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1 Communication

## 2 Tetramethoxystilbene-loaded liposomes restore 3 reactive oxygen species-mediated attenuation of 4 dilator responses in rat aortic vessels, *ex vivo*.

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22 **Abstract:** The methylated analogue of the polyphenol Resveratrol (RV), 2, 3', 4, 5'-  
23 Tetramethoxystilbene (TMS) displays potent antioxidant properties and is an effective Cytochrome  
24 P450 (CYP) 1B1 inhibitor. The bioavailability of TMS is low. Therefore, the use of liposomes for the  
25 encapsulation of TMS is a promising delivery modality for enhanced uptake into tissues. We  
26 examined the effect of delivery of TMS in liposomes, on the restoration of vasodilator responses of  
27 isolated aortic vessels after acute tension elevation, *ex vivo*. Aortic vessels from young male Wistars  
28 were isolated and endothelial-dependent (acetylcholine, ACh) and independent (sodium  
29 nitroprusside, SNP) responses assessed. Acute tension elevation (1 hour) significantly reduced ACh  
30 dilator responses, which were restored following incubation with superoxide dismutase or  
31 apocynin (an NADPH oxidase inhibitor). Incubation with TMS-loaded liposomes (mean diameter  
32  $157 \pm 6$  nm; PDI 0.097) significantly improved the attenuated dilator responses following tension  
33 elevation. Endothelial denudation or co-incubation with L-NNA (N $\omega$ -nitro-L-arginine; nitric oxide  
34 synthase inhibitor) resulted in loss of dilator function. Our findings suggest that TMS-loaded  
35 liposomes can restore attenuated endothelial-dependent dilator responses, induced by an oxidative  
36 environment, by reducing NADPH oxidase-derived ROS and potentiating the release of the  
37 vasodilator nitric oxide. TMS-loaded liposomes may be a promising therapeutic strategy to restore  
38 vasodilator function in vascular disease.

39 **Keywords:** liposomes; 2, 3', 4, 5'-Tetramethoxystilbene; endothelium; aorta; oxidative stress;  
40 reactive oxygen species; nitric oxide; vascular function.

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## 45 1. Introduction

46 The vascular endothelial lining of blood vessels plays a pivotal role in the synthesis and release  
47 of highly regulated vasoactive substances, including the potent vasodilator nitric oxide (NO). An  
48 imbalance in the release of endothelial-derived vasodilator and vasoconstrictor mediators causes  
49 endothelial dysfunction (ED), that results in a dominant pro-vasoconstrictor state due to the  
50 generation of reactive oxygen species (ROS) and consequent reduction in NO bioavailability [1, 2].  
51 ED is also associated with increased constriction, partly attributed to the decline in NO-mediated  
52 inhibition of potent vasoconstrictors, such 20-hydroxyeicosatetraenoic acid (20 HETE). The latter is  
53 a Cytochrome P450 (CYP) 1B1 eicosanoid, which has been shown to contribute to the development  
54 of hypertension [3]. Attractive supplements to current treatment strategies include plant-derived  
55 polyphenols such as Resveratrol (RV; 3, 5, 4'-trihydroxystilbene), due to its anti-oxidant properties  
56 and ability to regulate the endothelial nitric oxide synthase enzyme [eNOS] [4, 5]. Recent studies  
57 have been dedicated to developing derivatives of RV that have greater bioavailability and  
58 pharmacological activity than RV [6]. In particular, the methylated derivative of RV; 2, 3', 4, 5'-  
59 Tetramethoxystilbene (TMS), has been demonstrated to be 1000 times more potent in inhibiting  
60 CYP1B1 than RV in spontaneously hypertensive rats (SHR) [7]. Elevated CYP1B1 activity,  
61 endothelial dysfunction, ROS generation, NADPH oxidase activity and expression of NOX-1, ERK1  
62 / 2 and p38 MAPK were diminished by TMS in the aorta, heart and kidney of the SHR model [7, 8].  
63 Intravenous administration of TMS in rats shows moderate clearance ( $46.5 \pm 7.6$  mL / min / kg) [9]  
64 and better bioavailability (4.5%) [10], compared to RV, however, the absolute oral bioavailability of  
65 TMS remains low ( $6.31 \pm 3.30\%$ ) [9].

66 Liposomes are recognised as promising agents for drug delivery due to their biocompatibility  
67 and ability to cross physiological barriers [11, 12]. Liposomes have been clinically approved for  
68 medical applications, including anticancer treatment, owing to their higher therapeutic efficacy and  
69 low cytotoxicity. Major progress was made in their clinical application with the development of  
70 PEGylated liposomes. Displaying PEG (N-[amino (polyethylene glycol)] on the liposomal surface  
71 increases their circulation time by preventing opsonisation, uptake and elimination by phagocytes  
72 [12]. TMS encapsulated in PEGylated liposomes could thus serve as an attractive delivery system  
73 whilst preserving the stability of TMS and enhancing its bioavailability [13]. The aim of this study  
74 was to assess the potential of TMS-loaded liposomes to restore reduced vasodilator capacity of  
75 isolated aortic vessels exposed to acute tension elevation, *ex vivo*.

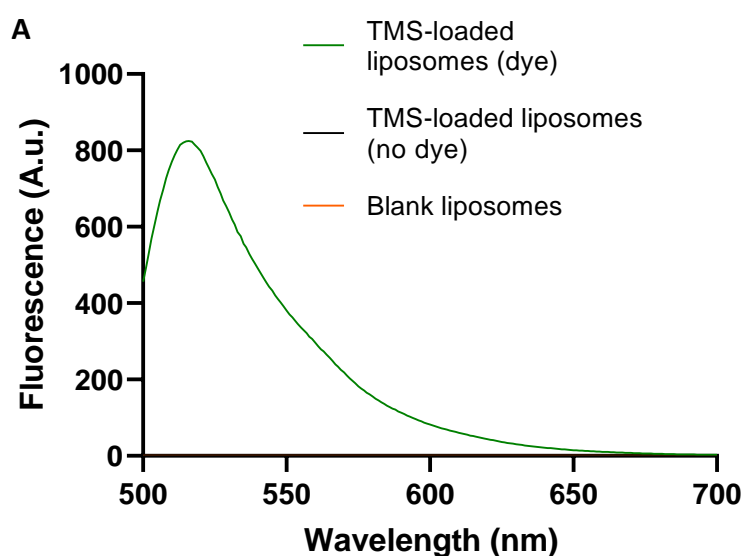
## 76 2. Results

### 77 2.1. Production and characterisation of TMS-loaded liposomes

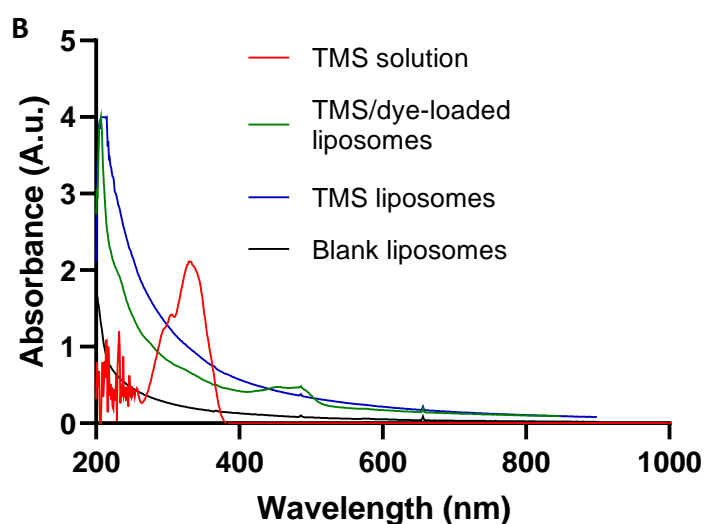
78 Phosphate buffered saline (PBS) containing liposomes (blank liposomes) had a mean diameter  
79 of  $182 \pm 3$  nm, polydispersity index (PDI) of 0.081 and an average zeta potential of  $-13.17 \pm 0.76$  mV.  
80 TMS-loaded liposomes had a mean diameter of  $157 \pm 6$  nm, PDI of 0.097 and an average zeta  
81 potential of  $-13.13 \pm 0.67$  mV, whereas liposomes containing the dye, 5(6)-carboxyfluorescein and  
82 TMS had a mean diameter of  $150 \pm 3$  nm, PDI of 0.070 and an average zeta potential of  $-15.80 \pm 1.31$   
83 mV. All measurements were conducted at pH 7.2-7.4.

84 Structural and functional characterisation of PEGylated liposomes were established using  
85 chemical techniques to confirm dye and drug entrapment within the liposomes. Fluorescence peaks  
86 were detected at 517 nm (corresponding to the dye) from TMS-dye encapsulated, but not blank or  
87 TMS-loaded liposomes (Figure 1A). Independent analysis of TMS solution identified two  
88 absorbance peaks at 302 and 326 nm, however, no peaks corresponding to TMS were  
89 distinguishable in either TMS- or TMS-dye encapsulated liposomes (Figure 1B). TMS has a similar  
90 infra-red profile to its well-characterised parent compound RV, with characteristic benzene valence  
91  $\nu(\text{C}=\text{C})$  vibrations identifiable at 1588 and 1570  $\text{cm}^{-1}$ . The strong, broad band characteristics of trans-

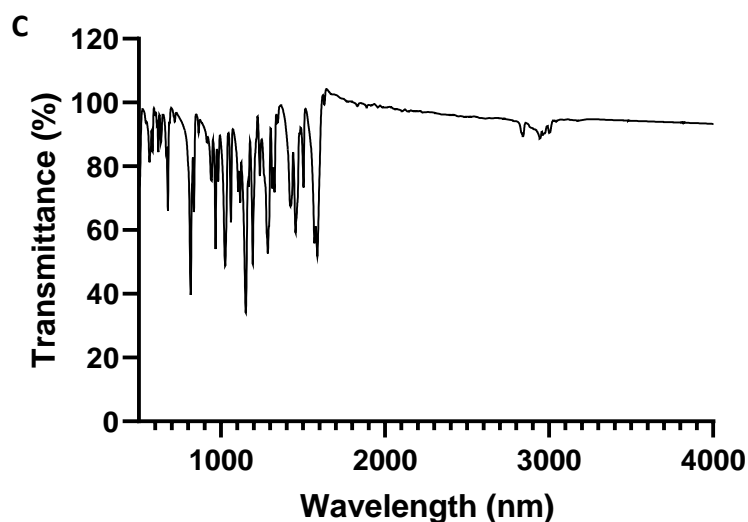
92 RV are noticeably absent between 3550-3200  $\text{cm}^{-1}$ ; however, several bands are identifiable at 2850,  
93 2952 and 3000  $\text{cm}^{-1}$ , corresponding to the valence vibration and stretching of O-CH<sub>3</sub> bonds within  
94 synthetically conjugated methyl groups. Additional CH<sub>3</sub> bending is observed at 1455  $\text{cm}^{-1}$ . Ether  
95 bonds (C-O-C) originating from phenolic groups are identifiable at 1026, 1152, 1195 and 1287  $\text{cm}^{-1}$ .  
96 The presence of TMS could not be identified in the liposomes due to the absence of corresponding  
97 peaks. The individual components of the liposomal structure, 1,2-distearoyl-sn-glycero-3-  
98 phosphocholine and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene  
99 glycol)-2000] ammonium salt were identifiable with peaks at 2950-2850 and 1730  $\text{cm}^{-1}$ ,  
100 corresponding with the presence of alkane (C-H) and carbonyl ester groups (C=O). The broad peak  
101 observed at 3500  $\text{cm}^{-1}$  are a result of OH groups found within fatty acids (cholesterol). Additional  
102 vibrations corresponding with a C-C bond at 1248  $\text{cm}^{-1}$  and a C-O bond from the phenolic group at  
103 1154  $\text{cm}^{-1}$  were identified within the spectrum (Figure 1C, D).



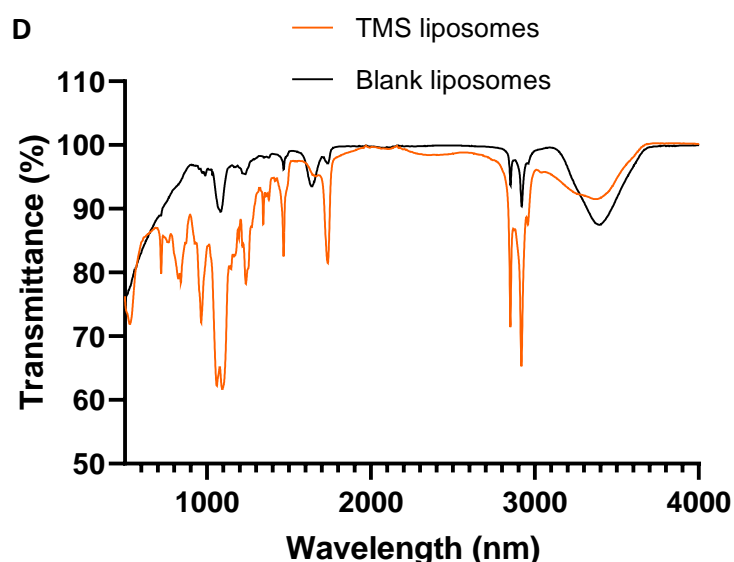
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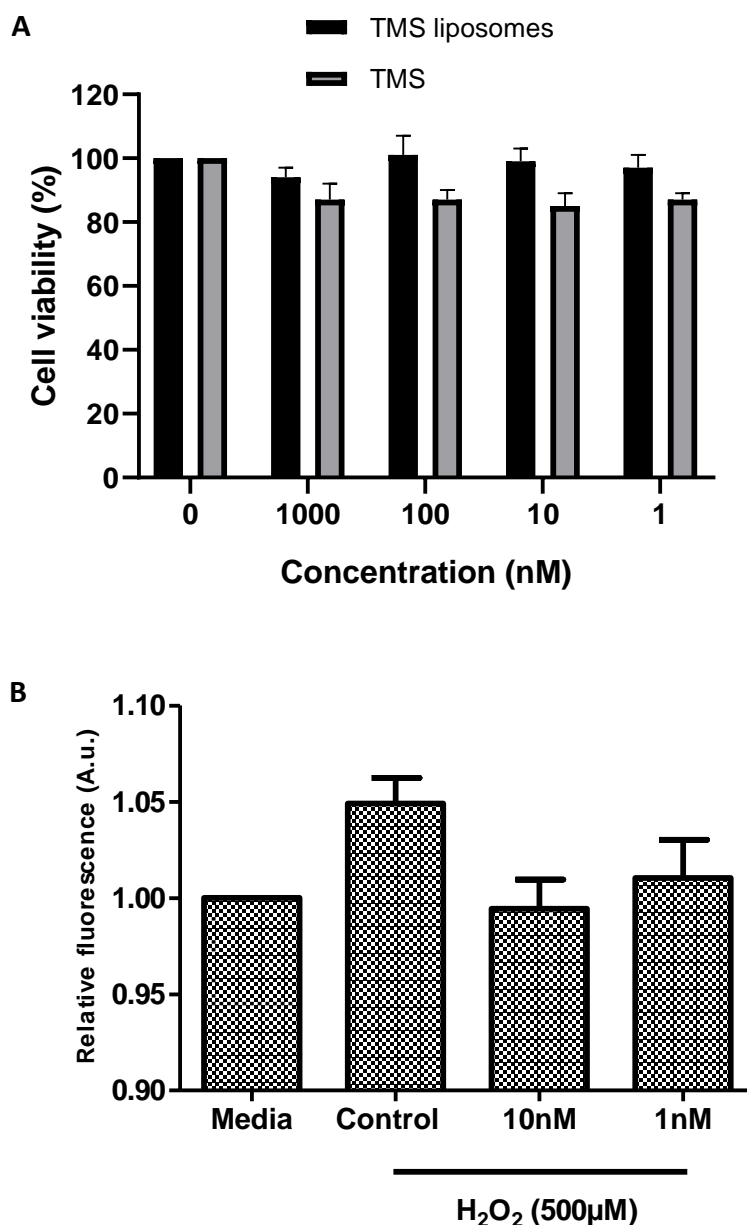


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108 **Figure 1.** Chemical characterisation of tetramethoxystilbene (TMS) and TMS - loaded liposomes.  
 109 Fluorescence - spectroscopy displaying spectra between 500 – 700 nm (A). Ultraviolet - visible  
 110 spectroscopy (UV - Vis) displaying absorbance spectra between 200 – 1000 nm (B). Fourier -  
 111 transform infrared spectroscopy (FTIR) profile of TMS - powder (C) and TMS - loaded liposomes  
 112 (D) displaying transmittance (%) ranging between 500 – 4000 nm.

### 113 2.2. TMS-loaded liposomes maintain cell viability, *in vitro*

114 Human coronary artery endothelial cell (HCAEC) viability did not differ significantly between  
 115 control cells and cells incubated with TMS or TMS-loaded liposomes for 24 hours (Figure 2A). Drug  
 116 encapsulation within liposomes has previously been shown to provide greater therapeutic efficacy  
 117 with low cytotoxicity levels in human retinal endothelial cells [14]. In order to replicate elevated  
 118 superoxide levels observed in the vasculature during oxidative stress, HCAECs were stimulated  
 119 with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) [15]. A reduction in fluorescence intensity, hence mitochondrial  
 120 superoxide production, was observed following incubation in TMS-loaded liposomes (Figure 2B).



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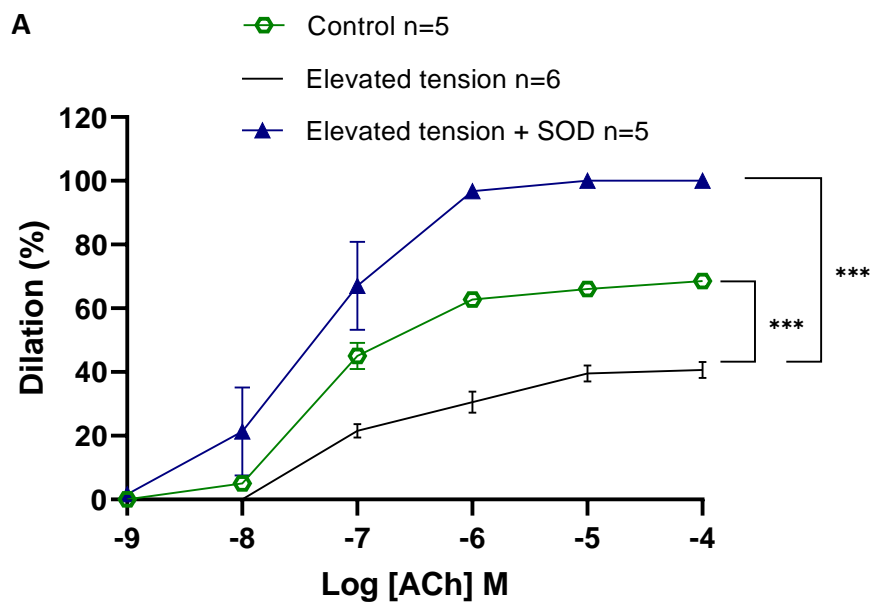
122

123 **Figure 2.** *In vitro* studies. Alamar blue cell viability assay performed in a 96 - well plate following  
 124 administration of cumulative doses of tetramethoxystilbene (TMS) and TMS - loaded liposomes for  
 125 24 hours (A). Mitochondrial superoxide generation in human coronary artery endothelial cells  
 126 measured using MitoSOX Red reagent. Assays performed in a 96 - well plate with cells treated with  
 127 TMS - loaded liposomes for 24 hours, followed by hydrogen peroxide ( $H_2O_2$ ) 500  $\mu M$  exposure.  
 128 Untreated cells received media alone (B). Data are presented as mean  $\pm$  standard error of mean, n =  
 129 3.

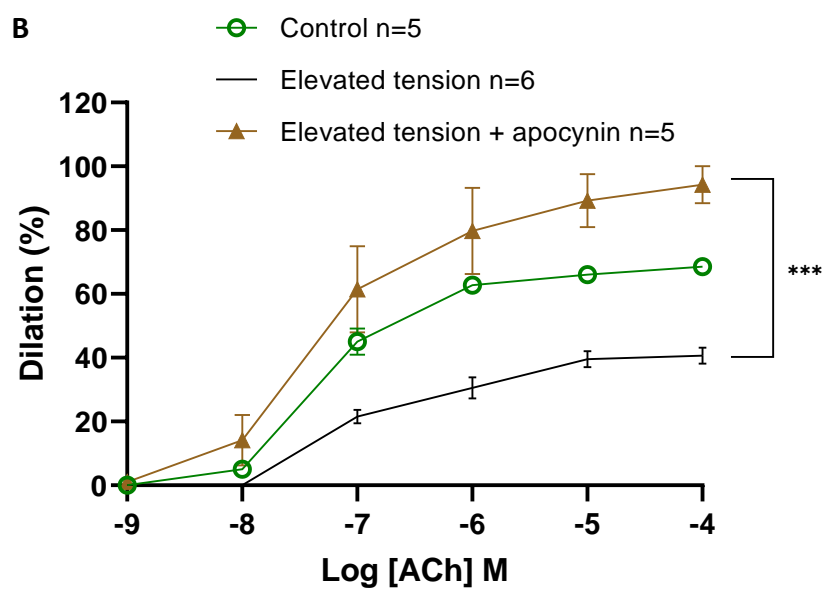
### 130 2.3. TMS-loaded liposomes restore endothelium-dependent dilation via Nitric oxide

131 All vessels constricted to high potassium (KPSS, 60 mM) and phenylephrine (Phe) ( $10^{-5}$  M)  
 132 solution. Acute tension elevation significantly attenuated endothelium-dependent dilation to  
 133 acetylcholine (ACh), which was restored following co-incubation with superoxide dismutase  
 134 (Figure 3A) or the NADPH oxidase inhibitor, apocynin (Figure 3B). This reduction in dilation  
 135 following tension elevation was significantly potentiated after incubation in TMS or TMS-loaded  
 136 liposomes. Incubation with blank liposomes did not alter ACh dilator responses (Figure 3C). There

137 was a significant reduction in dilator responses following inhibition of nitric oxide synthesis by L-  
138 NNA (Figure 3D). Vessels did not dilate to ACh following acute tension elevation and incubation  
139 with TMS or TMS-loaded liposomes after endothelial denudation (Figure 3E). TMS and TMS-  
140 loaded liposomes had no effect on endothelial-independent (SNP) dilation (data not shown).

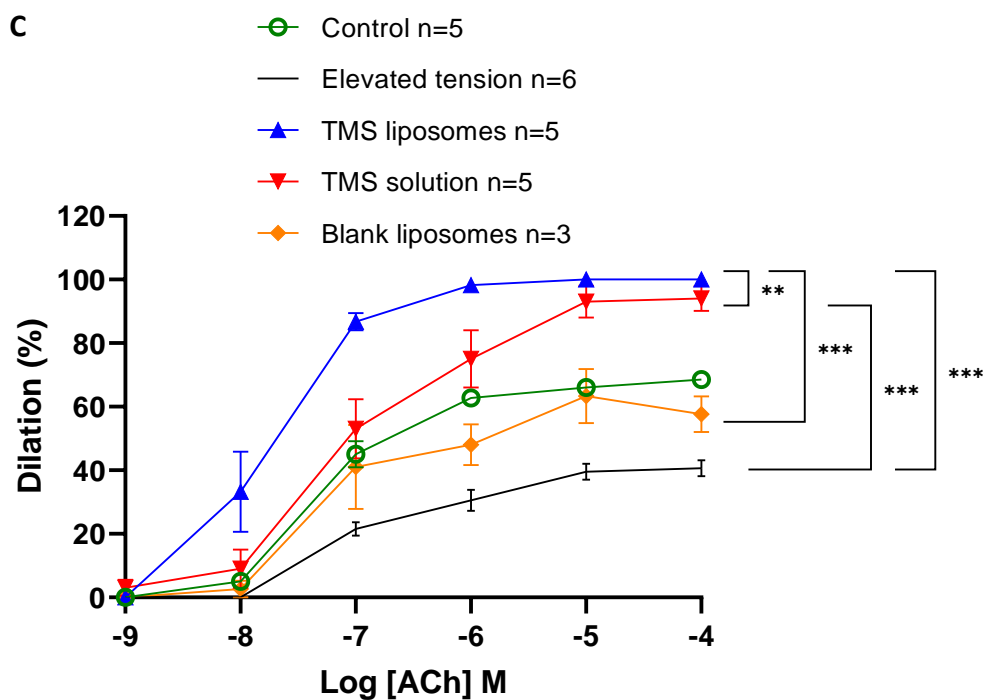


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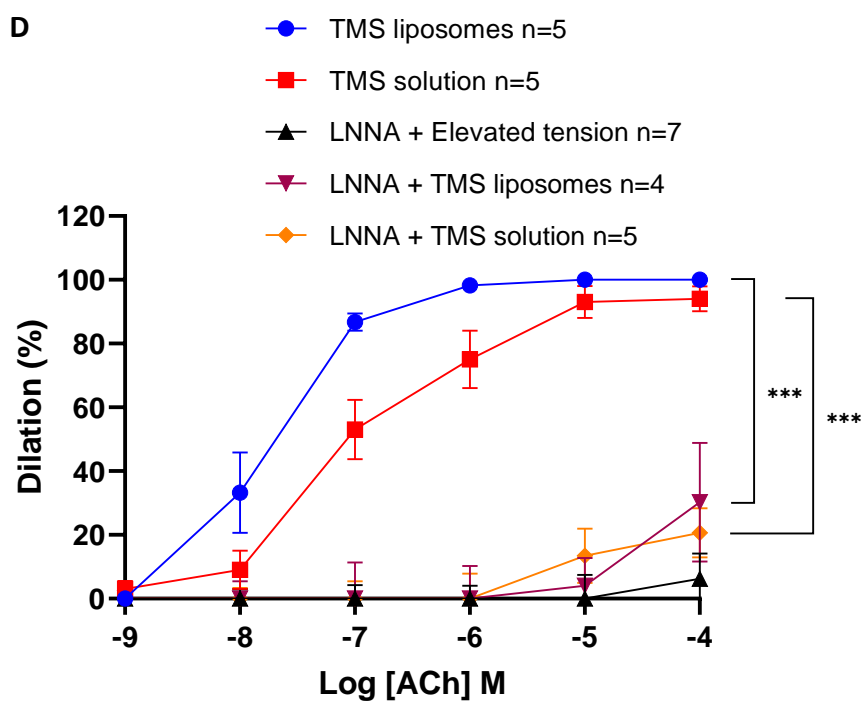


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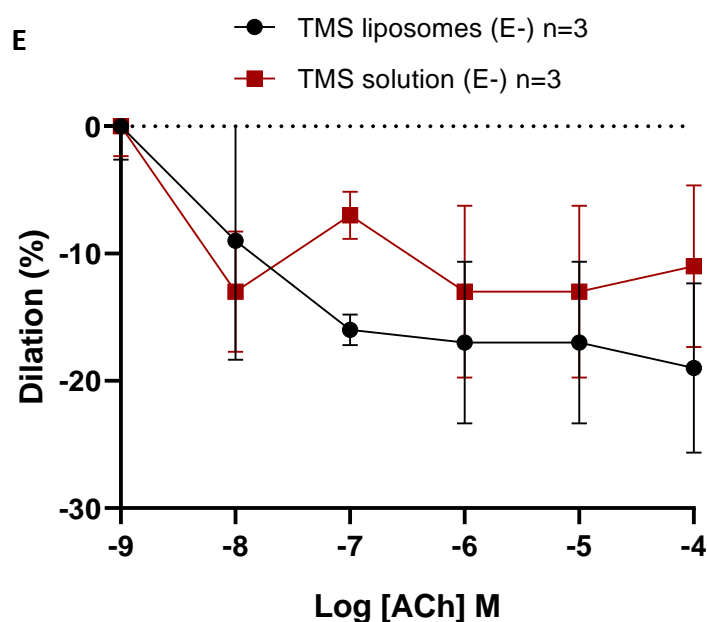
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148 **Figure 3.** Vascular responses. Endothelium - dependent acetylcholine (ACh) responses in control  
 149 vessels (control - 2g tension), following acute tension elevation (4g), acute tension elevation +  
 150 superoxide dismutase (SOD) (A) and tension elevation + apocynin (B) in phenylephrine pre-  
 151 constricted aortic vessels, *ex vivo*. The influence of tetramethoxystilbene (TMS), TMS - loaded  
 152 liposomes and blank liposomes (C), the inhibitor N $\omega$  - nitro - L-arginine (LNNA) (D) and  
 153 endothelial denudation (E) on endothelium - dependent ACh responses following acute tension  
 154 elevation in phenylephrine pre-constricted aortic vessels, *ex vivo*. \*\* = P < 0.01, \*\*\* = P < 0.001. Error  
 155 bars = standard error.

### 156 3. Discussion

157 We demonstrate that TMS, loaded within liposomes, can restore the reduced aortic vasodilator  
 158 response, induced by a high oxidative environment, by potentiating the NO pathway. We produced  
 159 TMS-loaded liposomes for direct uptake into the vasculature. These were characterised and dye  
 160 loading confirmed using a range of chemical techniques. Due to the limited content that can be  
 161 encapsulated within liposomes, a substantial absorbance peak would not be expected and may,  
 162 therefore, be obscured by more prominent absorption peaks, as seen at 207 nm. This intense  
 163 absorption towards the lower detection range has previously been observed in liposome samples  
 164 and has been attributed to the presence of cholesterol. Cholesterol and other well-known oxysterols  
 165 have been shown to absorb light at approximately 195 nm (vacuum-UV region). However, light  
 166 scattering results in the  $\lambda$  max shifting towards a longer wavelength, producing an effect commonly  
 167 referred to as false-energy. This results in a spectral 'redshift', with spectra now appearing in the  
 168 207 nm region [16]. Using FTIR we identified several peaks which corresponded with the parent  
 169 compound RV, confirming its derived nature. FTIR also confirmed the presence of constituent  
 170 compounds used to synthesise the liposomes; however, the spectrographic profile for TMS was not  
 171 distinguishable. Similar observations have been reported previously by several research groups,  
 172 where nanoparticles are capable of obscuring the absorption peaks of encapsulated drugs, showing  
 173 only the predominant parent compounds [17]. In the context of polyphenolic compounds, the  
 174 presence of OH groups are commonplace and would be expected at around 3500 cm<sup>-1</sup>. Despite  
 175 being synthesised from a polyphenolic parent compound, it is crucial to note that in TMS, OH  
 176 groups have been substituted for synthetically conjugated methyl groups, and as a result would not  
 177 expect valance vibration bands at 3500 cm<sup>-1</sup>. This, in addition to the presence of the same broad  
 178 peak on blank liposomes are suggestive that the vibration observed are solely from the lipid

179 components. The distribution and intensity of absorption (% transmission) bands (blank vs. TMS-  
180 loaded liposome) are a direct reflection of the range and quantity of chemical bonds present within  
181 a given sample. In some instances, this may reflect an increase or decrease in band intensity, as a  
182 result of sample modification and altered chemical composition. In this case, however, differences  
183 in intensity observed between samples can be attributed to different dilution ratios of stock  
184 samples.

185 Using isolated HCAECs in culture, we demonstrate that TMS-loaded liposomes can maintain  
186 cell viability. When the cells were exposed to H<sub>2</sub>O<sub>2</sub>, mitochondrial superoxide generation was  
187 reduced following 24 hour incubation with TMS-loaded liposomes. The lack of significance in  
188 reduction suggests that TMS-loaded liposomes may quench additional sources of ROS, including  
189 cytosolic superoxide, due to NADPH oxidase stimulation and NOS uncoupling [15]. Furthermore,  
190 TMS-loaded liposomes may also influence the upregulation of antioxidant enzymes and eNOS  
191 expression in a similar way to resveratrol [18, 19]. To demonstrate the vasodilator potential of the  
192 TMS-loaded liposomes, we used aortic vessels, exposed to elevated tension, to generate an  
193 oxidative environment. The upregulation of ROS sources particularly NADPH oxidase, decreases  
194 SOD activity and causes eNOS uncoupling [2]. Vessel incubation with the NADPH oxidase  
195 inhibitor apocynin significantly potentiated dilator responses following tension elevation. This is  
196 consistent with previous findings that NADPH oxidase is the major ROS generator in the  
197 vasculature [20]. Apocynin blocks the translocation of p47phox to the membrane, hence preventing  
198 the release of NADPH oxidase-derived superoxide [21].

199 Vessel incubation with TMS / TMS-loaded liposomes restored endothelium-dependent dilator  
200 responses following tension elevation even beyond the maximal dilation observed in control  
201 vessels. TMS has been documented to reduce the increase in NADPH oxidase-derived ROS in  
202 aortic, mesenteric and renal arteries of spontaneously hypertensive rats (SHR) primarily via  
203 inhibition of CYP1B1 activity. CYP1B1 has been reported to contribute to ED in SHR, with TMS  
204 reversing this effect as indicated by improvement in vasodilation in response to ACh [7]. It appears  
205 that ED in SHR and DOCA hypertensive models occurs largely as a result of elevated ROS  
206 generation, resulting from Arachidonic Acid (AA) metabolites such as 20-HETE that is produced by  
207 CYP1B1. This subsequently elevates NADPH oxidase activity and ROS levels resulting in the  
208 activation of signalling molecules including ERK1 / 2, p38 MAPK and c-Src [22]. This was prevented  
209 by TMS treatment [8]. The potentiation in dilator responses demonstrated by TMS can be  
210 attributable to increased eNOS phosphorylation, reduced NADPH oxidase-derived ROS and eNOS  
211 uncoupling via CYP1B1 inhibition, direct quenching of ROS and the activation of SIRT1 and / or  
212 AMPK pathway leading to elevated NO bioavailability. Inhibition of NO synthesis using L-NNA  
213 significantly attenuated the restored vasodilation by TMS / TMS-liposomes, suggesting its  
214 mechanism of action is mainly mediated via the NO pathway, which plays a major role in the  
215 maintenance of basal vasomotor tone in conduit vessels under normal but also under hypertensive  
216 conditions [23]. The dilator potential of TMS / TMS-liposomes was lost in denuded vessels, further  
217 suggesting that the potentiated dilation is mainly mediated via endothelial dependent mechanisms.  
218 The potentiation of dilator capacity by TMS-loaded liposomes to a higher extent than TMS solution,  
219 may be due to increased cellular uptake of liposomes, enhanced solubility and sustained release of  
220 encapsulated TMS [12] as compared to the rapid depletion / washout of TMS solution in the  
221 experimental setup. Therefore, we propose that the enhanced dilator effects of TMS-loaded  
222 liposomes is retained due to slow continued release of encapsulated TMS that has already been  
223 taken up by the cells.

#### 224 4. Conclusion

225 In the present study, we exposed isolated aortic vessels to elevated tension, *ex vivo*, to generate  
226 an oxidative environment, in order to examine the effects of TMS-loaded liposomes in restoring  
227 dilator capacity. We demonstrate that TMS-loaded liposomes can restore the attenuated

228 endothelial-dependent dilator responses, by reducing NADPH oxidase-derived ROS and  
229 potentiating NO mediated dilation. TMS-loaded liposomes may thus be a promising therapeutic  
230 strategy to improve vasodilator function in vascular disease.

## 231 5. Materials and Methods

### 232 5.1. Chemicals, Reagents and Materials

233 2, 3', 4, 5'-Tetramethoxystilbene (TMS) and all reagents were purchased from Sigma-Aldrich,  
234 UK. Physiological salt solution (PSS) had the following composition [mM]: 119 NaCl, 4.7 KCl, 1.2  
235 MgSO<sub>4</sub>·7H<sub>2</sub>O, de-ionised (dH<sub>2</sub>O), 25 NaHCO<sub>3</sub>, 1.17 KHPO<sub>4</sub>, 0.03 K<sub>2</sub>EDTA, 5.5 glucose, 1.6 CaCl<sub>2</sub>·H<sub>2</sub>O;  
236 pH 7.4.

### 237 5.2. Liposome Synthesis and Characterisation

238 Liposomes were synthesised using the thin lipid film process as previously described [24, 25].  
239 Briefly, the constituent lipids 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC; 32.5 mM, Avanti  
240 Polar Lipids), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-  
241 2000] ammonium salt [DSPE-PEG(200); 1.875 mM, Avanti Polar Lipids] and cholesterol (15 mM,  
242 Sigma Aldrich) were dissolved in 5 ml of chloroform in a round-bottom flask. Subsequent rotary  
243 evaporation (40°C, 270 mbar) removed excess chloroform and the lipid film was placed in a  
244 vacuum oven overnight (25°C, 0 mbar). The thin film was rehydrated with 1 ml of PBS, vortexed for  
245 10 minutes, heated to 55°C and vortexed for 5 minutes at 1 hour intervals for a minimum of 4 hours.  
246 The suspension containing large multilamellar vesicles was then extruded 11 times using a 1 ml  
247 Mini-Extruder (Avanti Polar Lipids) through a 200 nm polycarbonate membrane to produce  
248 unilamellar liposomes ~200 nm in diameter. To remove impurities, dialysis against PBS (8 x 500 ml;  
249 24 hours) in a Slide-A-Lyzer cassette with a molecular weight cutoff of 3.5 kD (Thermo Fisher  
250 Scientific) was performed. Liposomes were then stored at 4°C until use. The size distribution and  
251 polydispersity index of liposomes were measured by dynamic light scattering (DLS; 25°C;  
252 scattering angle of 173° Zetasizer Nano ZS, Model ZEN3600, Malvern Instruments). For TMS-  
253 loaded liposomes, the lipid film was rehydrated with 1 ml TMS (2 mM in PBS) heated to 55°C and  
254 extruded / dialysed as above, to give a final encapsulated concentration of approximately 1 mM  
255 TMS. For dual-loaded TMS / 5(6)-carboxyfluorescein liposomes a 1ml solution of TMS (2 mM)  
256 containing 5(6)-carboxyfluorescein (5.3 mM) was used to rehydrate the lipid film, to give a final  
257 concentration of 1 mM and 2.65 mM, respectively. UV-Vis spectrophotometer (Jenway 7305, Cole-  
258 Palmer) and fluorescence spectrometer (F-7000, Hitachi) was used to determine absorption and  
259 fluorescence spectra, respectively. The structure of liposomes was assessed using FTIR (Nicolet 380  
260 FT-IR Spectrometer, Thermo Scientific).

### 261 5.3. Cell Culture Studies

262 HCAECs were purchased from PromoCell (Heidelberg, Germany). Cells were grown in  
263 Endothelial Cell Growth Medium (MV2) supplemented with growth medium MV2 supplement  
264 pack, 100 µl / mL penicillin and 100 µl / mL streptomycin. Cells were maintained at 37°C with 4%  
265 CO<sub>2</sub> in a humidified incubator and subcultured 1:3 after reaching 80% confluency. The viability of  
266 HCAECs was determined using the fluorometric Alamar Blue Cell Viability assay. HCAECs were  
267 seeded in 96-well plates and exposed to increasing doses of TMS solution and TMS liposomes (1  
268 nM- 1 µM) for 24 hours. Fluorescence was measured at *ex.* 570 / *em.* 590 nm using a BioTek Synergy  
269 HT microplate reader. The generation of intracellular superoxide was quantified using MitoSOX  
270 Red Mitochondrial Superoxide Indicator (Thermo, UK). Briefly, HCAECs were cultured on 96-well  
271 plates at a density of 1 x 10<sup>4</sup> cells / well for 24 hours. Cells were then incubated with TMS-loaded  
272 liposomes (10 and 1 nM) for 24 hours, washed, then incubated with fresh medium in the presence  
273 of 500 µM H<sub>2</sub>O<sub>2</sub> for 30 minutes. Cells were incubated in the dark at 37°C with MitoSOX for 10

274 minutes. Fluorescence was measured at *ex.* 510 / *em.* 580 nm (BioTek Synergy HT microplate  
275 reader).

#### 276 5.4. Vascular Function Studies

277 Aortic vessels were excised from male Wistar rats (150-250 g) euthanized by stunning and  
278 cervical dislocation, following Institutional guidelines and in accordance with the 'Animals  
279 Scientific procedures act 1986'. The heart was isolated and kept in oxygenated PSS (95% O<sub>2</sub>: 5% CO<sub>2</sub>;  
280 4°C) in a dissection dish. The aortic vessel was gently pinned down at both ends and the underlying  
281 adipose tissue was removed using dissection scissors and forceps, under a binocular dissection  
282 microscope with a fibre optic light source (Olympus SZ61). The vessel was then cut into 3 mm rings  
283 and mounted between two fine steel wires in a calibrated organ-bath system, immersed in  
284 oxygenated PSS at 35°C, then placed under 2g tension using Harvard isometric strain transducer  
285 and tension recorded using Labchart 8 (Power lab, AD Instruments, UK), as previously described  
286 [26]. The viability of blood vessels was examined by ascertaining initial responses to high  
287 potassium solution (KPSS, 60 mM). Vessels that recorded constrictor responses of <1.0 g tension  
288 were not considered viable and were excluded from the study. The influence of TMS / TMS-loaded  
289 liposomes on blood vessel function was determined in response to vasodilator agonists including;  
290 acetylcholine (ACh) (10<sup>-9</sup>M - 10<sup>-4</sup>M), sodium nitroprusside (SNP) (100 nM) and the vasoconstrictor,  
291 Phenylephrine (Phe) (10<sup>-5</sup>M).

292 Endothelial-dependent (ACh) and independent (SNP) responses were examined following  
293 acute tension elevation (4g, 1 hour) in the presence / absence of TMS / TMS-loaded liposomes. In  
294 order to establish the minimal dose of TMS required to achieve maximal dilation, cumulative doses  
295 of TMS (1 pM-10 mM) were added to pre-constricted vessels with Phe. In order to assess the dilator  
296 effect of superoxide dismutase (SOD) and TMS / TMS-loaded liposomes, aortic rings were initially  
297 placed under elevated tension (4 g, 1 hour), followed by a 30 minute incubation with SOD (300 U /  
298 mL) or TMS / TMS-loaded liposomes (1 nM). Constrictor and dilator responses were investigated  
299 and recorded. To evaluate the dilator component involved in the action of TMS / TMS-loaded  
300 liposomes, inhibition studies were performed. LNNA (NO synthesis inhibitor; 100 µM) was used to  
301 evaluate the influence of NO following acute tension elevation with and without incubation with  
302 TMS / TMS-loaded liposomes. To assess the contribution of NADPH oxidase on the dilator function  
303 of TMS / TMS-loaded liposomes, apocynin (0.3 mM, 30 minutes) was used to block NADPH  
304 oxidase activity. Endothelial denudation was used to assess the endothelial dependent dilator  
305 component of TMS / TMS-loaded liposomes. To investigate any effects of the liposomes alone on  
306 vasodilation, aortic vessels were incubated with blank liposomes (drug-free) (1 nM) for 30 minutes  
307 after tension elevation. Constrictor and dilator responses to Phe and ACh was measured,  
308 respectively.

#### 309 5.5. Statistical Analysis

310 All values are presented as mean ± standard error. Data were tested for normal distribution  
311 using Shapiro-Wilk test. Results were analysed using a two-way ANOVA followed by Tukey's  
312 multiple comparisons post-test (IBM SPSS Statistics 25). Statistical significance is P < 0.05. \* = P <  
313 0.05, \*\* = P < 0.01, \*\*\* = P < 0.001. P > 0.05 was considered non-significant.

314

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316 investigation, AZ, CA; data curation, AZ, CA, LR, HD; writing—original draft preparation, AZ, CA, MA;  
317 writing—review and editing, AZ, CA, MA, APL, HD, YA; DW; LKH; supervision, MA, LKH, HD, APL, YA,  
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323

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