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Solid state ^{13}C NMR spectroscopy provides direct evidence for reaction between ethinyl estradiol and a silicone elastomer vaginal ring drug delivery system

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Abstract

Steroid molecules have a long history of incorporation into silicone elastomer materials for controlled release drug delivery applications. Previously, based on *in vitro* release testing and drug content data, we demonstrated indirectly that the contraceptive progestin levonorgestrel chemically and irreversibly binds to addition cure silicone elastomers via a hydrosilylation reaction between its ethynyl group and the hydrosilane groups in the silicone. Here, for the first time, we report that solid state ^{13}C nuclear magnetic resonance spectroscopy provides direct evidence for the irreversible binding of ethinyl estradiol (EE) – a steroid molecule containing an ethynyl functional group – to an addition cure silicone elastomer. By preparing silicone samples containing ^{13}C-labelled ethinyl estradiol, signals in the NMR spectra could readily be assigned to both the free and bound steroid. Additional depolymerisation studies, performed on an addition cure silicone elastomer system from which the unbound EE fraction was completely extracted, further confirmed the presence of bound EE through the formation of coloured reaction mixtures resulting from the reaction of bound EE and trifluoroacetic acid. These methods will be particularly useful in the ongoing development of new steroid-releasing silicone drug delivery devices, including various vaginal ring devices for contraception, HIV prevention and multipurpose prevention technology applications.

Keywords: Covalent Binding; 17α-ethinyl-estradiol; ^{13}C-labelled; Addition cure silicone elastomer; Nuclear Magnetic Resonance; Hydrosilylation

44 # Abbreviations

45 $CDCl_3$, deutrated chloroform; CP, cross-polarisation; DAC, dual asymmetric centrifuge;
46 DPV, dapivirine; EE, Ethinyl estradiol; EE-$^{13}C_2$, 17α-ethinyl-$^{13}C_2$-estradiol; HIV, human
47 immunodeficiency virus; LNG, levonorgestrel; NES, Nestorone; NMR, nuclear magnetic
48 resonance; ^{13}C-ssNMR, solid-state ^{13}C nuclear magnetic resonance spectroscopy; TOSS,
49 total suppression of spinning sidebands; TFA, trifluoroacetic acid;
50

1. Introduction

Following first demonstration in 1966 that steroid molecules could permeate through the walls of silicone rubber containers implanted in ewes,[1] a number of steroid-releasing silicone elastomer controlled release drug delivery devices have since reached market, including the subdermal implants Norplant®, Norplant II® and Jadelle® and the vaginal ring products Estring®, Femring®, Progering® and Fertiring®.[2–7] Several new silicone elastomer vaginal rings are currently in development, including a dapivirine-releasing ring for HIV prevention,[8–11] a combination dapivirine/maraviroc vaginal ring for HIV prevention,[12,13] a dapivirine/levonorgestrel (LNG) ring for combination HIV prevention and contraception,[14,15] a combination anastrozole/LNG ring as a novel therapy for treatment of endometriosis,[16–18] a vaginal ring releasing the progesterone receptor modulator ulipristal acetate for contraception,[19,20] and a Nestorone® (NES; segesterone acetate)/ethinyl estradiol ring for combination contraception.[21–24]

The NES/EE vaginal ring, currently under development by the Population Council (New York, USA), comprises a silicone elastomer ring body into which two steroid-containing silicone elastomer cores are inserted – one core contains only NES and the second both NES and EE (Figure 1). The ring body is manufactured from an addition cure silicone elastomer while the drug-loaded cores are prepared using a condensation cure silicone elastomer. The cure chemistries of these silicone elastomer systems are very different, with the condensation cure silicone often preferred for preparation of drug-loaded components due to its compatibility with a wide range of chemical functional groups.[11] In contrast, the platinum catalyst used in addition cure silicone elastomer systems is susceptible to reaction

with certain chemical functional groups leading to inhibition of cure. During stability testing of the NES/EE vaginal ring, significant loss of EE from the ring device was observed. Since EE could be fully recovered from NES+EE cores that had not been assembled into rings, even after long term storage, the loss of EE from the ring was attributed to reaction of EE with the addition cure silicone elastomer comprising the ring body through which the drug must permeate in order to be released.

Recently, as part of preclinical development of the dapivirine/LNG vaginal ring, we reported the irreversible binding of LNG in an addition cure silicone elastomer vaginal ring, resulting in significant loss of LNG and impacting LNG *in vitro* release.[14,15] Despite a lack of direct evidence, we hypothesized that the binding involved a hydrosilylation reaction between the LNG ethynyl group and the hydrosilane (Si–H) functionalised polydimethylsiloxane molecules in the elastomer system rendering the LNG covalently attached to the elastomer and incapable of release.[15] Normally, these hydrosilane groups react with the vinyl-functionalised groups ($Si–CH=CH_2$) in the polydimethylsiloxane molecules as part of the curing reaction (Figure 2).

Here, we report that solid state ^{13}C nuclear magnetic resonance spectroscopy (^{13}C-ssNMR) provides the first direct evidence for irreversible binding of EE to an addition cure silicone elastomer. By preparing silicone elastomer samples containing ^{13}C-labelled ethinyl estradiol (specifically, the ethynyl carbons are labelled and are therefore particularly sensitive to any reaction at this site), signals in the ^{13}C-ssNMR spectra could readily be assigned to both the free and bound steroid.

97 # 2. Experimental Section

98 ## 2.1 Materials

99 Addition cure silicone elastomer systems DDU-4331 (also known as MED4-4224) and
100 DDU-4320, and condensation cure silicone elastomer system DDU-4352 (also known as
101 MED-6603) were supplied by NuSil™ Technology LLC (Carpinteria, CA, USA).
102 Micronised ethinyl estradiol (EE) was supplied by Bayer AG (Bergkamen, Germany).
103 Non-micronised 17α-ethinyl-$^{13}C_2$-estradiol (20,21−$^{13}C_2$ labelled; 99.1% isotopic
104 enrichment) (EE-$^{13}C_2$) was purchased from Cambridge Isotope Laboratories, Inc.
105 (Andover, MA, USA). Particle size reduction of EE-$^{13}C_2$ was achieved by manual grinding
106 in a mortar and pestle. Deuterated chloroform ($CDCl_3$, 99.8 atom % D), acetone and
107 trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Gillingham, UK).

108

109 ## 2.2 Manufacture of Silicone Elastomer Samples

110 DDU-4331 silicone elastomer mixes without EE were prepared by mixing DDU-4331 Part
111 A and DDU-4331 Part B (9:1) in a DAC150 FVK-Z Speedmixer™ (3000 rpm, 30 s) to
112 obtain a homogeneous mixture. DDU-4320 elastomer mixes without EE were similarly
113 prepared by mixing DDU-4320 Part A and Part B (1:1). EE-loaded (2% w/w) DDU-4331
114 and DDU-4320 silicone elastomer mixes were similarly prepared except with extended
115 speedmixing at 3000 rpm for 60 s to achieve a homogeneous dispersion of the drug
116 powders in the silicone elastomer. The elastomer mixes were poured onto a glass plate
117 fitted with a thin-film cellulose acetate release liner and 1 mm spacers. After pouring, a
118 second acetate release liner and glass plate were set on top and the mixtures compressed to
119 form thin viscous films. Non-medicated DDU-4331 and DDU-4320 silicone elastomer

120 samples were cured in an oven at 150°C for 10 min or 90°C for 10 min, respectively. EE-
121 $^{13}C_2$-loaded DDU-4320 samples were cured in an oven at 90°C for 30 min. Despite
122 adjustments to the cure conditions (final temperature and time = 130°C for > 20 h), both
123 the EE-$^{13}C_2$ and EE-loaded DDU-4331 silicone samples only partly cured to form gum-
124 like consistency materials due to EE inhibition of the curing reaction.
125

126 ## 2.3 Solvent Extraction of EE from Cured Silicone Elastomer Samples

127 In order to increase sensitivity of detection for any bound EE using ^{13}C-ssNMR, the non-
128 bound EE fraction was extracted from the silicone elastomer samples using organic solvent.
129 Elastomer samples were placed in individually labelled glass vials. $CDCl_3$ or acetone (10–
130 40 mL, depending on EE loading) was added to each extraction flask. Flasks were sealed
131 and stored at ambient temperature for 24 h with periodic manual shaking. This extraction
132 protocol was repeated three times using fresh volumes of solvent to ensure complete
133 extraction of the non-bound EE. The elastomer samples were removed from the solvent
134 and allowed to evaporate to dryness in preparation for ^{13}C-ssNMR analysis. $CDCl_3$
135 extraction solutions were also retained for solution-state NMR analysis to verify that
136 extraction of the non-bound EE fraction had occurred.
137

138 ## 2.4 Trifluoroacetic Acid Depolymerisation of Cured Silicone Elastomers

139 Silicone elastomers are rapidly depolymerised to form a low viscosity colloidal liquid when
140 exposed to TFA. Also, under highly acidic conditions, steroid compounds are known to
141 react to form deeply coloured complexes.[25] DDU-4331 and DDU-4320 silicone elastomer
142 samples (~ 0.2 g) – with (2 % w/w) and without EE – were placed in individual glass vials.

TFA (10 mL) was added and the flasks immediately sealed.After 24 h, any colour change was noted and photographed. The colour intensity of the depolymerised samples was used to determine whether EE or EE$-^{13}C_2$ was detectable pre- and post-solvent extraction.

2.5 NMR Analysis

Various solution-state and solid-state ^{1}H-NMR and ^{13}C-NMR experiments were conducted to investigate binding of EE and EE$-^{13}C_2$ to DDU-4331 and DDU-4320 addition-cure silicone elastomer systems. In order to aid interpretation of the measured NMR spectra, MarvinSketch NMR Predictor software (ChemAxon, Budapest, Hungary) was used to produce simulated NMR spectra for EE, a model addition cure silicone elastomer and the reaction product for EE covalently bound to the silicone elastomer.

Solution-State NMR Analysis

For solution-state ^{1}H and ^{13}C-NMR analysis, samples were dissolved, dispersed or swollen in $CDCl_3$. Spectra were recorded using a Bruker DPX 400 MHz NMR spectrometer (Bruker UK Ltd., Coventry, UK). Chemical shifts were recorded in ppm (parts per million) with the chemical shift of the internal reference set to 77.0 ppm for ^{13}C-NMR and 7.26 ppm for ^{1}H-NMR with respect to $CDCl_3$. A series of reference spectra for the supplied APIs and DDU-4331 Part A and Part B silicone elastomer components were recorded to enable identification of characteristic API and silicone elastomer signals.

Solid-State NMR Analysis (ssNMR)

165 Solid-state NMR experiments were performed at the EPSRC National Solid-State NMR
166 Service at Durham University. ^{13}C-NMR experiments were performed using either a
167 Bruker Avance III HD spectrometer with a 4 mm (rotor o.d.) magic angle spinning (MAS)
168 probe or a Varian VNMRS spectrometer with a 6 mm (o.d.) rotor operating at 100.6 MHz.
169 Bruker Avance ^{13}C-NMR spectra were obtained using total suppression of spinning
170 sidebands (TOSS) and cross-polarisation (CP) with a 4 or 10 s recycle delay, 1 or 4 ms
171 contact time, ambient probe temperature (~25°C) and a sample spin-rate of 8 kHz. Between
172 250 and 20,000 repetitions were accumulated depending on the sample being analysed.
173 ^{13}C-NMR reference spectra (EE, EE−$^{13}C_2$ and DDU-4331) were obtained using the Varian
174 VNMRS spectrometer with TOSS (except for the EE API sample) and CP, a 1 or 30 s
175 recycle delay, 1 or 5 ms contact time, ambient probe temperature and a spin-rate of 6 kHz.
176 The number of scans accumulated for EE, EE−$^{13}C_2$ and DDU-4331 NMR samples varied
177 (72, 116 and 992 repetitions) depending on the sample being analysed.
178

179 # 3. Results and Discussion

180 To aid understanding of the decreased EE content recoveries observed during long-term
181 stability investigation of the NES/EE vaginal ring, a series of NMR studies on supplied
182 materials (APIs and silicone elastomer components) and cured silicone elastomer samples
183 (with and without API) were performed in order to seek direct evidence for binding of EE
184 to the DDU-4331 silicone elastomer. In previous preclinical studies involving the
185 dapivirine/LNG silicone elastomer ring device, we demonstrated indirect evidence – in the
186 form of content assay data – of irreversible covalent binding between the contraceptive
187 steroid LNG and an addition cure silicone elastomer (Nusil's DDU-4320).[15] We
188 hypothesised that the mechanism for LNG binding to the DDU-4320 silicone elastomer
189 was via a hydrosilylation reaction between the ethynyl groups (C≡C) of the LNG with the
190 Si–H groups on the silicone to produce a new carbon-carbon (C=C) double bond (Figure
191 2). The degree of LNG binding could be altered through modification of the temperature
192 and time used during the silicone elastomer curing process.[15] However, confirmation of the
193 covalent binding reaction could not be confirmed using ssNMR analysis as the bound LNG
194 fraction in the DDU-4320 silicone elastomer was below the level of detection for ssNMR.
195 In this study, the low natural abundance of the ^{13}C isotope (1.1%) combined with the low
196 fraction of EE that would potentially bind to the DDU-4331 silicone elastomer system
197 would similarly make detection of new covalent bonds between the EE ethynyl groups and
198 the silicone elastomer hydride groups extremely difficult. The use of ^{13}C-labelled EE
199 significantly increased the sensitivity of the ^{13}C NMR (by a factor of ~90 relative to its
200 natural 1.1% abundance), thereby improving the chances of detecting the EE + silicone
201 reaction product.

Sample Preparation

During preparation of the 2% w/w EE and EE–$^{13}C_2$ loaded DDU-4331 silicone samples, interaction between the EE and the DDU-4331 material inhibited the silicone curing reaction such that the resulting samples were tacky due to partial cure. These issues could not be resolved by reducing the EE loading or altering the elastomer cure temperature and time. Greater inhibition of cure was observed with the EE–$^{13}C_2$ material. Interestingly, incorporation of either 2% w/w labelled or unlabelled EE into the alternative addition cure silicone elastomer DDU-4320 (previously used for manufacture of dapivirine/LNG vaginal rings, where the LNG had a strong tendency to bind to the silicone elastomer) did not show any obvious inhibition of cure. This confirmed that the curing issues experienced were due to significant interaction between the DDU-4331 silicone system and EE.

NMR Analysis

^{13}C-NMR modelling software predicted that the purported reaction product between EE and a model addition cure silicone elastomer would contain new vinylene carbons (–CH=CH–) with chemical shifts in the 120–140 ppm range and disappearance of the ethynyl signals at ~74 and 100 ppm (Figure 3). Signals associated with the aromatic carbons are also expected to appear in 110–140 ppm region whilst the C–OH groups are predicted to appear at ~80 and 155 ppm. It should be noted that these predicted spectra cannot account for differences in chemical shift values associated with different stereochemical additions (syn-, anti- and α-adducts) of the terminal alkyne group to the hydrosilanes of the silicone

and therefore were only intended to be used as an indicator of changes in chemical shift values for the proposed reaction product.[26]

Solution-State NMR Reference Spectra

Solution-state ^{13}C-NMR spectra for EE, EE–$^{13}C_2$ and DDU-4331 silicone elastomer in $CDCl_3$ were recorded to identify reference signals. In all three solution-state ^{13}C-NMR reference spectra (Figures 4A–C), the triplet signal observed close to 77 ppm is due to the $CDCl_3$ solvent. In the EE ^{13}C-NMR reference spectra (Figure 4B), twenty different carbon signals associated with EE are observed. The signals at 74 and 87 ppm are assigned to the ethynyl carbons. Signals associated with C–OH carbons are observed at 80 ppm and 153 ppm, aromatic carbons in the region of 110–140 ppm, and C–C bonds in the region of 20–50 ppm. The ^{13}C-NMR reference spectra for the EE–$^{13}C_2$ (Figure 4C) displays similar chemical shifts to those of the EE reference spectra with the intense doublet signals at 74 and 88 ppm due to the ^{13}C-labelled ethynyl carbons. The presence of doublets, rather than single peaks, is due to spin-spin coupling between the neighbouring ^{13}C atoms. The apparent absence of the ~80 ppm C–OH signal in the EE–$^{13}C_2$ reference spectrum is due to its small size relative to the ^{13}C signals and/or deuterium substitution on the hydroxyl group causing an isotopic shift and resulting in the ^{13}C chemical shift of the C-OH group overlapping with the ^{13}C labelled ethynyl groups. The ^{13}C-NMR reference spectrum for the DDU-4331 silicone elastomer (Figure 4A; swollen sample in $CDCl_3$) displays a single large signal at ~0 ppm corresponding to the Si–CH_3 groups. The $CDCl_3$ solvent used during acquisition of the reference spectra did not contain tetramethylsilane (TMS) reference standard.

Solid-State NMR (ssNMR) Reference Spectra

^{13}C-ssNMR reference spectra were recorded for non-medicated cured DDU-4331 silicone elastomer, EE and EE–$^{13}C_2$ (Figures 4D–F, respectively). The narrow signals observed for the EE reference material (Figure 4B) are typical of crystalline molecules. Spinning side bands have not been supressed in this spectrum. Broader peaks observed in the EE–$^{13}C_2$ spectrum (Figure 4F), particularly for the labelled ethynyl groups at 75 and 85 ppm, are due to strong interactions between the two neighbouring ^{13}C atoms.[27] As previously observed with the solution-state spectra, the ^{13}C-ssNMR reference spectrum for the cured DDU-4331 elastomer displayed a single large (Si–CH_3) signal at 1.3 ppm (Figure 4D).

Interestingly, subtle differences in ^{13}C-ssNMR spectra for the labelled and unlabelled EE materials were observed when spectra were overlaid (overlaid spectra not shown). We suspect that these spectral differences may be the result of different polymorphic forms of EE. EE is known to exist in at least two polymorphic forms with melting points at 146°C and 183°C as well as multiple pseudo-polymorphs in the form of solvates and hydrates.[28–30] As the EE and EE-$^{13}C_2$ used in this study were obtained from two different suppliers (characterisation information was not provided), it is possible that differences in the synthetic processes caused the formation of different crystalline forms.

NMR Spectra of Silicone Elastomer Samples Containing EE or EE–$^{13}C_2$

Solvent extraction was performed on unlabelled EE-loaded silicone elastomer samples to remove the non-bound EE fraction prior to ^{13}C-ssNMR analysis. Solution-state ^{1}H and ^{13}C-

270 NMR analysis of the resulting extraction solutions showed a spectrum consistent with the
271 chemical structure of EE (spectra not shown), confirming successful extraction of the non-
272 bound EE portion. ^{13}C-ssNMR analysis of EE-loaded DDU-4331 samples (pre- and post-
273 extraction) proved inconclusive, i.e. no new signals suggestive of newly formed bonds
274 were observed.
275
276 As previously discussed, incorporation of EE into DDU-4331 resulted in significant
277 inhibition of cure with the ^{13}C-labelled EE samples curing to a lesser extent than the
278 unlabelled EE samples prepared with the same loading. This suggested that the EE–$^{13}C_2$
279 material had a greater propensity to inhibit the curing reaction of the DDU-4331 silicone
280 elastomer and we therefore hypothesised that more EE–$^{13}C_2$ compared to unlabelled EE
281 may be bound. From our previous studies, we know that curing conditions (temperature
282 and time) as well as API particle size can have a significant impact on binding of steroid-
283 type molecules to addition cure elastomers.[15] Although this was considered by the authors
284 – and was the reason that the EE–$^{13}C_2$ API was hand-milled to a finely powdered material
285 prior to incorporation into the silicone elastomer – the lack of particle size distribution
286 information for both EE materials meant no conclusions could be drawn. The presence of
287 different polymorphic forms of EE, with differing solubilities could be another explanation
288 for the different degrees of inhibition of curing observed for the ^{13}C-labelled and unlabelled
289 EE-loaded DDU-4331 samples. As only the solubilised portion of EE participates in the
290 covalent binding reaction with the silicone elastomer, any increase in the silicone solubility
291 of the API would cause a subsequent increase in the amount of dissolved EE molecules

292 available for participation in the binding reaction, resulting in an increase in the extent that
293 the silicone elastomer crosslinking reaction was inhibited.
294
295 Figure 5A shows the ^{13}C-ssNMR spectra for an EE–$^{13}C_2$ + DDU-4331 silicone sample
296 before solvent extraction. The chemical shifts associated with the ^{13}C-labelled ethynyl
297 groups are visible at 75 and 87 ppm. A second set of intense signals are observed at 125
298 and 153 ppm. These signals at 125 and 153 ppm are not observed in the EE–$^{13}C_2$ or DDU-
299 4331 reference spectra (Figures 4F and 4D, respectively) and are attributed to newly-
300 formed vinylene carbons produced from the hydrosilylation reaction between the ethynyl
301 groups of the EE and the hydrosilane groups within the silicone elastomer (Figure 2).
302
303 Analysis of the EE–$^{13}C_2$ + DDU-4331 material following acetone extraction showed that
304 the ethynyl signals (75 and 87 ppm) associated with the non-bound EE–$^{13}C_2$ were no longer
305 visible in the post-extraction sample (Figure 5B), confirming that the non-bound EE–$^{13}C_2$
306 fraction had been successfully removed via solvent extraction. More interestingly, the new
307 vinylene signals at 125 and 153 ppm were still observed and showed no reduction in
308 intensity when compared to the non-extracted sample (Figure 5A), clearly indicating that
309 they could not be removed from the silicone elastomer by solvent extraction and therefore
310 must be bound. Therefore, Figure 5A and 5B provide direct evidence for the formation of
311 the irreversible covalent bond between the ethynyl groups of the EE–$^{13}C_2$ and the
312 hydrosilane groups of the DDU-4331 addition cure silicone elastomer.
313

In addition to the DDU-4331 system, EE–$^{13}C_2$ loaded samples (2% w/w) were manufactured using DDU-4320 addition cure silicone elastomer. DDU-4320 has been successfully used for manufacture of a vaginal ring device containing LNG, a contraceptive steroid that also has a tendency to bind to addition cure silicones.[14,15] With this material, no inhibition of curing was observed. ^{13}C-ssNMR spectra for the EE–$^{13}C_2$ + DDU-4320 elastomer samples showed intense ^{13}C-labelled ethynyl carbons signals at 76 and 88 ppm (spectra not shown). However, no new signals indicative of EE–$^{13}C_2$ binding were observed. In fact, no EE–$^{13}C_2$ associated signals were observed in the spectrum of the silicone material after solvent extraction, indicating that the non-bound drug fraction had been successfully extracted and that any bound EE–$^{13}C_2$ was below the limits of detection. On reflection, the lack of curing issues observed with the EE–$^{13}C_2$ + DDU-4320 silicone elastomer indicated that the fraction of EE bound to the DDU-4320 was sufficiently low so as not to inhibit the curing reaction and therefore too low for detection by ssNMR. This reduced propensity for binding is most likely due to differences in the amounts of Si–H groups present in the two silicone elastomer systems.

Depolymerisation with Trifluoroacetic Acid

To supplement the NMR analysis, silicone elastomer depolymerisation experiments were performed using TFA as a qualitative measure of EE presence in cured silicone elastomer samples. TFA depolymerisation of steroid-containing silicone elastomer samples produces deeply coloured, low viscosity colloidal liquids that can be used as a rapid, highly sensitive assay for the presence of EE. The colour intensity of the EE + TFA reaction products was shown to be dependent on the initial EE concentration with a coloured reaction product

337 easily detected for elastomer samples containing as little as 0.05% w/w EE. Although, only
338 qualitative in nature, the TFA depolymerisation studies appeared to be more sensitive to
339 the presence of the EE than either solution-state or solid-state ^{13}C-NMR and provide a
340 valuable resource for detection of residual bound EE. Depolymerisation studies were
341 performed on a range of EE and EE–$^{13}C_2$ loaded silicone elastomer samples, both before
342 and after solvent extraction, as a means of visually identifying the presence of bound EE.
343
344 TFA depolymerisation solutions obtained from a series of DDU-4331 and DDU-4320
345 elastomer samples, with and without EE–$^{13}C_2$, are shown in Figures 6A & 6B.
346 Depolymerisation of a non-medicated DDU-4331 control sample produced a brilliant white
347 solution (Figure 6A). This white colour is attributed to the titanium dioxide (TiO_2) re-
348 enforcing filler used in this particular silicone material. Addition of a 2% w/w EE–$^{13}C_2$ +
349 DDU-4331 silicone elastomer samples to TFA produced a deep red-brown solution
350 indicative of the presence of a large quantity of EE–$^{13}C_2$ (bound and non-bound) (Figure
351 6A). Interestingly, despite extensive solvent extraction with multiple large volumes of
352 acetone, depolymerisation of the extracted EE–$^{13}C_2$ + DDU-4331 silicone material
353 produced a coloured solution that was only slightly paler than that of the pre-extraction
354 sample. This suggested that a large proportion of EE–$^{13}C_2$ was still present in the silicone
355 elastomer, and presumably in the bound state. These results strongly support the findings
356 of the ^{13}Css-NMR experiments (Figure 5). A similar trend was observed for the unlabelled
357 EE + DDU-4331 silicone samples (image not shown) despite the fact that solid-state NMR
358 analysis of this material proved inconclusive.
359

360 The coloured reaction products obtained for a non-medicated DDU-4320 silicone
361 elastomer sample, an EE-$^{13}C_2$ + DDU-4320 sample pre-extraction and two EE-$^{13}C_2$ + DDU-
362 4320 silicone elastomer samples extracted in either $CDCl_3$ or acetone are shown in Figure
363 6B. The image shows an off-white solution for the non-medicated DDU-4320 elastomer
364 sample, an amber coloured solution for the EE-$^{13}C_2$ + DDU-4320 sample and pale orange
365 coloured solutions for both the acetone and $CDCl_3$ extracted EE-$^{13}C_2$ + DDU-4320
366 samples. The paler solutions observed for the $CDCl_3$ and acetone extracted samples suggest
367 that although a significant fraction of the non-bound drug had been removed during the
368 extraction process, a detectable fraction of EE-$^{13}C_2$ still remained either in the bound or
369 unbound state. As mentioned previously, no evidence of EE-$^{13}C_2$ was observed in the
370 ^{13}Css-NMR spectra obtained for this extracted silicone material (spectra not shown)
371 indicating that the non-bound drug fraction had been removed and any bound drug was
372 below the limits of detection. These findings suggest that the TFA colour indicating assay
373 is particularly sensitive to the presence of low levels of steroids when compared to either
374 solution-state or solid-state NMR.
375

376 # 4. Conclusions

377 In this study, we demonstrate for the first time evidence for the covalent and irreversible
378 binding of a contraceptive steroid to an addition cure silicone elastomer system. Using ^{13}C-
379 ssNMR analysis of DDU-4331 silicone elastomer samples containing EE–$^{13}C_2$, we
380 observed NMR signals due to the ^{13}C-labelled ethynyl groups (75 and 87 ppm) as well as
381 additional signals associated with newly formed ^{13}C-labelled vinylene carbons (125 and
382 153 ppm). These new signals – not observed in the API or silicone elastomer reference

383 spectra – indicated the formation of a new C=C bond resulting from the hydrosilylation
384 reaction between the EE−$^{13}C_2$ and the silicone elastomer. In order to confirm that these new
385 signals resulted from bound EE, the elastomer samples were subjected to acetone
386 extraction. Analysis of the extracted elastomer samples showed the complete
387 disappearance of the ethynyl-associated signals but no reduction in the new vinylene
388 signals, indicating that they could not be removed by the solvent extraction process and
389 therefore must be bound. These new vinylene chemical shifts provide conclusive evidence
390 for covalent and irreversible binding of EE−$^{13}C_2$ to the hydrosilane groups of the DDU-
391 4331 addition cure silicone elastomer system. These results were further confirmed by the
392 findings of a TFA depolymerisation assay that demonstrated formation of strongly
393 coloured reaction products only in the presence of bound EE.

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Author Contributions

All authors contributed to the design of experiments and analysis of the data. C.F.M and D.A. conducted the experimental work. The manuscript was drafted by R.K.M and C.F.M, with input from other authors.

Declarations of Interest

None

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533 **FIGURE CAPTIONS**

534

535 **Figure 1.** A – Photograph of Nestorone® / ethinyl estradiol vaginal ring; B – Schematic of
536 ring showing dimensions and location of drug-loaded cores; C – chemical structure of the
537 progestin Nestorone®; D – chemical structure of the estrogen ethinyl estradiol

538

539 **Figure 2**. Schematic showing the platinum-catalysed hydrosilylation reaction between
540 ethinyl estradiol and the poly(dimethylsiloxane-co-methylhydrosilane) component of an
541 addition cure silicone elastomer system.

542

543 **Figure 3.** Simulated ^{13}C-NMR spectra for ethinyl estradiol and platinum-catalysed
544 hydrosilylation reaction product between ethinyl estradiol and a model methylhydrosilane
545 molecule.

546

547 **Figure 4.** A – solution state ^{13}C-NMR spectrum of silicone elastomer DDU-4331; B –
548 solution state ^{13}C-NMR spectrum of ethinyl estradiol; C – solution state ^{13}C-NMR
549 spectrum of 17α-ethinyl-$^{13}C_2$-estradiol (20,21−$^{13}C_2$ labelled); D – solid state ^{13}C-NMR
550 spectrum of silicone elastomer DDU-4331; E – solid state ^{13}C-NMR spectrum of ethinyl
551 estradiol; F – solid state ^{13}C-NMR spectrum of 17α-ethinyl-$^{13}C_2$-estradiol (20,21−$^{13}C_2$
552 labelled).

553

554

555 **Figure 5.** A – solid-state ^{13}C-NMR spectrum of a 2% w/w EE-$^{13}C_2$ + DDU-4331 silicone
556 elastomer sample before acetone solvent extraction; B – solid-state ^{13}C-NMR spectrum of
557 a 2% w/w EE-$^{13}C_2$ + DDU-4331 silicone elastomer sample after acetone solvent
558 extraction
559
560 **Figure 6**. A – TFA depolymerisation solutions containing blank DDU-4331 silicone, 2%
561 w/w EE−$^{13}C_2$ +DDU-4331 silicone and 2% w/w EE−$^{13}C_2$+DDU-4331 extracted silicone
562 samples; B – TFA depolymerisation solutions containing blank DDU-4320 silicone, 2%
563 w/w EE−$^{13}C_2$+DDU-4320 silicone, 2% w/w EE−$^{13}C_2$+DDU-4320 deuterated chloroform
564 extracted silicone and 2% w/w EE−$^{13}C_2$ +DDU-4320 acetone extracted silicone samples
565 (samples listed from left to right)
566