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Design, Synthesis and Characterization of 1,2-Phenylenediamine Functionalized Poly (Maleic Anhydride-*alt*-Vinyl Acetate) as a Potential New Bioactive Formulation

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ABSTRACT

This study includes the design, synthesis and characterization of 1,2-phenylenediamine (*o*-PDA) functionalized maleic anhydride (MA)-vinyl acetate (VA) copolymer-based conjugate to develop a new formulation. The phenylenediamine molecule is a fluorescent dye that allows designing new chemotherapeutic polymeric molecules. Poly(maleic anhydride-*alt*-vinyl acetate) [Poly(MA-*alt*-VA)] was obtained via charge transfer complex (CTC) radical polymerization presence of methyl ethyl ketone (MEK), utilizing benzoyl peroxide (BPO) free-radical initiator at 80 °C, as a potential functional polymeric carrier. Structural characterization of the surface functionalized poly(MA-*alt*-VA)/1,2-PDA conjugate was performed by Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) and Nuclear Magnetic Resonance (^{1}H-NMR). Spectroscopic methods and water solubility results confirmed that the conjugation took place successfully after the ring opening reaction by the amide mechanism.

Keywords:
poly(MA-*alt*-VA); Surface modification; 1,2-phenylenediamine; ATR-FTIR; ^{1}H-NMR.

INTRODUCTION

Polymers, whether natural or artificial, is a material made up of large molecules obtained by linking together smaller repeating chemical units. Polymer-based materials have become interesting in different research areas, such as: automobile and construction industries, electronics, food packaging, aviation and aerospace design [1,2]. In addition polymers are commonly preferred for biomedical purposes, especially in health science, of disease diagnosis and treatment [3-5]. Polymer based substances to be used as carriers for the pharmaceutically active ingredients, for example drugs, for the controlled delivery to produce functional useful biomaterials. Covalent polymer-drug conjugates called polymer based synthetic drugs or pharmaceutically active compounds were first researched by Helmut Ringsdorf in 1975. The model known by his surname is based on a strong interaction, such as a covalent bond between the drug and a polymer backbone [6,7].

Synthetic polymers are produced as targeted functional biomaterials using different reaction types. The water solubility of polymers makes them attractive for most applications, such as: rational polymer based drug fabrication and controlled drug delivery systems. Maleic anhydride (MA) copolymers, used for drug carrier tools because of its unique functionalization property, can be produced by the free radical chain polymerization method under mild conditions. MA-containing macromolecules often form biocompatible (non-toxic, non-immunogenic, and biodegradable) copolymers and display many biological activities [8].

MA, $C_4H_2O_3$, is a well-known electron-acceptor monomer that can be chemically undergo structural modification by its highly reactive functional anhydride moiety with nucleophiles such as amine (RNH2) or hydroxyl groups (ROH) derivatives [9,10] by the ring opening reaction. The unique functional property of MA unit allows manufacturing polymeric structures with desired functionalities through various reactions especially for the designing of biocompatible drug carrier.

This study primarily focuses on the synthesis of MA-containing alternating copolymers owing to the

electron-accepting monomer and electron-acceptor monomer properties of MA and VA, respectively and then the 1,2-Phenylenediamine functionalized conjugate form of the obtained copolymer. Phenylenediamine (PDA) molecule is an aniline derivative (Molar Mass: 108.14 g/mol Chemical Formula: 1,2-$(NH_2)_2C_6H_4$, Solubility 54 g/l) that various heterocyclic compounds can be designed [11]. For example, PDA can be utilized in the synthesis of various benzimidazole derivatives that are main structural units of several biologically active drug substances and also pigments [12].

Maleic anhydride-vinyl acetate alternating copolymer (Scheme 1), poly(MA-*alt*-VA), was synthetically conjugated, through its functional anhydride moiety by the combining of fluorescent molecule 1,2-phenylenediamine (Scheme 2) to design of novel biologically active compound.

The conjugated product was symbolically named pMAVA/1,2-PDA for copolymer/nucleophilic reagent pair representation. Copolymer and conjugate structure characterized by ATR-FTIR and ^{1}H-NMR measurements. In addition water solubility test was also implemented. Spectroscopic enlightening results and water solubility confirmed that the poly(MA-*alt*-VA)/1,2-PDA fluorescent based conjugation took place successfully after the nucleophilic ring opening reaction according to amide mechanism. A linear polymer characterized by a repetition of amide groups along the backbone chain is called a polyamide.

Considering the chemical structure and water solubility of the novel designed poly(MA-*alt*-VA)/1,2-PDA fluorescent based conjugate, it is necessary to perform its pharmacological tests on living cells such as bacteria, tumor, and virus etc.

MATERIALS AND METHODS

Chemicals and Instrument

Chemicals

Maleic anhydride (MA, further purification was done by recrystallization from dry benzene before copolymerization), methyl ethyl ketone (MEK), and benzoyl peroxide (BPO) were supplied from Merck (Schuchardt, Germany). Ethanol (95 %) was provided by Carlo-Erba (Rodano, Italy). Vinyl acetate (VA, 86.09 g.mol^{-1}), petroleum ether, and ethyl acetate were provided from Sigma-Aldrich (St. Louis, USA). The fluorescent molecule 1,2-phenylenediamine was supplied from Sigma. The reagents and chemicals used were of analytical quality.

Instrument

The FTIR spectrum of poly(MA-*alt*-VA) copolymer and poly(MA-*alt*-VA)/1,2-PDA conjugate were recorded on a FTIR spectrophotometer (Bruker Mode: Tensor II) at 400–4000 cm^{-1}. Nuclear magnetic resonance, ^{1}H-NMR, measurements were recorded at 400 MHz (JEOL, JNM-ECZ400S/L1). Analyzes were performed after 6 mg of each of the samples were taken and prepared in 0.8 mL of DMSO-d6.

Synthesis of p[MA-*alt*-VA] Copolymer

Maleic anhydride-Vinyl acetate alternating copolymer was synthesized using the *in situ* charge transfer complex (CTC) radical polymerization method (Table 1). Briefly, poly(MA-*alt*-VA) was produced by free-radical polymerization of the monomers (monomer feed at 1:1 mole ratios for MA:VA pair) in MEK following the free radical initiation (generated by BPO) for 24h at 80 °C. Removal of residues or undesirable chemicals such as vinyl acetate, BPO, and pol(vinyl acetate) from an extract was implemented by reacting the residue with ethyl acetate for overnight. Copolymer precipitation was carried out with excess of cold petroleum ether. Then the liquid phase removed from the precipitate with vacuum filtration system, and incubated in drying oven under vacuum at 50 °C for 24 hours for purification [13].

Synthesis of poly(MA-*alt*-VA)/1,2-PDA Conjugate

Copolymer/fluorescent based conjugate, poly(MA-*alt*-VA)/1,2-PDA was obtained by the modification ~1:1molar ratio of the poly(MA-*alt*-VA) with 1,2-phenylenediamine (potential to be modified to fluorescence molecule), in anhydrous *N,N*-Dimethylformamide (DMF), with basic amide transformation reaction catalytic activator triethylamine (TEA or Et_3N) for 6h at 40 °C under a reflux system (Table 1) [14]. Poly(MA-*alt*-VA)/1,2-PDA conjugate, was treated with ethanol for precipitation, at -20 °C for 30 minutes and incubated under vacuum for further 24h at 50 °C until the used solvents removed to obtain powder form [14,15]. Poly(MA-*alt*-VA) powder (0.5 mmol, 92 mg) was mixed with DMF (2.5 mL) 15 min. at 35°C, then catalytic activator, TEA (20 µL), was slowly dripped to homogeneous DMF solution of poly(MA-*alt*-VA) at constant speed with continuous mixing. Fluorescent molecule, PDA, (0.5 mmol, 54.07 mg) was prepared in DMF (2 mL) and finally added drop by drop to the poly(MA-*alt*-VA)-DMF mixture at cold condition (~20 °C) [14]. Obtained final solution was shaken continuously for ~30 minutes incubation at 25 °C, following for further ~5.5 h at 40 °C by shaking

until viscous mixture obtained. Final homogeneous mixture cooled to room temperature within 1h. Then it was treated with cold ethyl alcohol, stored at -20 °C for 30 min. to gain an orange colored powder form. Finally, liquid phase immediately separated, product was precipitated with high-speed (6000 rpm) centrifugation for 10 min. and solid particles were powdered, purified under vacuum with incubation at 50 °C for 24h [14,16].

Table 1. Reaction components for the pMAVA alternating copolymer and the pMAVA/1,2-PDA conjugate.

Sample	Mole proportions	Initiator-catalyst	Solvents	Time (h)	Temperature (°C)
pMAVA	MA:VA (1:1)	BPO	MEK	24	80
pMAVA/ 1,2-PDA	MAVA:1,2-PDA (1:1)	TEA	DMF	6	40

*BPO: benzoyl peroxide; MEK: methyl ethyl ketone

Water solubility test

Water-soluble polymers have been widely used in the clinics for the surface modification of biomaterials, and as carriers of drugs. This study focuses on water-soluble copolymer and its highly water-soluble 1,2-PDA conjugate in powdered formulation was incubated in HPLC-grade water (1 g/mL) at 25 °C for 1 h [17]. Solubility in water was tested after 1 h by mechanical mixing and centrifugation of the solution at 6000 rpm.

Proposed reaction mechanisms for copolymer and conjugate

The reaction mechanisms are proposed according to the spectroscopic measurement and water solubility test for the structural characterization of the designed alternating copolymer and florescent based-conjugate.

RESULTS AND DISCUSSION

Copolymer and modification product were structurally characterized by ATR-FTIR and ^{1}H-NMR spectroscopic methods.

FTIR Analysis

In our previous study, a detailed IR spectrum for poly(MA-*alt*-VA) was given [14]. Briefly, functional polymeric carrier (Fig. 1a) had the anhydride moiety at 1857, and 1781 cm^{-1}, related to symmetric and asymmetric carbonyl (-C=O) characteristic stretching peaks of MA-anhydride, respectively [18,19]. Characteristic modes appeared at 1025 and 933 cm^{-1} defined as the stretching modes of C–O–C on MA moiety [20]. The stretching vibrations of $-CH_3$ and $-CH_2$ groups on VA unit were recorded at 1373 and 1432 cm^{-1}, respectively [21,22]. Distinctive $-COCH_3$ stretching modes of VA-fragment detected at 1094 cm^{-1} [18]. Peak detected around at 1216 cm^{-1} was attributed to the main ester groups (CO–O–C) on VA units [23]. These spectroscopic data confirmed the existence of the pMAVA copolymer structure [18–22].

Characteristic peaks of the 1,2-PDA detected clearly in the pMAVA/1,2-PDA spectrum. The spectra show vibration bands at 3384-3363 cm^{-1} related to the free NH_2 stretching (Fig. 1b). The ring mode positions (at 1590 and 1498 cm^{-1}) were found to be identical to 1,2-disubstituted benzene ring. Ring bending vibration around ~690 cm^{-1} was not detected in the spectra, as characteristic of ortho-substituted rings, only C-H wagging appears at 743 cm^{-1} that it was accepted as diagnostic peak for 1,2-disubstituted rings that in general this vibration falls between the range of 770 and 735 cm^{-1} (Fig. 1b) . Characteristic aromatic, C=C, vibrations were also recorded at 1590 cm^{-1} [24]. Characteristic absorption band appears around 3300-3400 cm^{-1} defined as the -N-H stretching of 1,2-PDA molecule. Vibrations in the 650-900 cm^{-1} region are indicative for substituent position in the aromatic ring. The vibration mode appeared at 804 cm^{-1} could be attributed to the 1,2-disubstituted ring [25-27]. In addition, –NH stretching vibration of $-NH_2$ around at 3000–3700 cm^{-1} was also detected.

Characteristic vibrations of the anhydride ring for poly(MA-*alt*-VA) were disappeared completely in poly(MA-*alt*-VA)/1,2-PDA spectrum (Fig. 1c). This result revealed that furan rings were opened by ring-opening reaction following the amine nucleophilic attack of 1,2-PDA [28]. The product resulting in the ring opening reaction is usually a carboxyl functionality with an amide or ester moiety [28] (Fig. 1c). Moreover, vibrations of carbonyl belong to MA-anhydride moiety (C–O–C: at 1857 cm^{-1} and C=O at 1781 cm^{-1}) were disappeared and shifted to 1641 cm^{-1} (CO–N–H) and 1520 cm^{-1} following the amide and acid binary new formation [14]. These findings clearly demonstrated that a new structure formed as an amide linkages, mediated by bending a –NH and stretching a –CN (C–N–H) fragments according to –CONHR amide functionality with mono-substituted position. (Fig. 1b). The remaining frequencies assignment of are as follows: a C–C bending frequency at 602 cm^{-1}, a -C–O stretch was detected at 1223 cm^{-1}, typical vibration of

C–O–C belongs to VA fragment, were noticed at 1024 and 933 cm^{-1} [20], aromatic C–H and C=C absorption bands on 1,2-PDA, at 751 and 801 cm^{-1} [29]. A bending =C–H (out-of plane) vibration was appeared at 751 cm^{-1} belongs to aromatic ring [30–32]. In addition, characteristic carboxylic acids group demonstrated a strong and wide vibration band ascribed to O–H stretching. Characteristic O–H stretch belongs to carboxylic acid recorded as a broad vibration band at 3300-2500 cm^{-1} region, centered approximately at 2916 cm^{-1} and also 1730 cm^{-1} ascribes to typical carbonyl (C=O) stretch of carboxylic acid functions. On the other hand be-

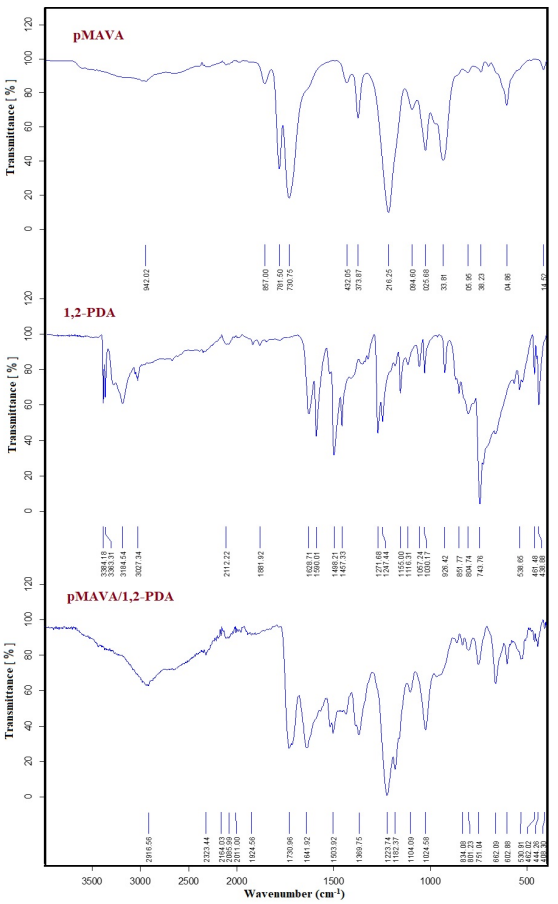


Figure 1. FTIR spectrum of pMAVA copolymer, 1,2-PDA molecule and pMAVA-1,2-PDA conjugate.

cause 1,2-PDA molecule having two primary amino (-NH_2) group and one of them conjugated with copolymer backbone as turned to amide group, the other one is remain as free amine group appeared at 1641 cm^{-1}. According to FTIR results it can be concluded that pMAVA polymeric material chemically modified by 1,2-PDA via amine-amide transformation reactions.

^{1}H-NMR Analysis

In the lights of related previous study [33] ^{1}H-NMR results also confirmed the functional group identification obtained by FTIR spectroscopy for pMAVA carrier. Typical absorption bands detected for pMAVA in ^{1}H-NMR spectra (Fig. 2) can be summarized as follows: two protons belongs to MA fragment at 3–4 ppm (as 3.96 and 3.97 ppm), $-CH_2$ protons of VA moiety at ~2.61 ppm, the multiplet signal of –CH, (attached to an oxygen) at ~5.07–5.61 ppm [34,35], and typical methyl protons, close to C=O function, at 1.85–1.97 ppm [18,36,37].

The amide and carboxyl group formed as a result of conjugation for novel pMAVA/1,2-PDA product was corroborated by ^{1}H-NMR (Fig. 3). Anhydride ring signals of pMAVA, at 3 ppm, shifted sharply in pMAVA/1,2-PDA spectrum to 2.45, 2.7, 2.8 and 3.0, 3.25 ppm, defined as anhydride –CH protons [38] (Fig. 3). CH_2 signals were detected at 1.85 and ~2.8 ppm ascribes to VA moiety [39,40], and $-CH_3$ protons

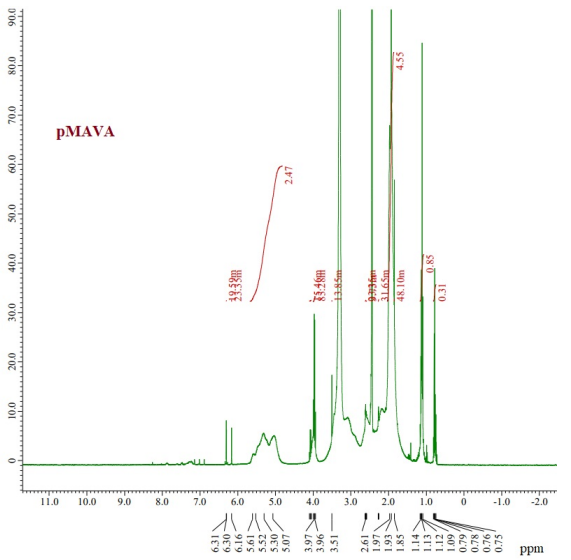


Figure 2. ^{1}H-NMR spectrum of pMAVA copolymer.

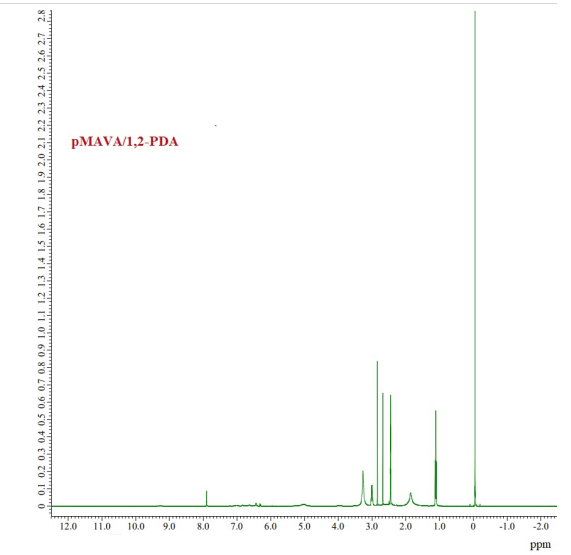


Figure 3. ^{1}H-NMR spectrum of pMAVA-1,2-PDA conjugate.

were appeared at ~ 1.1 ppm attributed to the VA [41]. The characteristic protons associated with the aromatic rings of 1,2-PDA groups were observed to form a broad peak at about at 6.3 and 6.4 ppm (Fig. 4) [40,42,43]. Amide functional protons, arising from –NH (–CONH–) fragment, were appeared at 7.9 ppm, proved that 1,2-PDA florescent dye molecule was covalently attached to pMAVA copolymer chain by an amide functionalization [44].

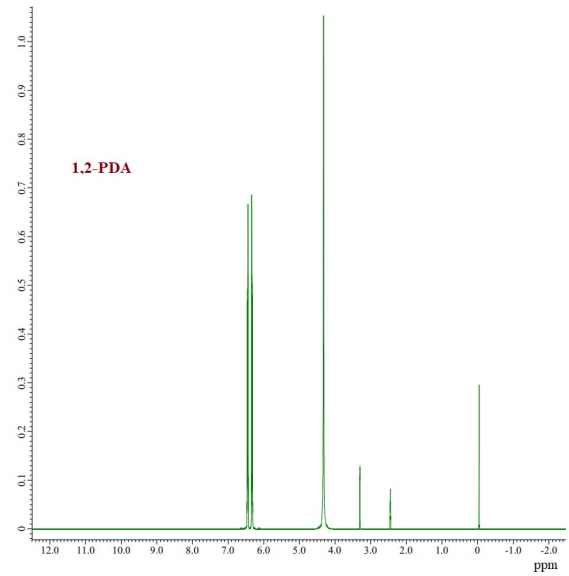
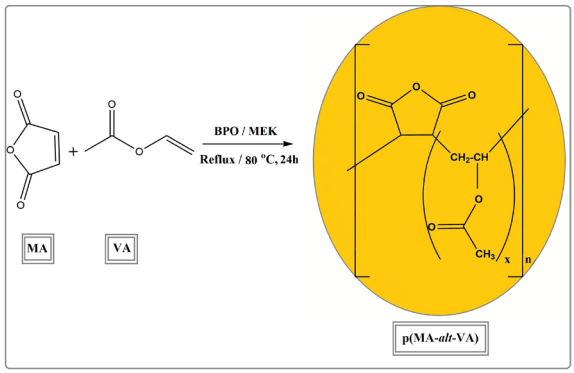


Figure 4. ^{1}H-NMR spectrum of 1,2-PDA.

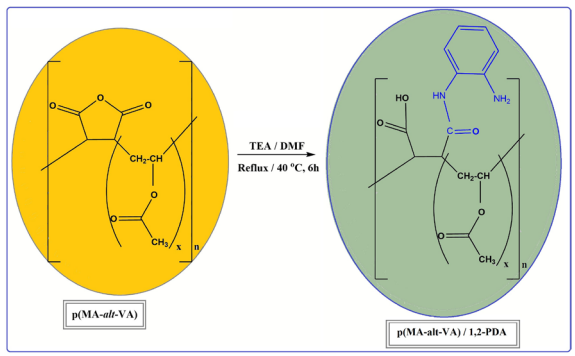
Water solubility test

In this study 1,2-phenylenediamine, known as a fluorescent dye, selected as a nucleophilic conjugation agent that allows formulating chemotherapeutic polymeric molecules. Conjugation of drugs as a polymeric prodrugs via chemical modification play an extremely crucial roles in clinical therapeutic applications for efficient and controlled drug delivery. In particular, drug solubility in aqueous medium is one of the critical parameters to provide the required pharmaceutically active dose for systemic circulation to reach the desired therapeutic effect.

poly(MA-*alt*-VA)/1,2-PDA conjugate in dried powder form was used for water solubility test. pMAVA soluble in water and 1,2-PDA is a small molecule that solubility increases from cold to hot water. Furthermore, 1,2-PDA soluble in alcohol, ether and chloroform. Although having an aromatic unit and also hydrophobicity of the carrier pMAVA/1,2-PDA dissolved in water. Water solubility and solubility test demonstrated that poly(MA-*alt*-VA)/1,2-PDA conjugate dissolved in water very quickly and very well in any ratio. Rational design of polymer-drug conjugates for drug delivery water solubility test is the first step for evaluation of the conjugate stability property.



Scheme 1. Synthesis reaction representation of pMAVA copolymer.



Scheme 1. Synthesis reaction representation of pMAVA/1,2-PDA conjugate.

Proposed reaction mechanisms for copolymer and conjugate

Spectroscopic analysis results, FTIR and ^{1}H-NMR, clearly showed that the mechanism of synthesis reaction for MA containing copolymer is compatible with radical initiated free-radical polymerization and conjugation occurs by nucleophilic ring-opening reaction. (Scheme 1 and 2).

CONCLUSION

Polymeric and polymer-based materials are well known biomaterials for controlled and targeted drug release/uptake product developments for biomedical applications.

In the present study 1,2-phenylenediamine (has the potential to transform into fluorescent molecules) used for the conjugation of poly(MA-*alt*-VA) to obtain water-soluble conjugate, poly(MA-*alt*-VA)/1,2-PDA, has been successfully achieved.

PDA is an amino di-substituted benzene molecule used in the production of various paints. In particular, its potential use in sensitive immune sensor for cancer biomarker has attracted attention in recent years. Structural characterization of the surface functionalized poly(MA-*alt*-VA)/1,2-PDA conjugate was performed by ATR-FTIR and ^{1}H-NMR.

Spectroscopic analysis and water solubility results suggested that the conjugation took place successfully after the ring opening reaction by the amide mechanism. 1,2-PDA, proposed as a conjugation reagent, is an important amino derivative molecule used in the design of cancer biomarkers and also in the production of various dyes.

The results indicate that the pMAVA/1.2-PDA fluorescent-based conjugate needs further investigation to better assess its potential fluorescent probe capacity.

CONFLICT OF INTEREST

The author of the manuscript solemnly declare that no scientific and/or financial conflicts of interest exist with other people or institutions.

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