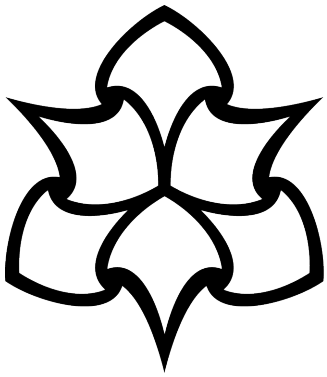

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Communication

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

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

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

1. Introduction

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

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

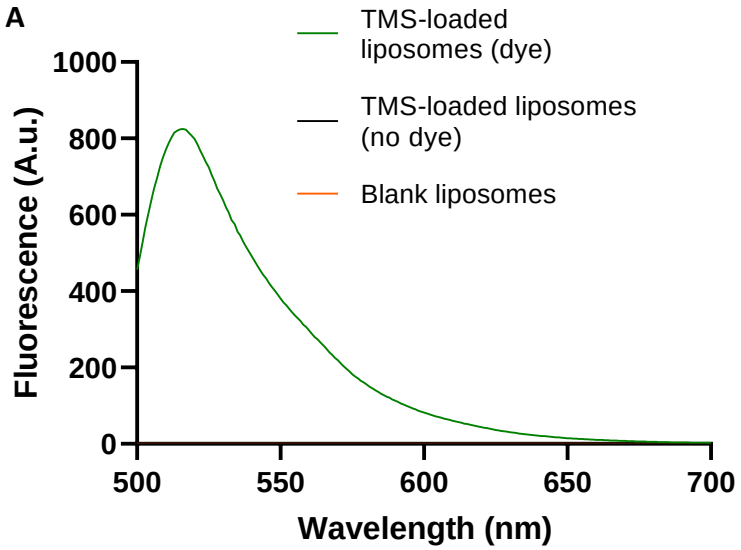
2. Results

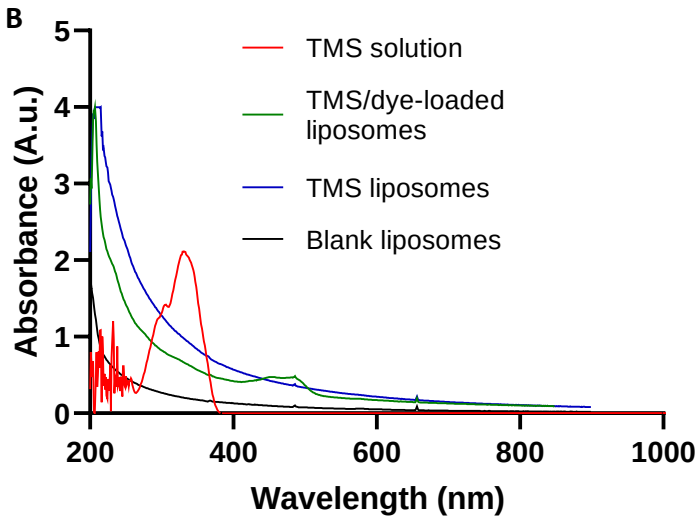
2.1. Production and characterisation of TMS-loaded liposomes

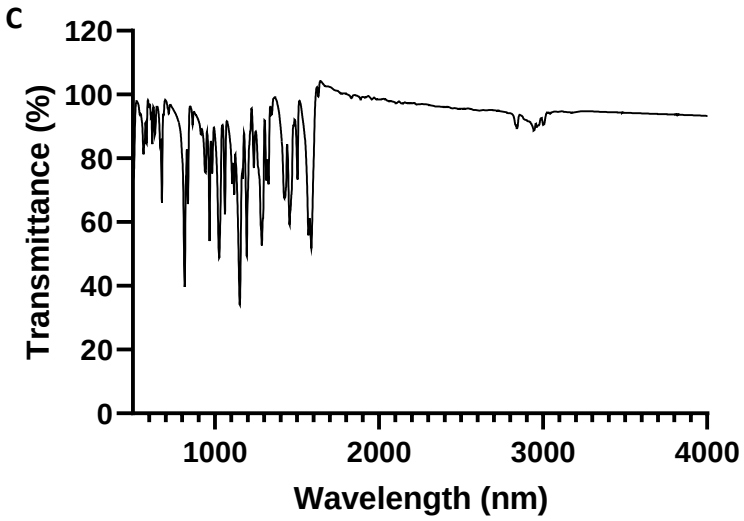
Phosphate buffered saline (PBS) containing liposomes (blank liposomes) had a mean diameter of 182 ± 3 nm, polydispersity index (PDI) of 0.081 and an average zeta potential of -13.17 ± 0.76 mV. TMS-loaded liposomes had a mean diameter of 157 ± 6 nm, PDI of 0.097 and an average zeta potential of -13.13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

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

RV are noticeably absent between 3550-3200 cm^{-1}; however, several bands are identifiable at 2850, 2952 and 3000 cm^{-1}, corresponding to the valence vibration and stretching of O-CH_3 bonds within synthetically conjugated methyl groups. Additional CH_3 bending is observed at 1455 cm^{-1}. Ether bonds (C-O-C) originating from phenolic groups are identifiable at 1026, 1152, 1195 and 1287 cm^{-1}. The presence of TMS could not be identified in the liposomes due to the absence of corresponding peaks. The individual components of the liposomal structure, 1,2-distearoyl-sn-glycero-3-phosphocholine and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] ammonium salt were identifiable with peaks at 2950-2850 and 1730 cm^{-1}, corresponding with the presence of alkane (C-H) and carbonyl ester groups (C=O). The broad peak observed at 3500 cm^{-1} are a result of OH grounds found within fatty acids (cholesterol). Additional vibrations corresponding with a C-C bond at 1248 cm^{-1} and a C-O bond from the phenolic group at 1154 cm^{-1} were identified within the spectrum (Figure 1C, D).







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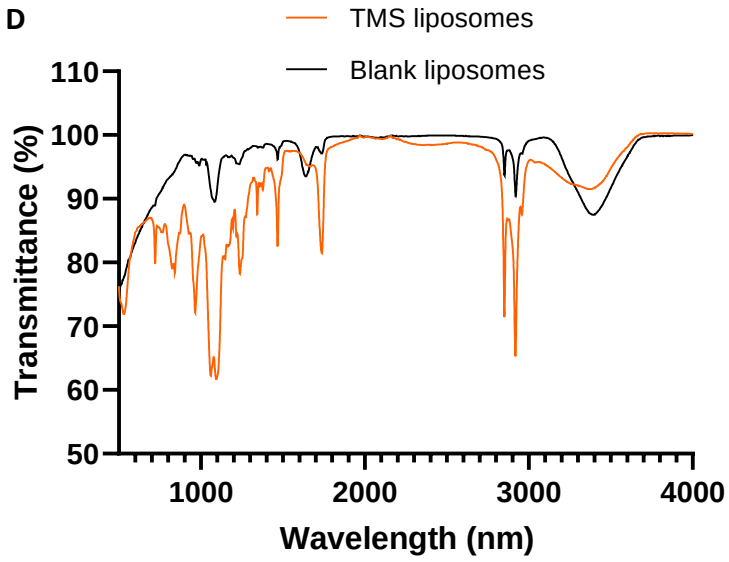
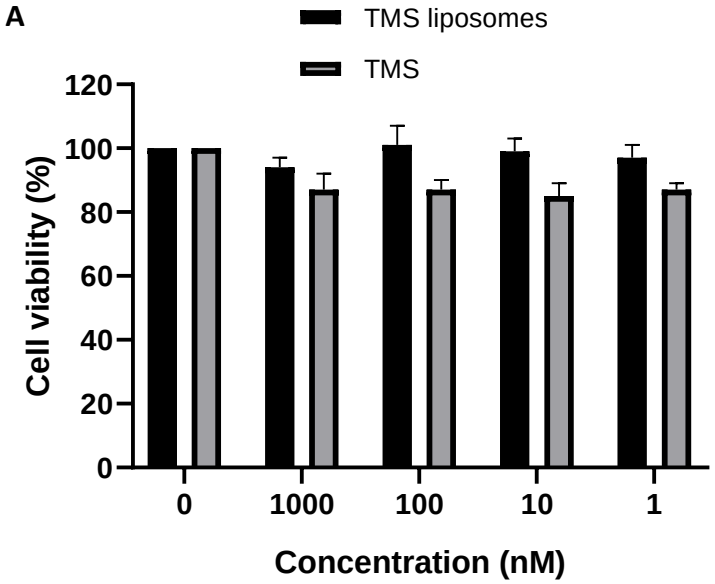


107

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_2O_2) [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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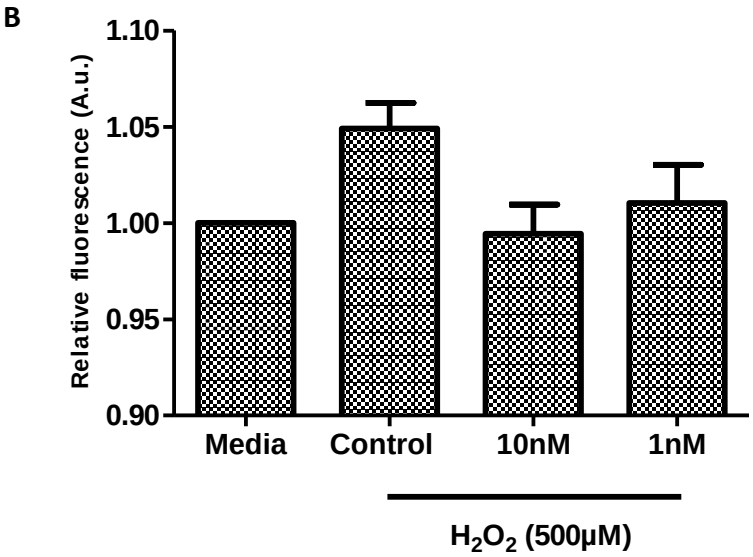

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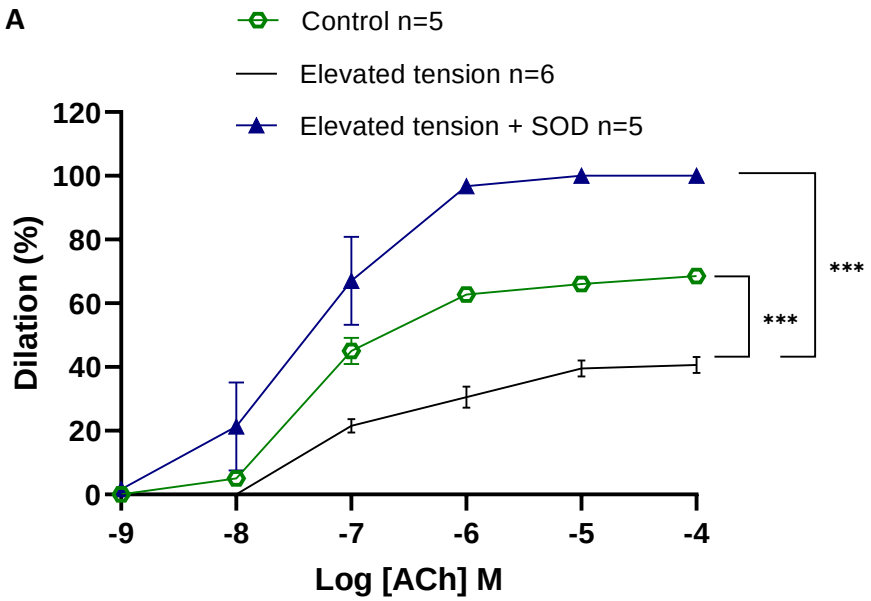
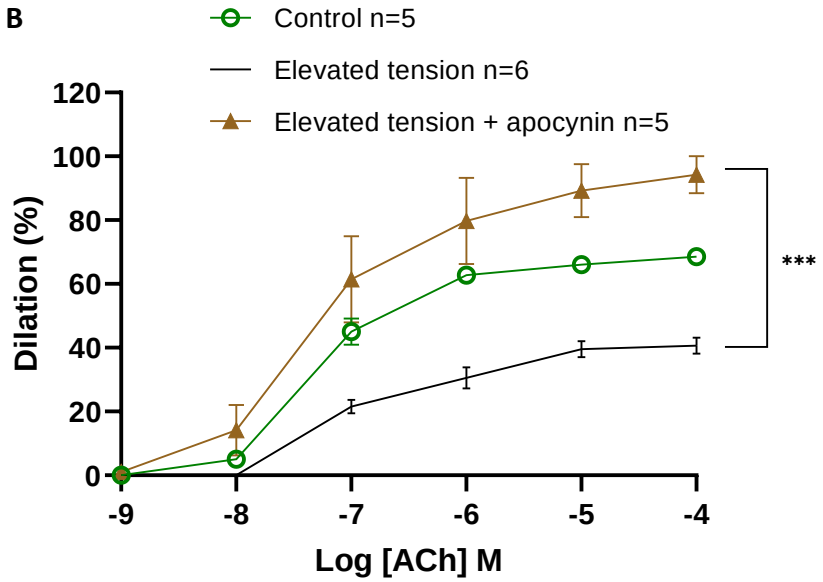
123 **Figure 2**. *In vitro* studies. Alamar blue cell viability assay performed in a 96 - well plate following
124 administration of cumulative doses of tetramethoxystilbene (TMS) and TMS - loaded liposomes for
125 24 hours **(A)**. Mitochondrial superoxide generation in human coronary artery endothelial 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 μM exposure.
128 Untreated cells received media alone **(B)**. Data are presented as mean ± standard error of mean, n =
129 3.

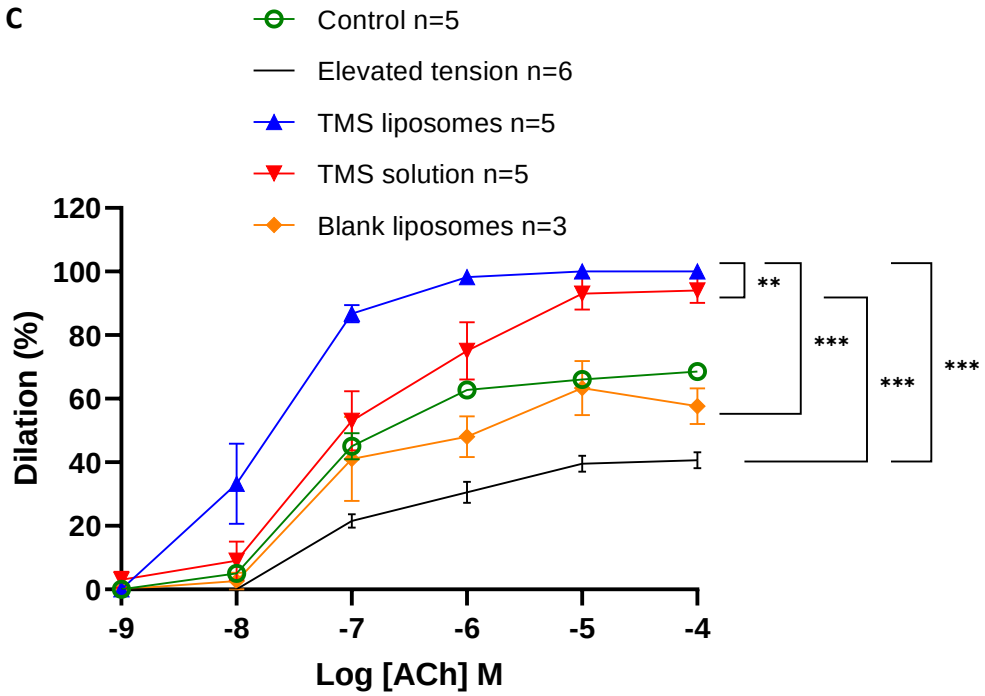
130 *2.3. TMS-loaded liposomes restore endothelium-dependent dilation via Nitric oxide*

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

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






C
Control n=5
Elevated tension n=6
TMS liposomes n=5
TMS solution n=5
Blank liposomes n=3
Dilation (%)
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
Log [ACh] M
**
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***
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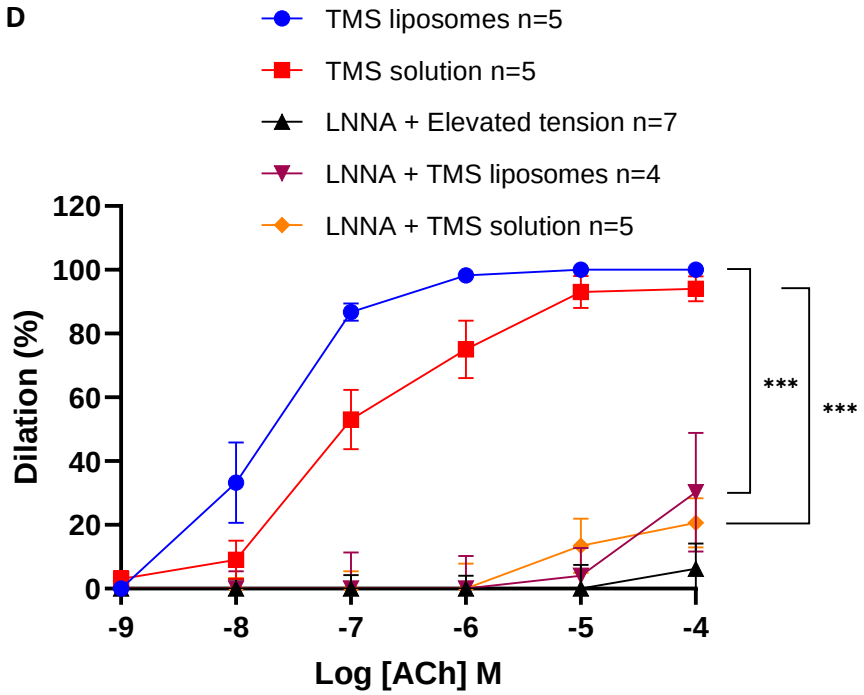
144


D
TMS liposomes n=5
TMS solution n=5
LNNA + Elevated tension n=7
LNNA + TMS liposomes n=4
LNNA + TMS solution n=5
Dilation (%)
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
Log [ACh] M
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145

146

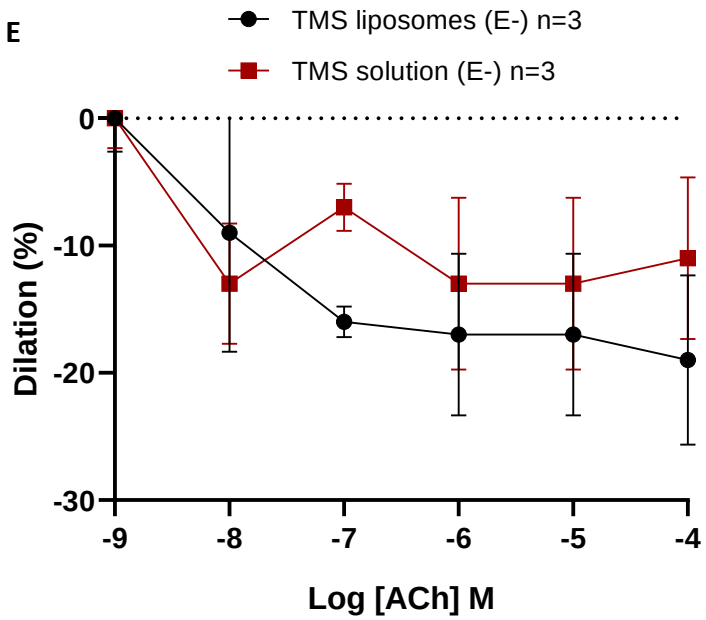

Figure 3. Vascular responses. Endothelium - dependent acetylcholine (ACh) responses in control vessels (control - 2g tension), following acute tension elevation (4g), acute tension elevation + superoxide dismutase (SOD) **(A)** and tension elevation + apocynin **(B)** in phenylephrine pre-constricted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

3. Discussion

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

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

Using isolated HCAECs in culture, we demonstrate that TMS-loaded liposomes can maintain cell viability. When the cells were exposed to H_2O_2, mitochondrial superoxide generation was reduced following 24 hour incubation with TMS-loaded liposomes. The lack of significance in reduction suggests that TMS-loaded liposomes may quench additional sources of ROS, including cytosolic superoxide, due to NADPH oxidase stimulation and NOS uncoupling [15]. Furthermore, TMS-loaded liposomes may also influence the upregulation of antioxidant enzymes and eNOS expression in a similar way to resveratrol [18, 19]. To demonstrate the vasodilator potential of the TMS-loaded liposomes, we used aortic vessels, exposed to elevated tension, to generate an oxidative environment. The upregulation of ROS sources particularly NADPH oxidase, decreases SOD activity and causes eNOS uncoupling [2]. Vessel incubation with the NADPH oxidase inhibitor apocynin significantly potentiated dilator responses following tension elevation. This is consistent with previous findings that NADPH oxidase is the major ROS generator in the vasculature [20]. Apocynin blocks the translocation of p47phox to the membrane, hence preventing the release of NADPH oxidase-derived superoxide [21].

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

4. Conclusion

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

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

5. Materials and Methods

5.1. Chemicals, Reagents and Materials

2, 3′, 4, 5′-Tetramethoxystilbene (TMS) and all reagents were purchased from Sigma-Aldrich, UK. Physiological salt solution (PSS) had the following composition [mM]: 119 NaCl, 4.7 KCl, 1.2 $MgSO_4 7H_2O$, de-ionised (dH_2O), 25 $NAHCO_3$, 1.17 $KHPO_4$, 0.03 K_2EDTA, 5.5 glucose, 1.6 $CaCl_2H_2O$; *pH* 7.4.

5.2. Liposome Synthesis and Characterisation

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

5.3. Cell Culture Studies

HCAECs were purchased from PromoCell (Heidelberg, Germany). Cells were grown in Endothelial Cell Growth Medium (MV2) supplemented with growth medium MV2 supplement pack, 100 µl / mL penicillin and 100 µl / mL streptomycin. Cells were maintained at 37°C with 4% CO_2 in a humidified incubator and subcultured 1:3 after reaching 80% confluency. The viability of HCAECs was determined using the fluorometric Alamar Blue Cell Viability assay. HCAECs were seeded in 96-well plates and exposed to increasing doses of TMS solution and TMS liposomes (1 nM- 1 µM) for 24 hours. Fluorescence was measured at *ex*. 570 / *em*. 590 nm using a BioTek Synergy HT microplate reader. The generation of intracellular superoxide was quantified using MitoSOX Red Mitochondrial Superoxide Indicator (Thermo, UK). Briefly, HCAECs were cultured on 96-well plates at a density of 1 x 10^4 cells / well for 24 hours. Cells were then incubated with TMS-loaded liposomes (10 and 1 nM) for 24 hours, washed, then incubated with fresh medium in the presence of 500 µM H_2O_2 for 30 minutes. Cells were incubated in the dark at 37°C with MitoSOX for 10

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

5.4. Vascular Function Studies

Aortic vessels were excised from male Wistar rats (150-250 g) euthanized by stunning and cervical dislocation, following Institutional guidelines and in accordance with the 'Animals Scientific procedures act 1986'. The heart was isolated and kept in oxygenated PSS (95% O_2: 5% CO_2; 4˚C) in a dissection dish. The aortic vessel was gently pinned down at both ends and the underlying adipose tissue was removed using dissection scissors and forceps, under a binocular dissection microscope with a fibre optic light source (Olympus SZ61). The vessel was then cut into 3 mm rings and mounted between two fine steel wires in a calibrated organ-bath system, immersed in oxygenated PSS at 35˚C, then placed under 2g tension using Harvard isometric strain transducer and tension recorded using Labchart 8 (Power lab, AD Instruments, UK), as previously described [26]. The viability of blood vessels was examined by ascertaining initial responses to high potassium solution (KPSS, 60 mM). Vessels that recorded constrictor responses of <1.0 g tension were not considered viable and were excluded from the study. The influence of TMS / TMS-loaded liposomes on blood vessel function was determined in response to vasodilator agonists including; acetylcholine (ACh) (10^{-9} M - 10^{-4} M), sodium nitroprusside (SNP) (100 nM) and the vasoconstrictor, Phenylephrine (Phe) (10^{-5} M).

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

5.5. Statistical Analysis

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

Author Contributions: Conceptualization, MA, LKH; methodology, AZ, CA, LR, FB; formal analysis, AZ, CA; investigation, AZ, CA; data curation, AZ, CA, LR, HD; writing—original draft preparation, AZ, CA, MA; writing—review and editing, AZ, CA, MA, APL, HD, YA; DW; LKH; supervision, MA, LKH, HD, APL, YA, DW; funding acquisition, MA, LKH, YA.

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