Poly(amidoamine) dendrimers grafted on electrospun poly(acrylic acid)/poly(vinyl alcohol) membranes for host–guest encapsulation of antioxidant thymol†

Georgiana Amariei,a Karina Boltes,a Pedro Letón,a Isabel Iriepa,b Ignacio Moraleda,b and Roberto Rosal*a

Amino-terminated fifth generation poly(amidoamine) (PAMAM G5-NH₂) dendrimers were grafted onto the surface of poly(acrylic acid)/poly(vinyl alcohol) (PAA/PVA) electrospun fibres with the purpose of creating a host–guest architecture for the controlled delivery of a natural antioxidant, thymol. The nanofibers were stabilized by esterification crosslinking to produce a water insoluble non-woven membrane. The functionalization with PAMAM G5-NH₂ led to dendrimer loadings in the 7.4 × 10⁻⁷–2.25 × 10⁻⁶ mol dendrimer per g membrane range. The resulting materials were characterized using SEM, ATR-FTIR and surface ζ-potential measurements. The loading capacity for thymol reached 2.5 × 10⁻⁴ mol thymol per g membrane. The membranes were tested for thymol release in different aqueous and non-aqueous food simulants. Computational modelling was used to get a further insight into the host–guest association of thymol and PAMAM G5-NH₂ molecules through docking studies. For this purpose, we examined the molecular level details of the dendrimer–guest complex, calculated the number of included or attached molecules, the exact location of thymol in host–guest complexes and the local environment around the thymol molecules. Docking studies showed that PAMAM-G5-NH₂ dendrimers can encapsulate thymol molecules through hydrophobic interactions and hydrogen bonding. The maximum amount of thymol molecules theoretically encapsulated was 16, while another 25 could be hosted at the dendrimer surface through interaction with the outer part or the dendritic branches. The experimental value was 37 ± 5, in agreement with theoretical predictions.

Introduction

Electrospinning is a simple and cost-effective method for producing fibres below the micron size. Many polymeric nanofibres have already been produced using this technique for a variety of application areas.¹ In electrospinning, a thin jet of dissolved or molten polymer is accelerated due to the electric field forced using a high voltage source to a collector electrode. In the whipping-like path to the collector, the solvent evaporates or the polymer solidifies yielding an ordered array of fibres or a disordered nonwoven membrane depending on the collector geometry.² Electrospun materials can be used to create carriers for insoluble molecules by simple dispersion into the polymeric matrices.³ Many systems based on biodegradable and non-biodegradable polymers have been proposed based on this idea.⁴ The release of hosted molecules can be diffusion controlled or triggered by means of polymers responsive to environmental variables like pH.⁵ The critical aspects for the formulation of electrospun carriers are the compatibility between the drug and the polymer and the solubility of both in the electrospinning solution.⁴ If the compatibility between the drug and the polymer does not allow direct co-electrospinning, other possibilities exist such as coaxial electrospinning, which allows one-step encapsulation of labile or non-solvent compatible compounds without using solvents or emulsifiers.⁷ Another possibility for formulating electrospun fibers in drug delivery applications are host–guest inclusion complexes such as those prepared from cyclodextrins.⁸ Dendrimers are nano-sized, radially symmetric, essentially monodispersed hyper-branched polymers consisting of a central core, from which radially branched monomers grow forming successive shells referred to as generations (denoted by G).⁹
consists of a compact layer of surface end-groups that can be easily functionalized.\textsuperscript{10} The useful properties of dendrimers include chemical stability, low viscosity and high solubility, owing to which they have been proposed for a number of applications that include catalytic, sensing, environmental and biomedical uses.\textsuperscript{11} The branched structure of dendrimers leads to the formation of relatively large internal cavities, which can offer unique microenvironments allowing different chemical species to be accommodated within.\textsuperscript{12} Overall, dendrimers exhibit a molecular architecture capable of interacting with a wide variety of molecules either by encapsulation, which generally involves electrostatic, hydrophobic and hydrogen bond interactions, or by surface attachment by electrostatic interactions or covalent bonding. The interacting mode of the ligands depends not only on the structure of the dendritic core and ligands but also on pH because the dendrimers can undergo pH responsive conformational changes upon protonation of internal and core moieties.\textsuperscript{13} The host–guest capacity of the dendrimers arising from their internal cavities and surface structures makes them very attractive for creating drug delivery materials.\textsuperscript{14,15}

Thymol (5-methyl-2-isopropylphenol) is a natural monoterpenic phenol derivative of cymene found in thyme extracts with natural antioxidant and antimicrobial properties. Its use is supported by the fact that thymol is listed by the Food and Drug Administration (FDA) as a food additive on the GRAS (Generally Recognized As Safe) list. The use of thymol and other natural extracts faces the problem of the ease of degradation of many of the extracted compounds, their limited water solubility and their short term bioactivity due to their volatile character.\textsuperscript{16} Thymol has been included in different kinds of films for active packaging to extend the shelf life of food products.\textsuperscript{17–19} The hydrophobicity of thymol has been overcome by incorporating it into submicron emulsions with and without a carrier oil.\textsuperscript{20} The encapsulation of thymol in water-dispersible zein nanocarriers has also been proposed for ensuring sustained activity in aqueous medium.\textsuperscript{21} Another important feature of encapsulated systems is that the inclusion of hydrophobic antioxidant compounds may enhance their antioxidant performance due to the protective effect offered by host–guest architectures against the degradation of the active molecules.\textsuperscript{22}

In this work, we prepared electrospun membranes modified with amino terminated poly(amidoamine) fifth generation dendrimers (PAMAM G5-NH\textsubscript{2}) for their use as thymol carriers using the host–guest capacity of dendrimers. The dendrimers were grafted to electrospun fibers made of poly(acrylic acid) (PAA) and polyvinyl alcohol (PVA). PAA and PVA are non-toxic water-soluble polymers that can be electrospun without organic solvents that produce insoluble PAA/PVA membranes by cross-linking esterification.\textsuperscript{23} Poly(vinyl alcohol) and poly(acrylic acid) are two important materials that can be used to form hydrogels for tissue engineering scaffolds and for the design of drug delivery systems.\textsuperscript{24} Hydrogel forming polymers are desired to be non-toxic to the cells they are in contact while serving as scaffolds or delivering active compounds to the surrounding tissues. The toxicity usually comes from the release of toxic leachable compounds or unreacted cross-linking agents.\textsuperscript{25} Most stable hydrogels are non-toxic and do not induce immune responses when properly prepared, but they do need to be modified in order to promote cellular adhesion if serving as scaffolds. In this regard, PVA has been modified with the fibronectin protein to promote functionality.\textsuperscript{26} Poly(acrylic acid), however, has been shown to depress cell growth due to its acidic environment.\textsuperscript{27} Cell adhesion and growth has been enhanced in this case by immobilizing collagen on the PAA surface.\textsuperscript{28} Another interesting feature of PAA/PVA fibres is the intrinsic antimicrobial activity of poly(acrylic acid) polymers, which has been recently studied demonstrating that the binding by PAA of the divalent cations that stabilize prokaryotic membranes was the cause for the impairment of bacterial cells in contact with PAA/PVA membranes.\textsuperscript{23,29}

Computational modelling was used to get a further insight into the host–guest association of thymol and PAMAM G5-NH\textsubscript{2} molecules through docking studies. Our contribution emphasizes the nature and relative strength of the intermolecular interactions responsible for the dendrimer–target interactions and binding mechanisms. Despite recent advances in cheminformatics, the amount of molecular modelling research is limited due to the lack of understanding of interactions at the atomic level. Computer strategies have the potential to minimize laborious and expensive laboratory experiments by guiding experimentation based on rational design. For this purpose, we examined the molecular level details of the dendrimer–guest complex, calculated the number of included or attached molecules, the exact location of thymol in host–guest complexes and the local environment around the thymol molecules. The results were compared with the experimental loading capacity of membranes grafted with PAMAM G5-NH\textsubscript{2}.

**Experimental**

**Chemicals**

Poly(vinyl alcohol) (PVA, MW 89–98 kDa, 99+% hydrolysed) and poly(acrylic acid) (PAA, MW 450 kDa) from Sigma Aldrich were used to prepare the polymeric fibrous matrix. PAMAM dendrimer generation 5 (PAMAM G5-NH\textsubscript{2}), 4.57 wt% solution in water, was purchased from Dendritech (Midland, MI, USA). N,N-Dicyclohexylcarbodiimide (DCC, MW 206.33, 99%) and thymol (99+%) were obtained from Sigma Aldrich. N,N-Dimethylformamide (DMF, 99%) and absolute ethanol (EtOH) were obtained from Scharlab (Spain). Ultrapure water (Millipore Milli-Q System) with a resistivity of at least 18 M\textohm cm at 25 °C was used during the fibre production and modification processes.

**Preparation of electrospun fibres**

The PAA/PVA electrospun fibres used to attach dendrimers were prepared as described elsewhere.\textsuperscript{23} Briefly, the spinning solution was a polymeric mixture of 8 wt% PAA and 15 wt% PVA in ultrapure water. Two formulations with a final PAA/PVA weight ratio of 83 : 17 and 35 : 65 were produced to study the effect of different amounts of carboxylic acid groups on the functionalization process. The formulations refer to the weight ratio of PAA and PVA,
83/17 meaning 83 wt% PAA and 17 wt% PVA in the final, solvent free, material. The solutions were stirred for 2 h at 25 °C and degassed before electrospinning. The solution was drawn into a 5 mL syringe equipped with a 23-gauge stainless steel blunt-tip needle and electrospun using the following parameters: voltage 23 kV, working distance 23 cm, flow rate 0.8 mL h⁻¹, RH 40%, temperature 25 °C. A drum collector (PDrC:3000, Yflow, Spain) rotating at 100 rpm was used. The electrospun fibres were collected on aluminium foil and dried at 50 °C for 24 h. After production, the nanofibres were cross-linked by heating at 140 °C for 30 min. The crosslinked fibres were washed with distilled water and dried under vacuum (10 kPa, 50 °C, 24 h) for dendrimer grafting. The stability of the crosslinked nanofibers was studied by immersion in water. For this, accurately weighed pieces of crosslinked electrospun membranes were immersed in water. The NPOC (Non-Purgeable Organic Carbon) of the resulting solutions was analysed using a Total Organic Carbon (TOC) analyser and the results are expressed as wt% release of soluble substances.

Membrane functionalization by dendrimer grafting

The amine-terminated groups of PAMAM G5-NH₂ were used to attach dendrimers to PAA/PVA fibres via amide formation with their surface carboxyl groups by means of the coupling agent DCC. To check the efficiency of PAMAM G5-NH₂ insertion on membrane fibres, two different amounts of dendrimers were tested using two kinds of fibres with different concentrations of carboxyl groups. Reaction conditions and the specific formulation for each sample are given in Table 1. The amount of carboxyl groups per unit mass of the dry PAA/PVA membrane was determined using titration as described below. For the functionalization process, accurately weighed pieces of about 100 mg of the dry PAA/PVA base membrane were immersed in a PAMAM G5-NH₂ dendrimer solution in DMF containing DCC with a molar ratio of 1 : 1 with respect to the available carboxyl groups (7.9 mmol g⁻¹ and 12.5 mmol g⁻¹ for 35 : 65 and 83 : 17 PAA/PVA formulations respectively). The mixture was kept under constant shaking (100 rpm) for 24 h at room temperature. After the amide formation reaction, the membranes were washed with DMF and water and kept for 16 h in distilled water to remove unattached dendrimer molecules, residual reagents and functionalization by-products. Finally, the membranes were vacuum dried at 50 °C for 24 h. The amount of dendrimer grafted per gram of the mats was calculated based on the total nitrogen content of the samples.

Table 1  PAMAM G5-NH₂-functionalized membranes prepared in this work

<table>
<thead>
<tr>
<th>Membrane</th>
<th>PAA/PVA formulation (w/w)</th>
<th>-COOH amount (mmol COOH per g membrane)</th>
<th>PAMAM-G5-NH₂ amount used (mmol G5 per mg membrane)</th>
<th>PAMAM-G5-NH₂ content (mmol G5 per mg membrane)</th>
</tr>
</thead>
<tbody>
<tr>
<td>83/17 (neat)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G5(−)@83/17</td>
<td>83/17</td>
<td>12.5 ± 0.7</td>
<td>—</td>
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<tr>
<td>G5(+)@83/17</td>
<td>—</td>
<td>1.15 × 10⁻⁶</td>
<td>—</td>
<td>1.06 × 10⁻⁶ ± 4.1 × 10⁻⁷</td>
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<tr>
<td>35/65 (neat)</td>
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<tr>
<td>G5(−)@35/65</td>
<td>35/65</td>
<td>7.9 ± 0.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>G5(+)@35/65</td>
<td>—</td>
<td>1.15 × 10⁻⁶</td>
<td>—</td>
<td>7.4 × 10⁻⁷ ± 1.5 × 10⁻⁷</td>
</tr>
</tbody>
</table>

Thymol loading and migration tests

The dendrimer-modified membranes were loaded with thymol by displacing the ethanol with water from a solution of thymol in ethanol. Control experiments were performed using unmodified PAA/PVA membranes. Accurately weighted pieces of approx. 3.5 × 2.5 cm (~50 mg) of neat and dendrimer-functionalized PAA/PVA membranes were immersed into 5 mL ethanol solution (75% v/v) containing 50 mg of thymol for 20 h at room temperature. After that, water was slowly dropped until a final volume of 20 mL. Finally, all samples were rinsed three times with water and dried at room temperature for 24 h. To determine the loading/encapsulation performance, the dry samples were extracted twice with pure ethanol in an orbital shaker. Thymol concentration was measured using UV spectrophotometry at the maximum absorbance of thymol (275 nm) using a freshly prepared calibration curve. A second contact with thymol was performed to assess the reusability of PAMAM G5-NH₂ dendrimer-functionalized membranes. To this end, after the total extraction of thymol with ethanol, the membranes were washed with water for 24 h, dried at 50 °C, and reloaded with thymol using the same procedure described before.

Migration tests with food contact materials were performed using thymol-loaded PAMAM G5-NH₂ dendrimer-functionalized membranes. The assessments were performed by total immersion into five food simulants, according to European Standard EN 13130-2005 (Materials and articles in contact with foodstuffs. Plastics substances subject to limitation. Guide to test methods for the specific migration of substances from plastics to foods and food simulants and the determination of substances in plastics and the selection of conditions of exposure to food simulants): distilled water, pH 5.6 (A); acetic acid 30 g L⁻¹, pH 2.4 (B); and ethanol 100 mL L⁻¹, pH 4.0 (C) as aqueous food simulants whereas ethanol 950 mL L⁻¹ (D) and isooctane (E) were used as non-aqueous fatty food simulants as established as substitutes to olive oil in the Standard EN-13130-1:2004. All simulants were non-buffered, and the indicated pH corresponds to the average value along the release experiments indicated below. Samples of approx. 50 mg were immersed in 15 mL of each food simulant in a sealed vessel that was gently agitated (60 rpm) in an orbital shaker. Thymol migration from controls and thymol-loaded dendrimer-functionalized membranes was monitored until equilibrium at prescribed times: 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h, 5 d and 15 d. Thymol was analysed using UV spectrophotometry as described before. Neat PAA/PVA membranes were used as reference samples. All experiments were performed in triplicate.
Computational procedure

Thymol was assembled within Discovery Studio, version 2.1, software package, using standard bond lengths and bond angles. With the CHARMM force field and partial atomic charges, the molecular geometry of thymol was energy-minimized using the adopted-based Newton–Raphson algorithm. Structures were considered fully optimized when the energy changes between iterations were less than 0.01 kcal mol\(^{-1}\). The initial dendrimer structure was obtained from the study by Maingi et al. using the structure where the terminal amine groups are protonated to mimic PAMAM dendrimers under neutral pH conditions. Docking calculations were performed using the program Autodock. The best scoring docked conformation was selected in each case. The 3-dimensional parameters for docking the ligand to the dendrimer were determined using the GridBox option. The docking procedure was applied to the whole dendrimer target, without imposing docking sites. This process is considered as blind docking. For this, the centre of the grid box was chosen in such a way that the search space for thymol limits to the interior region of the PAMAM G5-NH\(_2\) dendrimer. Besides, multiple docking experiments have been used focusing on the dendrimer surface. For blind and focused dockings, the grid centre coordinates are included in Table 2 with grid points separated by 1 Å. Default parameters were used except num_modes, which was set to 40. Thymol was docked 50 times onto the PAMAM-G5 molecule and the docking results generated were directly loaded into Discovery Studio, version 2.1 for analysis. The PAMAM-G5-NH\(_2\)-functionalized PAA/PVA nanofibres were electrospun as revealed by the amount of non-crosslinked organic matter took place. The titration of free carboxyl groups yielded 12.5 ± 0.7 and 7.9 ± 0.3 mmol COOH per g of membrane for 83:17 and 35:65 PAA/PVA nanofibres respectively (Table 1). Both membranes were used as base materials for dendrimer functionalization, which was performed using two different amounts of PAMAM-G5-NH\(_2\). The amount of dendrimer graft per unit mass of membrane is shown in Table 1 and was larger for 83:17 than 35:65 PAA/PVA base membranes, as expected from their higher amount of carboxyl groups (+4.6 mmol COOH per g membrane). The yield of PAMAM G5-NH\(_2\) increased with the amount of dendrimer in the functionalization solution. By doubling the concentration of the dendrimer, the final content of PAMAM G5-NH\(_2\) in the final membranes roughly doubled reaching the maximum value of 2.25 × 10\(^{-6}\) ± 3.0 × 10\(^{-7}\) mmol PAMAM G5-NH\(_2\) per mg membrane for G5(+)@83/17 membranes. It has been reported that the stable by-product N-acylurea from the carbodiimide reaction with carboxyl groups formed only in the presence of an excess of carbodiimide. Based on that, we used an equimolar amount of DCC with respect to the carboxyl groups in PAA/PVA membranes. The dicyclohexylurea that could have been formed was removed during the washing process of functionalized membranes as

Results and discussion

PAMAM G5-NH\(_2\)-functionalized PAA/PVA nanofibres

The base PAA/PVA electrospun membranes were stabilized by crosslinking esterification for 30 min at 140 °C, after which they were kept in water until constant weight. The stability of electrospun as revealed by the amount of non-crosslinked materials showed that the two formulations used in this study released <10 mg NPOC per g membrane (equivalent to <1 wt%) after 24 h in water and <0.5 mg NPOC per g membrane (equivalent to <0.05 wt%) after 48 h. The membranes used in this work were water preconditioned for 48 h, after which no significant release of organic matter took place. The titration of free carboxyl groups yielded 12.5 ± 0.7 and 7.9 ± 0.3 mmol COOH per g of membrane for 83:17 and 35:65 PAA/PVA membranes respectively (Table 1). Both membranes were used as base materials for dendrimer functionalization, which was performed using two different amounts of PAMAM-G5-NH\(_2\). The amount of dendrimer grafted per unit mass of membrane is shown in Table 1 and was larger for 83:17 than 35:65 PAA/PVA base membranes, as expected from their higher amount of carboxyl groups (+4.6 mmol COOH per g membrane). The yield of PAMAM G5-NH\(_2\) increased with the amount of dendrimer in the functionalization solution. By doubling the concentration of the dendrimer, the final content of PAMAM G5-NH\(_2\) in the final membranes roughly doubled reaching the maximum value of 2.25 × 10\(^{-6}\) ± 3.0 × 10\(^{-7}\) mmol PAMAM G5-NH\(_2\) per mg membrane for G5(+)@83/17 membranes. It has been reported that the stable by-product N-acylurea from the carbodiimide reaction with carboxyl groups formed only in the presence of an excess of carbodiimide. Based on that, we used an equimolar amount of DCC with respect to the carboxyl groups in PAA/PVA membranes. The dicyclohexylurea that could have been formed was removed during the washing process of functionalized membranes as

### Table 2 Coordinates of the grid box for blind and focused dockings (spacing 1 Å)

<table>
<thead>
<tr>
<th></th>
<th>x center</th>
<th>y center</th>
<th>z center</th>
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<th>Size y</th>
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<td>58.175</td>
<td>27.377</td>
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revealed by FTIR spectra. The zeta potential of membrane fibres after grafting the PAMAM G5-NH$_2$ dendrimer were less negative, increasing about 15 mV from the more negative values obtained for neat PAA/PVA membranes. Amine-terminated PAMAM dendrimers bear a positive charge at slightly acidic pH, but the large excess of carboxyl groups in PAA/PVA membranes made them overall negative. The values of surface zeta-potential are listed in Table 3.

Fig. 1 presents the FT-IR spectra of PAA/PVA nanofibres before and after modification with the PAMAM G5-NH$_2$ dendrimer. FTIR spectra of neat PAA/PVA membranes revealed the characteristic peaks associated with PAA and PVA. The broad O–H stretching band (3200–3600 cm$^{-1}$) appears as the most characteristic feature of alcohols. The C–H alkyl stretching band (2850–3000 cm$^{-1}$) is also clear as well as the peak at 1142 cm$^{-1}$, which is attributed to the C–O stretching in the crystalline domains of PVA. The characteristic carboxyl stretching frequency of PAA is observed at 1700 cm$^{-1}$, while the symmetric and antisymmetric stretching frequencies of the carboxylate ion appeared at 1420 and 1560 cm$^{-1}$.

Table 3  Surface zeta-potential and thymol loading capacity of PAMAM G5–NH$_2$ functionalized membranes

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Surface zeta-potential (mV, pH 6)</th>
<th>Thymol loading 1st charge (mol thymol per g membrane)</th>
<th>Thymol loading 4th charge (mol thymol per g membrane)</th>
<th>PAMAM G5-NH$_2$ loading (mol thymol per mol G5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>83/17 (neat)</td>
<td>$-44.5 \pm 7.6$</td>
<td>$4.56 \times 10^{-3} \pm 3 \times 10^{-7}$</td>
<td>$3.84 \times 10^{-3} \pm 1.8 \times 10^{-6}$</td>
<td>$-2.4$</td>
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<td>G5(−)@83/17</td>
<td>$-31.1 \pm 0.6$</td>
<td>$1.20 \times 10^{-4} \pm 3.3 \times 10^{-5}$</td>
<td>$1.30 \times 10^{-4} \pm 1.7 \times 10^{-5}$</td>
<td>$39 \pm 3$</td>
</tr>
<tr>
<td>G5(+)@83/17</td>
<td>$-30.4 \pm 0.1$</td>
<td>$2.05 \times 10^{-4} \pm 7 \times 10^{-6}$</td>
<td>$1.94 \times 10^{-4} \pm 1.4 \times 10^{-5}$</td>
<td>$41 \pm 4$</td>
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<tr>
<td>35/65 (neat)</td>
<td>$-35.2 \pm 0.2$</td>
<td>$1.84 \times 10^{-5} \pm 2 \times 10^{-7}$</td>
<td>$1.62 \times 10^{-5} \pm 4.7 \times 10^{-6}$</td>
<td>$-2.4$</td>
</tr>
<tr>
<td>G5(−)@35/65</td>
<td>$-25.9 \pm 6.2$</td>
<td>$5.7 \times 10^{-5} \pm 1.8 \times 10^{-5}$</td>
<td>$5.6 \times 10^{-5} \pm 1.8 \times 10^{-5}$</td>
<td>$31 \pm 8$</td>
</tr>
<tr>
<td>G5(+)@35/65</td>
<td>$-22.3 \pm 0.4$</td>
<td>$1.30 \times 10^{-4} \pm 2.4 \times 10^{-5}$</td>
<td>$1.36 \times 10^{-4} \pm 2.8 \times 10^{-5}$</td>
<td>$39 \pm 4$</td>
</tr>
</tbody>
</table>

$^a$ Difference from the amount adsorbed by neat PAA/PVA membranes.

Also evidences the attachment of dendrimers to PAA/PVA fibers. The decrease in the carboxyl signals at 1700 cm$^{-1}$ and 1420 cm$^{-1}$ was consistent with the amide formation reaction. The presence of thymol in electrospun hybrid fibers after encapsulation was also confirmed by FTIR analysis (Fig. S1, ESI$^+$. A broad band between 3400 and 3100 cm$^{-1}$ corresponded to the phenolic OH stretching. The peaks at 1360 and 1241 cm$^{-1}$ can be attributed to the OH in-plane bending vibration and the C–O stretching in phenol, respectively. The bands at 804 and 738 cm$^{-1}$ were due to out of plane aromatic C–H wagging vibrations, while the expected band between 2955 and 2863 cm$^{-1}$ related to CH$_3$ stretching was overlapped with the dominant bands corresponding to the CH$_3$ and CH$_2$ symmetric and asymmetric stretching vibrations of the base material.

Fig. 2 shows the SEM micrographs of electrospun 83/17 (A), G5(+)@83/17 (B), 35/65 (C) and G5(+)@35/65 (D) membranes. A and C represent specimens before functionalization while B and D correspond to membranes functionalized with the highest amount of PAMAM G5-NH$_2$. The average fibre diameters, obtained from SEM micrographs, were 254 ± 65 nm and 320 ± 52 nm for 83/17 and 35/65 membranes, respectively. The fibers were uniform, straight and well-defined without beading or other

![Fig. 1 FTIR spectra of neat and PAMAM G5–NH$_2$-functionalized membranes.](image1)

![Fig. 2 SEM images of PAA/PVA (83/17, A and 35/65, C) membranes and PAMAM G5–NH$_2$-functionalized membranes G(+)@83/17 (B) and G(+)@35/65 (D).](image2)
apparent flaws. After dendrimer functionalization and washing (Fig. 2B and D) some fibre merging between adjacent fibers at their contact points was observed, but the fibrous morphology of membranes was retained. An additional set of SEM micrographs is presented in Fig. S2 (ESI†) showing all functionalized membranes unloaded and thymol loaded. No significant differences have been observed between specimens before and after the inclusion of thymol.

Thymol loading and release profiles

Table 3 shows the results of the total amount of thymol loaded into neat PAA/PVA and dendrimer-modified membranes. Measurements were performed after four consecutive extractions in ethanol for 24 h at 25 °C and ensured the complete removal of thymol from loaded membranes as demonstrated by the absence of thymol in successive re-extractions under the same conditions. A certain amount of thymol was adsorbed on neat PAA/PVA membranes, which accounted for 12–18% of the total amount of G5(+) membranes, which were those loaded with the higher amount of dendrimers. The most probable reason is the formation of hydrogen bonds due to the existence of numerous carboxylic acid and hydroxyl end groups on the membrane surface. The amount of thymol was higher for membranes with a higher content of PAMAM G5-NH₂ dendrimers being expected. The maximum amount of thymol hosted amounted to 2.05 × 10⁻⁴ mol thymol per g membrane for G5(+)@83/17 membranes after subtracting the thymol retained by the PAA/PVA base membrane, which was 4.56 × 10⁻⁵ mol thymol per g membrane. For the whole set of membranes, the hosted amounts (excluding PAA/PVA fibers) were in the 5.7 × 10⁻⁵–2.05 × 10⁻⁴ mol thymol per g membrane range, which corresponded to an average value of 37 molecules of thymol per molecule PAMAM G5-NH₂ with an uncertainty of ±5 and without significant differences among membrane type and PAMAM-G5-NH₂ loading (Table 3). The recharge capacity of modified membranes was also tested for 4 consecutive recharge assays. The results are shown in Table 3 and Fig. S3 (ESI†). No statistically significant decline in the thymol loading capacity could be assessed for any of the tested membranes along the 4 cycles of charge-release.

Fig. 3 presents the percent release of thymol when G5(+)@35/65 membranes, fully loaded with thymol, were put in contact with the five food simulants indicated before. The membrane used for this purpose was based on 35/65 PAA/PVA formulation because in this case thymol loading was below the overall migration limit of 10 mg dm⁻² established by EU Commission Regulation 10/2011. Taking into account the geometry of functionalized membranes, the loading for G5(+)@83/17 was >30 mg dm⁻², whereas for G5(+)@35/65 the maximum loading was 9.1 mg dm⁻². The difference is most probably due to the lower swelling observed in membranes with a higher PAA content.²³

The release of thymol was very rapid at low pH (simulant B) with >90% of the thymol released before the first measurement (2 h). The extended dendritic conformation and the weaker character of hydrophobic interactions in acetic acid solution promoted the fast discharge of thymol from the dendrimer hosting sites. In diluted ethanol (simulant C), roughly 20% of the thymol initially loaded remained after 15 days, the other 80% being released in less than 2 h. Similar behaviour was observed in water (simulant A), but the amount of thymol retained at the end of the assay was higher, about 40%. The higher solubility of thymol in acetic acid and absolute ethanol might be responsible for the variation in the released rate.²⁰ The protonation of surface amines at low pH and the subsequent loss of their ability to interact with thymol molecules together with the opening of the dendrimer box under acidic conditions would be the reason for the fast release at low pH.²¹²² In 95 vol% ethanol (simulant D) the release of thymol followed a two-stage profile. After a relatively rapid release during the first 4 h, thymol passed to the solution at an essentially constant rate of ~8 × 10⁻⁴ mmol thymol per g membrane per day. Practically no release of thymol was observed in isooctane (simulant E). The loading of thymol in the functionalized membranes is expected to be driven by a combination of hydrogen bonding and hydrophobic interactions.¹⁵ The rapid release phase observed for simulant D, which represented approx. 3.4 × 10⁻⁵ mol thymol per g membrane in 4 h, could be attributed to the less tightly bound molecules attached to the external surface of the PAA/PVA fibers (~12% of the total amount of thymol) or the external dendritic core. The slow release phase would represent the thymol encapsulated inside dendrimer cavities of forming more stable complexes with the outer functional groups of the dendrimer molecules. At the end of the release and repeated release experiments, the tested mats did not show any apparent loss of capacity.

Computational modelling

The purpose of using molecular modelling to understand the PAMAM G5-NH₂ interaction with thymol was to obtain a reliable complex stoichiometry and a suitable binding mode for the ligand–dendrimer association between the dendrimer and thymol using docking simulations. Host molecule binding can be the result of both covalent and non-covalent interactions at the surface

Fig. 3  Release of thymol with the time in contact with different media: water (A), acetic acid 30 g L⁻¹ (B), ethanol 100 mL L⁻¹ (C), ethanol 950 mL L⁻¹ (D) and isooctane (E). The membrane, G5(+)@35/65, was previously loaded with thymol as indicated in Table 3. Thymol concentration was recorded for 15 days, the latter point representing 360 h.

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and in the internal void spaces. The computational approach consists of a blind docking run that covers the complete dendrimer and a set of independent docking runs carried out on small boxes centred on the dendrimer surface (focused docking). For the docking simulations, the initial dendrimer structure chosen was that in which the terminal amine groups are protonated to mimic the PAMAM dendrimer under neutral pH conditions. Under these conditions, the –OH groups of thymol molecules are not ionized and, therefore, the interactions that are established are of the hydrogen bond type and non-electrostatic. The first modelling strategy provided information related to the internal cavities and can be used to estimate the number of host molecules the dendrimer can encapsulate. From the focused docking experiments, the thymol molecules attached onto the dendrimer surface could be estimated. The coordinates containing the information about the size and position of the grid box used in blind and focused dockings are tabulated in Table 2.

PAMAM G5-NH$_2$ was blindly docked with thymol using Autodock Vina. This technique was used to determine appropriate binding sites present in the interior of the dendrimer and the whole structure of this is covered under an imaginary 3D grid box for docking. Fig. 4 and Fig. S4 (ESI†) show the docking model of thymol in association with the dendrimer. The results indicated that the PAMAM G5-NH$_2$ dendrimer could accept up to 16 molecules with binding energies calculated using the docking score in the (−3.7) to (−4.8) kcal mol$^{-1}$ range. This result can be attributed to the interaction of thymol molecules with the hydrophobic internal cavities of PAMAM dendrimers that drive aromatic non-polar molecules to penetrate the dendrimer cages. Thymol molecules were located in protected pocket-like structures formed between or under dendrimer branches. The docking calculations showed that the interaction took place mainly with the hydrophobic parts of the dendrimer plus a minor hydrophilic interaction via hydroxyl groups.

The affinity of the complex involves hydrophilic and hydrophobic interactions, which are explicitly shown in Fig. 5. Hydrophilic interactions arise from the formation of hydrogen bonds involving hydroxyl groups of thymol molecules. Hydrophobic interactions involve the phenyl, the methyl and isopropyl groups. This hypothesis is in agreement with previous studies. The location near the tertiary amines and between branches of the dendrimer is the same association site as previously proposed from experiments with other guest molecules including 2-naphthol. Kleinman et al. proposed that the alcohol from 2-naphthol interacts with the tertiary amine of PAMAM dendrimers by hydrogen bonds.$^{53}$ Yang et al. showed that one encapsulation site for phenylbutazone was between dendrimer branches near the tertiary amines.$^{54}$ In light of these results, the location around the tertiary amine of PAMAM dendrimers appears to be a favourable association site for hydrophobic aromatic groups in general.

PAMAM G5-NH$_2$ at neutral to slightly acidic pH contains most of the tertiary amines in non-protonated form. The hydroxyl group of the benzene skeleton participates as a hydrogen bond acceptor in the interaction with the carbonyl and amino groups located within the dendrimer. One important factor in the dendrimer-guest interaction is the location of the guest association Fig. 4 Docking model of thymol molecules (blue) associated with PAMAM G5-NH$_2$ dendrimers (yellow). (A) Thymol molecules and dendrimers represented as sticks. (B) Thymol molecules represented as surfaces.

Fig. 5 Representation view of the interaction between thymol (blue) and PAMAM-G5 dendrimers, indicating the role of the hydrogen bonds in the capture. Intermolecular hydrogen bonds are drawn as dashed green lines.
sites within the dendrimer structure. At neutral pH conditions, the open structure of the PAMAM G5-NH₂ dendrimer allows 4 thymol molecules to diffuse to locations near the dendrimer centre (Fig. S5, ESI†). The other 12 molecules of thymol were located near the tertiary amines and between dendrimer branches.

Focussed docking experiments determined the number and position of the thymol molecules attached to the periphery of PAMAMG5-NH₂. The results showed that 25 additional thymol molecules could be hosted at the dendrimer surface where the docking contacts involve the outer part or the dendritic branches. Overall, modelling studies showed that 41 (16 + 25) thymol molecules can be hosted either on the dendrimer surface or in internal cavities (Fig. 6).

The binding energies showed that the interaction between thymol and the dendrimer surface led to less stable complexes than those of thymol encapsulated inside dendritic cavities. The binding energies calculated using the docking score were in the (−2.4) to (−3.4) kcal mol⁻¹ range, ~30% lower than those of thymol in internal cages, but strong enough to explain the experimental results of maximum thymol loading (excluding the amount retained by PAA/PVA fibres) of 35 ± 5 molecules of thymol per molecule of PAMAM G5-NH₂ (Table 3). In diluted ethanol (simulant C), the thymol retained in the membrane after 15 days would correspond to the molecules encapsulated inside the dendrimer, representing about 33% of the total amount of thymol, plus the more tightly bound ones hosted in the outer parts of the dendrimer molecule. The difference between simulants A and C can be explained by the higher pH of the former, which resulted in a tighter dendritic conformation. The behaviour with non-aqueous fatty food simulants is significant, as it represents an improvement over many active agent carriers that generally suffer from a severe burst release in fatty simulants.55–57

PAMAM dendrimers have a large number of cationic surface groups. The pKₐ of primary amines in the outer layer of PAMAM dendrimers is in the 9.0–10.8 range.58 Consequently, surface protonated amines of PAMAM G5-NH₂ offer binding sites for hydrogen bonds with thymol molecules.59 The internal tertiary amines of PAMAM G5-NH₂ do not undergo complete protonation under slightly acidic conditions, due to their weaker basic character.60 Their corresponding pKₐ was reported to be in the 5.8–6.7 range.58,61 Consequently, the hydrogen bonding of thymol to the surface of PAMAM G5-NH₂ would be favoured in all cases at the pH (6.0 ± 0.5) of the loading thymol–ethanol solution.62 At the same time, the electrostatic repulsions (excluded-volume interactions and Coulombic repulsions) between the protonated primary and tertiary amines cause an increase in the radius of gyration and the stretching out of dendritic branches, which makes the internal hydrophobic cavities more accessible to thymol molecules.13,63

These findings were in good agreement with the results reported by Buczkowski et al., who studied the interactions of 5-fluorouracil with PAMAM G5-NH₂ and PAMAM G5-OH dendrimers.66 The authors determined that PAMAM G5-NH₂ is able to guest about 70 5-fluorouracil molecules, three times more than its hydroxyl PAMAM G5-OH counterpart. This fact confirms the importance of the interaction with surface amine groups for the host–guest association of ligand molecules to dendrimers. The interaction with internal tertiary amine groups would be playing a minor role. However, the more hydrophobic interior cavities can retain the thymol molecules with higher binding energies than the surface of the dendrimer.64

Conclusions

We prepared electrospun membranes from the blends of poly(acrylic acid) and poly(vinyl alcohol), which were stabilized by esterification crosslinking and functionalized by covalent bonding of amino terminated poly(amidoamine) fifth generation dendrimers (PAMAM G5-NH₂). The purpose was to use the
membranes for the host–guest encapsulation of the antioxidant, thymol.

The electrospun membranes consisted of uniform, straight and well-defined fibers that preserved their fibrous morphology after washing and functionalization. The amount of grafted dendrimers reached $2.25 \times 10^{-6} \pm 3.0 \times 10^{-7}$ mmol PAMAM G5-NH$_2$ per mg membrane and was higher for membranes with a higher content of poly(acrylic acid).

The amount of thymol loaded reached a maximum of $2.51 \times 10^{-4}$ mol thymol per g membrane, from which $4.56 \times 10^{-5}$ mol thymol per g membrane corresponded to the PAA/PVA base membrane. For the whole set of membranes, the amount of thymol hosted by the dendrimer represented an average of 37 ± 5 molecules of thymol per molecule of PAMAM G5-NH$_2$. The membranes could be recharged without losing their capacity.

Computational modelling allowed determining the molecular details of the dendrimer–guest complex and the calculation of the rate of ethanol the release of thymol took place at a relatively constant base membrane. For the whole set of membranes, the amount of thymol loaded reached a maximum of 3.0 mol thymol per g membrane, from which 4.56 molecules of thymol per molecule of PAMAM G5-NH$_2$.

The membranes could be recharged without losing their capacity. Upon contact between thymol-loaded membranes and food simulants, we showed that the release behaviour was mainly driven by the pH of the medium, which determines the opening of the dendrimer box upon amine protonation. In 95 vol% ethanol the release of thymol took place at a relatively constant rate of $\sim 8 \times 10^{-4}$ mmol thymol per g membrane per day after an initial release of the less tightly bound thymol molecules.

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