, UK). The instrument is equipped with a nitrogen laser, operated at a wave-

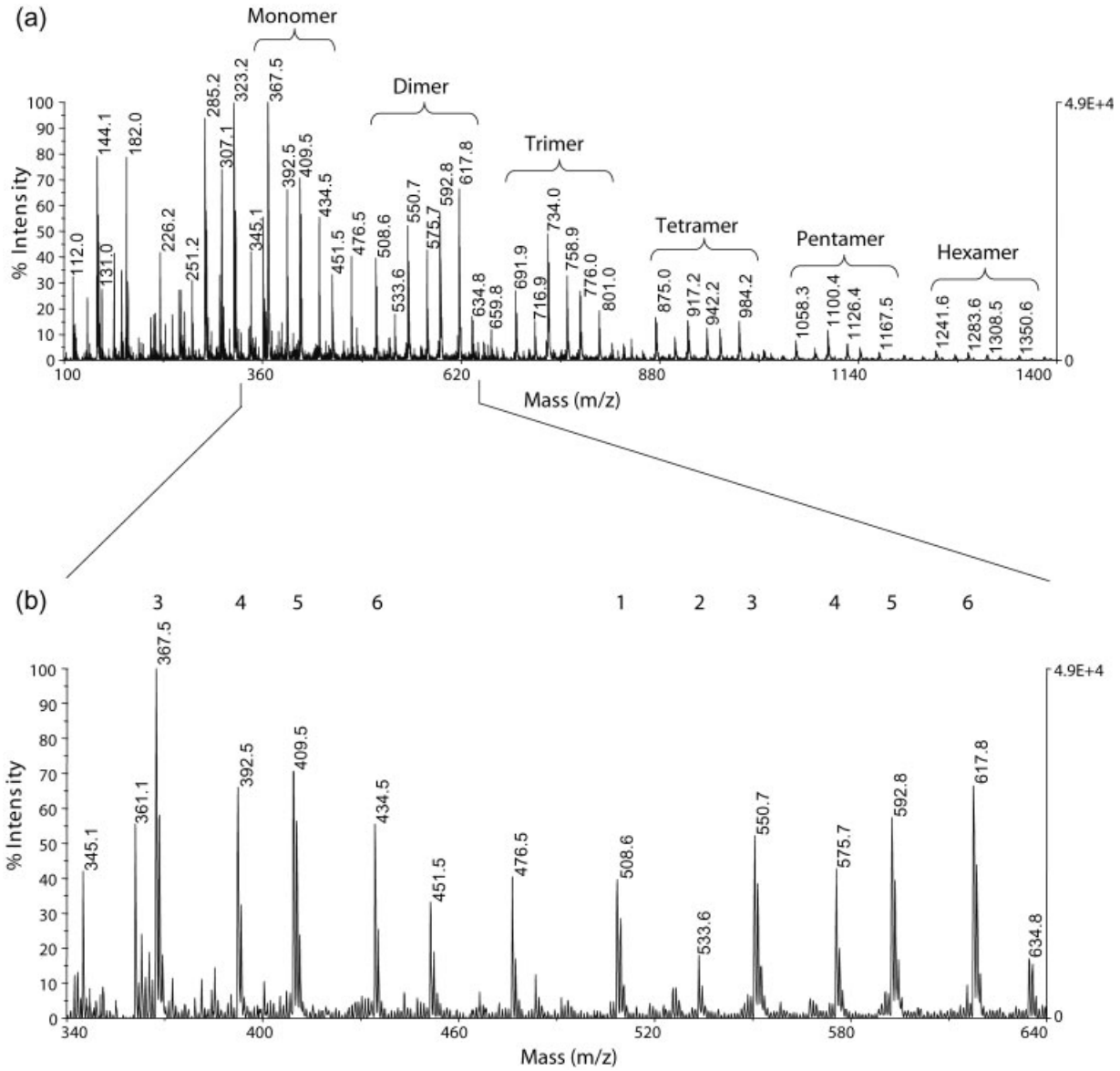


Figure 2 (a) MALDI-TOF mass spectrum of standard PHMB (Cosmocil CQ); (b) expanded view of standard PHMB mass spectrum between 340 and 640 Da, revealing different families of PHMB oligomers (labeled 1–6).

length of 337 nm and a pulse time of 3 ns. Spectra were recorded in reflector mode, with an acceleration voltage of 20 kV. Dried-down PHMB samples were resuspended in 10 μL of matrix solution (6-aza-2-thiothymine; 10 mg mL^{-1} in methanol, Sigma-Aldrich, Gillingham, UK). Either 0.5 or 1 μL of the sample–matrix mixture was spotted onto the sample plate. Generally, 100–150 laser shots were averaged to produce mass spectra. Values of *m/z* from monoisotopic peaks are provided.

During PSD fragmentation of precursor ions, PHMB samples were prepared in the same manner except that α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, Gillingham, UK) was used as the matrix. PSD spectra were determined by selection of the ion of interest within a 10 Da width pulsar mass window. Resultant PSD mass spectra were calibrated using a Sequazyme peptide mass standards kit (Applied Biosystems), using a manufacturer-supplied instruments setting file.

RESULTS AND DISCUSSION

PHMB analysis by MALDI-TOF mass spectrometry

A typical MALDI-TOF mass spectrum resulting from analysis of standard PHMB (Cosmocil CQ) is shown in Figure 2(a), depicting the observed $[M + H^+]$ ions. MALDI-TOF spectra of improved signal intensity, without loss of any molecular ions, were observed after SPE concentration and purification of PHMB samples (data not shown). Several signal clusters can

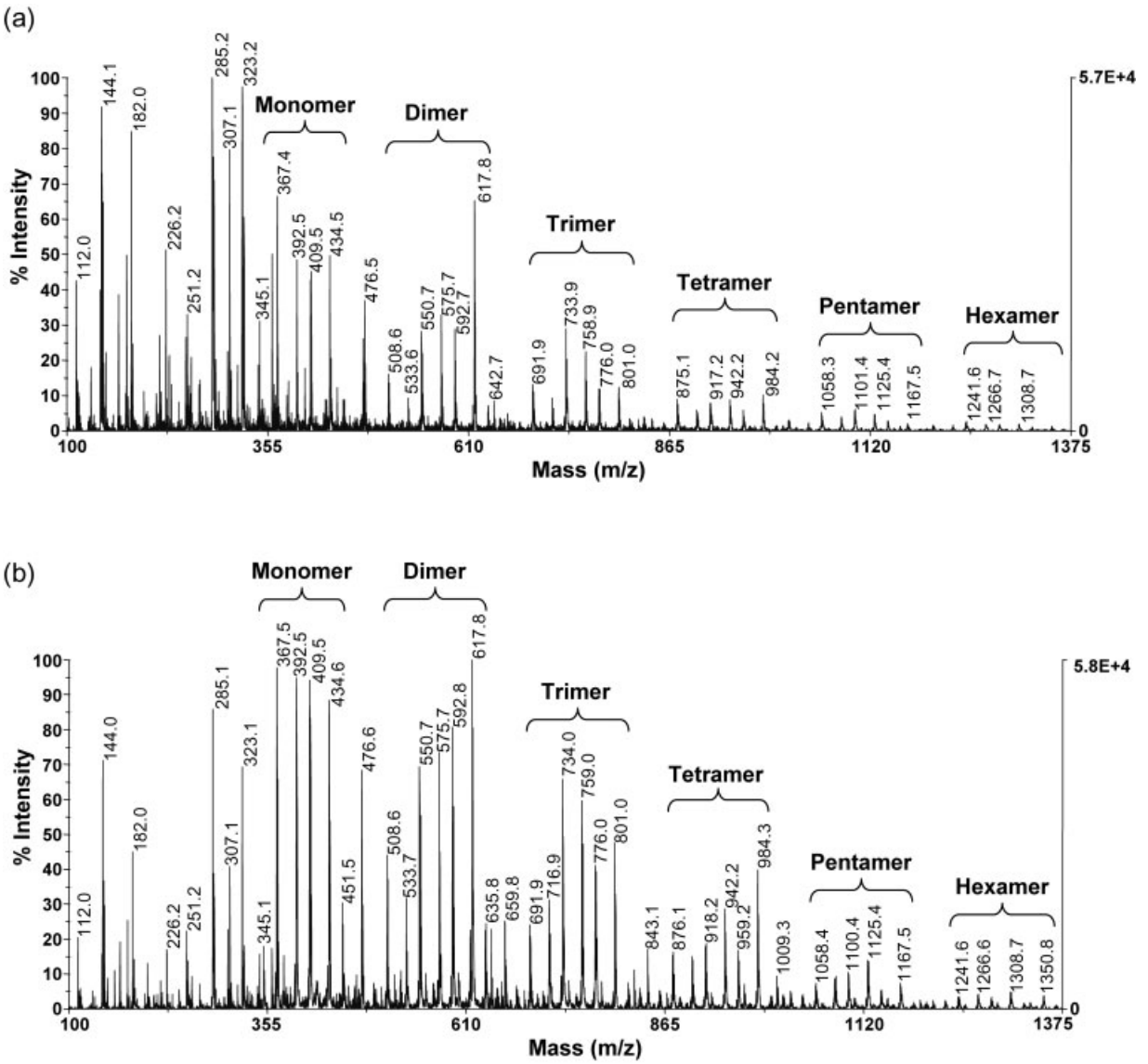
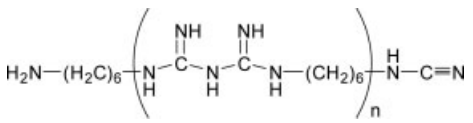
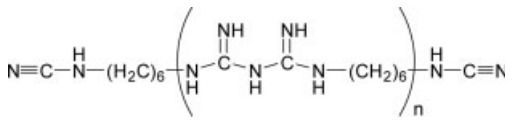
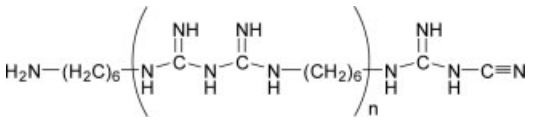
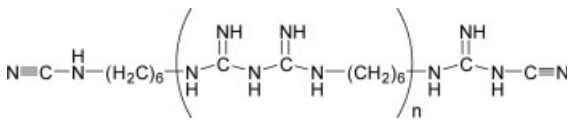
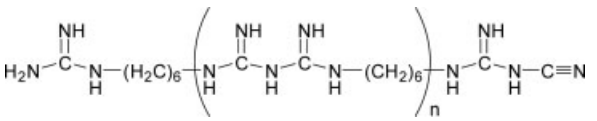
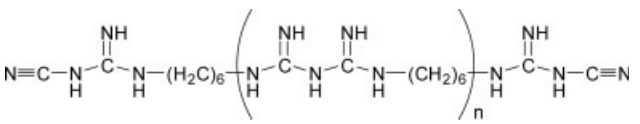


Figure 3 (a) MALDI-TOF mass spectrum of PHMB molecular weight fraction 1 (M_w of 4000 determined by GPC). (b) MALDI-TOF mass spectrum of PHMB molecular weight fraction 6 (M_w of 1375 determined by GPC).

be depicted from the spectrum, which represent groups of PHMB oligomers containing the same number of biguanide-hexamethylene repeating units. The degree of signal intensity is observed to decrease with increasing mass, such that only species of $n \leq 6$ can be observed by this method. This number of PHMB species is observed on MALDI-MS analysis of all polydisperse PHMB molecular weight fractions (determined to have M_w values of 4000, 3975, 3375, 2800, 1725, and 1375 Da by GPC analysis). The only observed difference between the MALDI-TOF mass spectra on analysis of the molecular weight samples is an increase in signal intensity on decrease of PHMB sample M_w (Fig. 3). Therefore, it is considered that the reported MALDI-MS method is only able to detect PHMB oligomers of $n \leq 6$, rather than the PHMB preparations being composed of only this range of oligomers.

The most prominent molecular ion on MALDI analysis of PHMB is that observed at *m/z* 367, which represents the detection of a PHMB monomer, terminated with one cyanoguanidine (CG) and one amine (A) end-group. In all, six different PHMB product types are detected by MALDI-TOF MS [Fig. 2(b)]. These may be summarized as possessing biguanide-hexamethylene (BG) repeating units terminated with one A and one cyanoamine (CA) end-group (1), two CA end-groups (2), A and one CG end-group (3), one CA and one CG end-group (4), one guanidine (G) and one CG end-group (5) and two CG end-groups (6). Species 1–6 have nominal masses of $(16 + 84 + (183)_n + 41)$, $(41 + 84 + (183)_n + 41)$, $(16 + 84 + (183)_n + 83)$,

TABLE I
Structural Assignment of Ions Belonging to the Six Main Product Types, Which Appear in the Recorded MALDI-TOF Mass Spectra of Standard ^{14}N-PHMB (Cosmocil CQ) and ^{15}N-PHMB

Structure	*n*	$[M+H]^+$ ^{14}N-PHMB	$[M+H]^+$ ^{15}N-PHMB
	1	n.d.	n.d.
	2	508.6	n.d.
	3	691.9	n.d.
	4	875.0	n.d.
	5	1058.3	n.d.
	6	1241.2	n.d.
	1	n.d.	n.d.
	2	533.6	533.6, 535.6
	3	716.9	720.9
	4	900.1	904.1
	5	1083.4	n.d.
	6	1267.6	n.d.
	1	367.5	367.4, 369.5
	2	550.7	550.7, 552.7, 554.6
	3	734.0	735.9, 737.9, 738.9
	4	917.2	921.1, 923.1
	5	1100.4	1107.4
	6	1283.6	n.d.
	1	392.5	392.5, 394.5
	2	575.7	575.7, 577.7
	3	758.9	758.9, 760.9, 762.9, 764.9
	4	942.2	946.2, 948.2
	5	1125.3	1131.4
	6	1308.5	n.d.
	1	409.5	409.5, 411.5
	2	592.8	592.7, 594.7, 596.7
	3	776.0	778.0, 780.0, 782.0
	4	959.2	963.2, 965.2
	5	1143.5	1149.4
	6	n.d.	n.d.
	1	434.5	434.5, 436.5
	2	617.8	617.8, 619.8, 621.8
	3	801.0	801.0, 803.0, 805.0, 807.0
	4	984.2	986.2, 988.2
	5	1167.5	1173.5
	6	1350.6	n.d.

In the case of ^{15}N-PHMB, the most prominent molecular ion detected is underlined; n.d., not detected.

$(41 + 84 + (183)_n + 83)$, $(58 + 84 + (183)_n + 83)$, and $(83 + 84 + (183)_n + 83)$ Da respectively. Structural assignments for each detected PHMB molecular ion are provided in Table I. Some deviation from the above theoretical masses is observed, suggested to be due to variable protonation of biguanide units when combined with the matrix.

On examination of MALDI-TOF mass spectra, other signals can be assigned to PHMB-derived species not described by the six families detailed earlier. Ions at *m/z* 451 and 634 are suggested to be PHMB oligomers (possessing two and three BG repeating units respectively) that lack any end-groups. In addition, ions at *m/z* 476 and 659 remain unassigned, although we suggest they could be some form of cyclized polybiguanide. Previously, East et al.[2] had shown that branched forms of PHMB, containing substituted melamines, can be formed during synthesis by either cyclization of BG and CA groups, or by condensation of CG and CA end-groups.

Confirmation that product types 1–6 are PHMB species was achieved by comparison of MALDI-TOF spectra on analysis of standard and ^{15}N-labeled PHMBs. During synthesis of ^{15}N-labeled PHMB, inclusion of label is achieved by the polycondensation of ^{15}N-labeled HMD with unlabeled HMBDA. Therefore, an ordered and sequential inclusion of ^{15}N into the growing polymer should be expected, with the label position alternating between *N*-1 and *N*-5 along the polymer chain (Fig. 4). For example, condensation

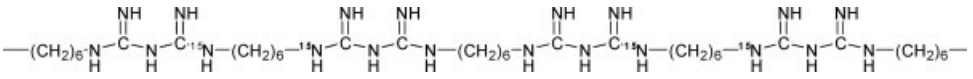


Figure 4 Theoretical location of ^{15}N label in ^{15}N-PHMB oligomers.

of one molecule of HMD and HMBDA should result in a species containing one BG repeating unit; terminated with one A and one CG end-group (molecular ions expected to be observed at *m/z* 367 [^{14}N] or 369 [^{15}N]). Subsequently, should HMBDA react with the free A end group, a two BG containing species with two CG end-groups (molecular ions expected to be observed at *m/z* 617 [^{14}N] or 619 [^{15}N]) will be formed. Therefore, if this hypothesis for PHMB synthesis held true, only one signal (at *m/z* +2 compared to that observed for standard PHMB) should be observed for ^{15}N PHMB species containing one or two BG repeat-

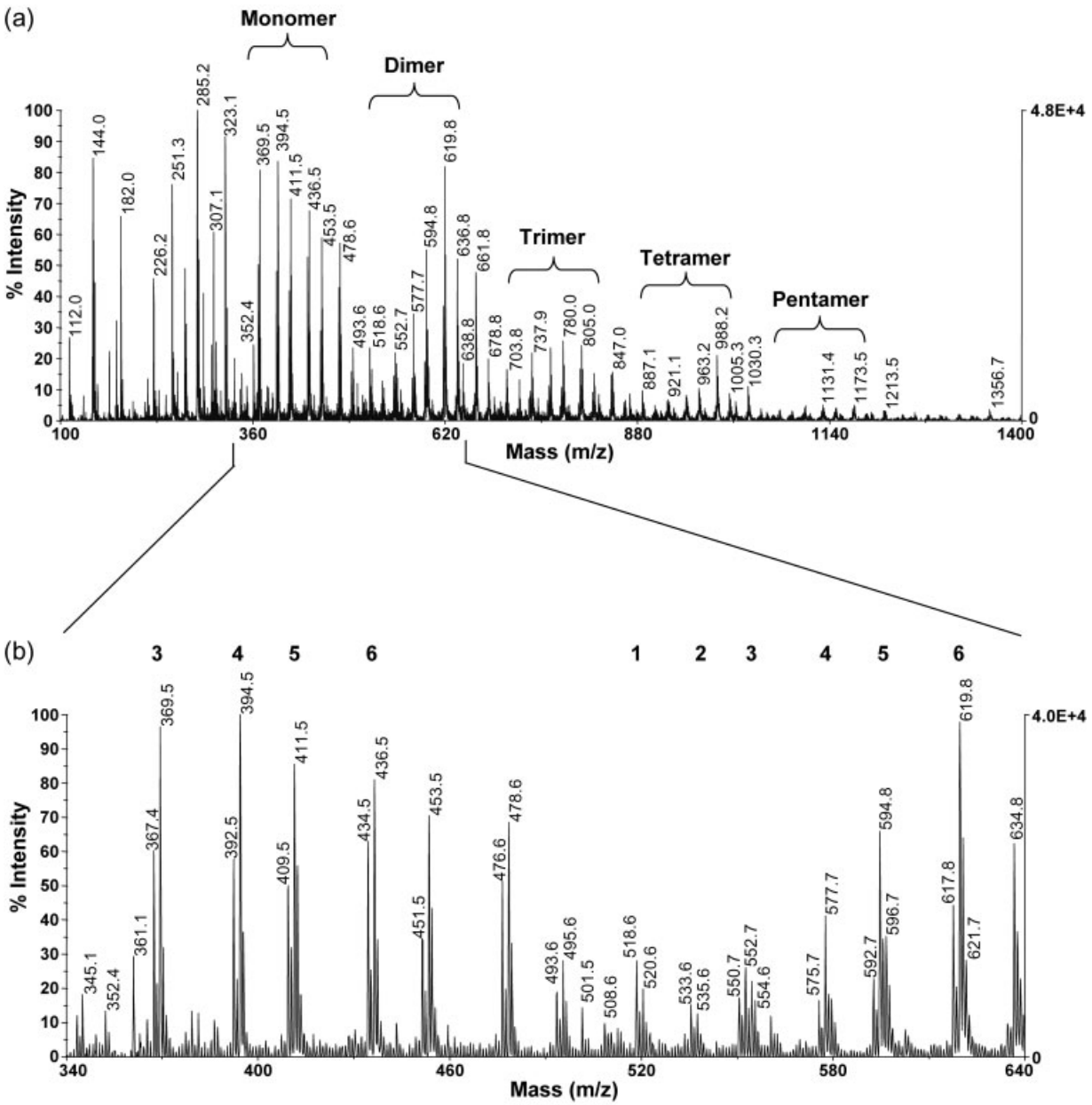


Figure 5 (a) MALDI-TOF mass spectrum of ^{15}N-labelled PHMB; (b) expanded view of ^{15}N-labelled PHMB mass spectrum between 340 and 640 Da, revealing different families of PHMB oligomers (labeled 1–6).

ing units. Similarly, for ^{15}N PHMB species containing three or four BG repeating units, only one signal (at $m/z + 4$) should be observed.

On examination of MALDI-TOF mass spectra for ^{15}N PHMB [Fig. 5(a)], it is observed that the molecular ion of greatest intensity for each polybiguanide species does indeed follow the expected rule for amount of ^{15}N incorporation (underlined molecular ions in Table I). However, other molecular ions for each oligomeric species are often detected, which is indicative of variable incorporation of ^{15}N into the molecule. For example, PHMB monomer species exhibit doublet signals, with an increase in *m/z* value compared to standard PHMB of either 0 or 2 [Fig. 5(b) and Table I]. PHMB dimer species exhibit doublet or triplet signals, with an increase in *m/z* value of 0, 2, or 4 [Fig. 5(b) and Table I]. The incorporation of ^{15}N is observed for five of the six PHMB product types. Interestingly, product type one (A and CA end-groups) is not detected in the ^{15}N-PHMB sample, which may be due to the different scale of synthesis employed during preparation of this sample.

Interpretation of polymerization process from MALDI-TOF MS data

While detection of signals with increased *m/z* values unequivocally confirms the assignment of PHMB species, the variation in ^{15}N incorporation can be explained by the existence of an equilibrium between polymerization and depolymerization during PHMB synthesis.[2] Shapiro et al.[10] has previously shown that a BG group may be cleaved under synthetic conditions to form two species possessing either (i) a CG and an A end-group; or (ii) a G and CA end-group (depicted for molecular ion at *m/z* 367 in Fig. 6). First, the existence of this process serves to explain the detection of G and CA end-groups in the PHMB samples. Second, the newly formed depolymerization species will be available to react with other free end-groups in the synthetic mixture to form new polybiguanide species. In the case of ^{15}N-PHMB synthesis, subsequent polyaddition reactions involving cleaved portions from larger PHMB oligomers will result in variation in ^{15}N position within species, and is concluded to be the reason for detection of multiple signals for ^{15}N-labeled PHMB.

Also, detection of several PHMB species by MALDI-TOF MS can only be explained by the existence of an equilibrium during synthesis. For example, of the detected one BG-containing species, only the molecular ion at *m/z* 367 (A and CG end-groups) can be explained by simple condensation of one molecule of HMD and HMBDA. Therefore, the other one-BG-containing species must be formed by either (i) release of the entire species by thermal decomposition of a larger PHMB oligomer at two points in the chain; or (ii) release of two fragments from a larger PHMB oligomer, and subsequent condensation of these.

In addition, all of the detected PHMB product types are terminated with either CG and/or CA end-groups. During melt polymerization of the standard polymer when, as in our case, a 1 : 1 (w/w) ratio of HMD to HMBDA are combined, it should be expected that the majority of CG groups be consumed to form BG groups.[2] However, the continued dominance of CG end-groups is again evidence of the polymerization–depolymerization process during synthesis, as CA and CG groups will be produced from cleavage of BG.

PSD fragmentation of PHMB

PSD MALDI-MS allows for specific fragmentation of an intact molecular ion in the field-free drift region of the TOF mass analyzer. Therefore, fragmentation of the ion will provide confirmation that the correct assignment of an ion has been made. On completion of PSD analysis, detected fragmentation ions of defined *m/z* can be assigned to specific portions of the parent ion molecular structure. PSD fragmentation has generally been used to elucidate peptide structure, although it has recently been applied to analysis of synthetic polymers.[11–13]

On PSD analysis of the precursor ion at *m/z* 367 (Fig. 7), extensive fragmentation is observed. All fragments are presumed to be the result of C—N bond cleavage at various points along the backbone of the molecule. Figure 8(a) outlines the presumed source of all fragmentation ions formed as a result of in-chain

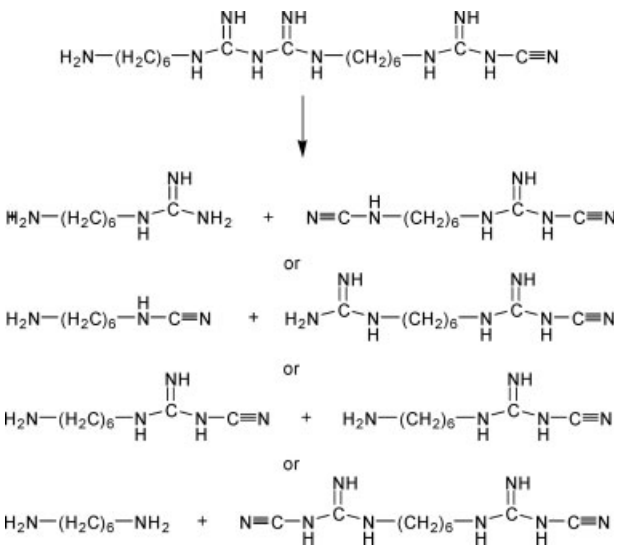


Figure 6 Theoretical formation of end-groups from thermal depolymerization of biguanide moiety. Cleavage of the biguanide group is depicted for the PHMB species detected at *m/z* 367.

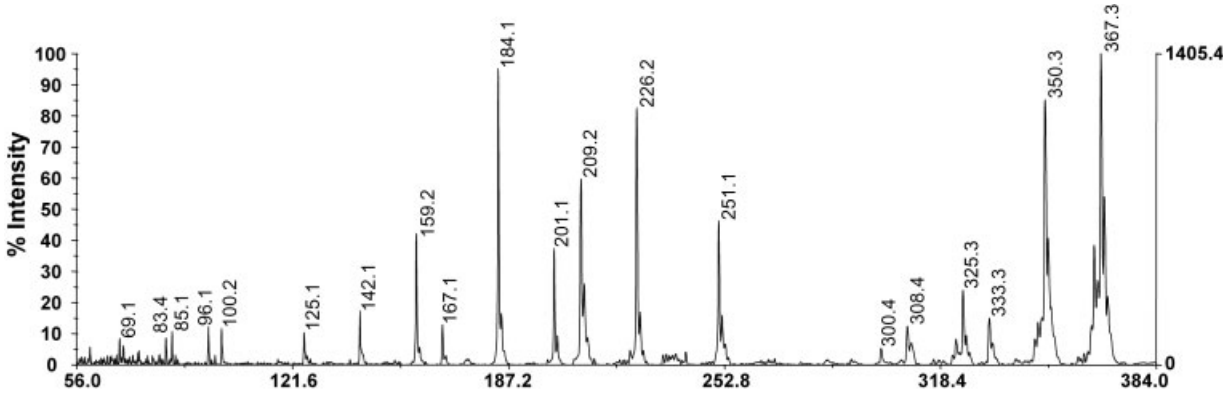


Figure 7 PSD spectrum of standard PHMB precursor ion at *m/z* 367.

C—N bond cleavage. Figure 8(b) outlines the presumed source of all fragment ions that are a direct result of imine group removal in conjunction with C—N bond cleavage at other points in the molecule. Collectively, the obtained data demonstrate the correct assignment of molecular structure for the principal precursor ion for the PHMB sample.

CONCLUSIONS

It has been shown that MALDI-TOF MS is able to characterize PHMB molecular structure in a manner hitherto impossible by other analytical methods. The developed MALDI-MS method is able to detected PHMB oligomers of $n \leq 6$. Six different PHMB prod-

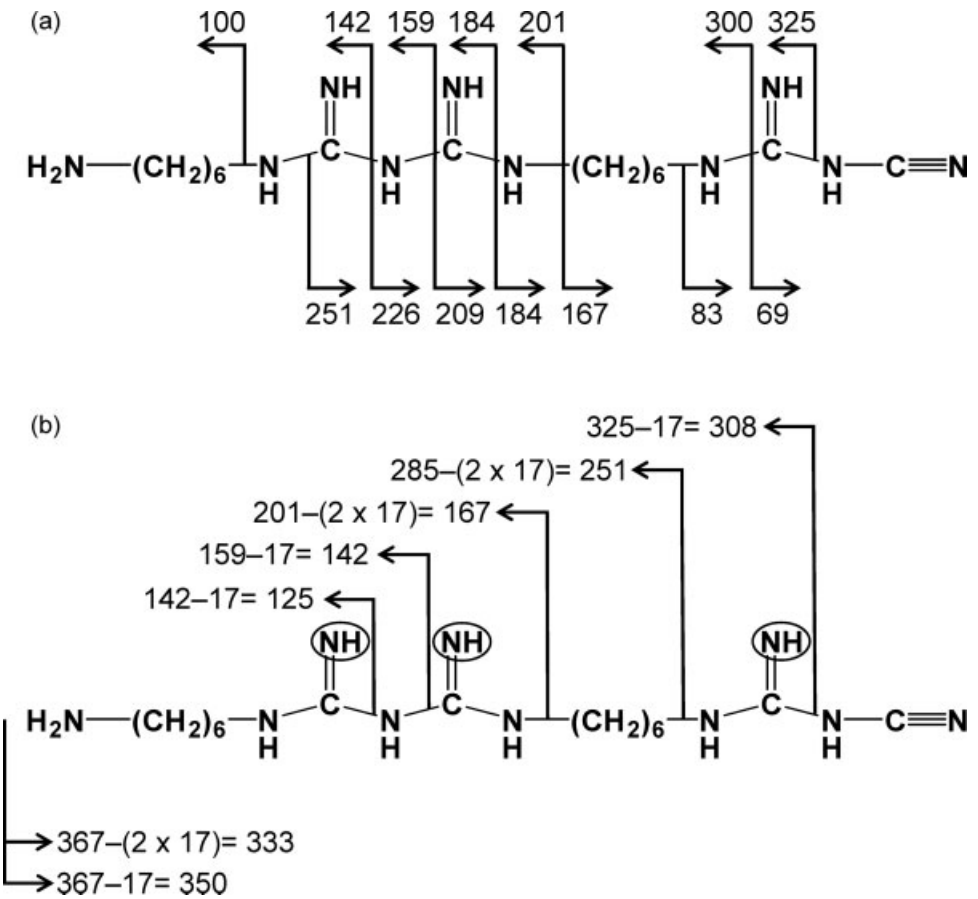


Figure 8 Fragmentation pattern of standard PHMB precursor ion at *m/z* 367 resulting from (a) in-chain C—N bond cleavage; and (b) in-chain C—N bond cleavage in combination with removal of imine groupings (sites for possible loss of imine groups indicated in ovals).

uct types (distinguished by end-group identities) are observed to be present in the biocide. PSD fragmentation of the main PHMB precursor ion at *m/z* 367 has confirmed the correct structural assignment of PHMB molecular ions on obtained MALDI-TOF mass spectra. In addition, comparison of MALDI-TOF spectra for standard and ^{15}N-labeled PHMBs has provided confirmation that observed molecular ions are PHMB species, and has also provided direct evidence for the existence of an equilibrium between polymerization and depolymerization during PHMB synthesis.

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