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Knowledge-Based Tailoring Gelatin-Based Materials by Functionalization with Tyrosine-Derived Groups[a]

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Molecular models of gelatin-based materials formed the basis for the knowledge-based design of a polymer system with tyrosine-derived side groups enabling π–π interactions and hydrogen bonds and in this way creating physical netpoints. The models were validated by comparison with experimental data. Both analyses showed the desired physical interactions of desaminotyrosine (DAT) and desaminotyrosyl tyrosine (DATT) side chains. Gelatin was functionalized with DAT and DATT at 80 mol-% of the free amino groups. The functionalized gelatins had reduced helical conformations due to sterical hinderance and interchain contacts, and systematic changes of macroscopical properties, such as a clear reduction in the degree of swelling, were observed.

[a] Supporting information for this article is available at the bottom of the article's abstract page, which can be accessed from the journal's homepage at http://www.macros.wiley-vch.de, or from the author.
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Introduction

The development of biocompatible polymers based on repeating units of biopolymers such as proteinogenic amino acids is a major aim of biomaterial research from its beginning. Poly (amino acids) represent one approach,[1] but copolymers from different amino acids have the risk of unwanted bioactivity of the polymer and fragments of it. Therefore, alternative strategies have employed amino acid derivatives in polymers such as tyrosine-derived MDI analogues in polycarbonates and pseudo poly (amino acids).[2] On the other hand, starting material synthesis from biopolymers such as gelatin, collagen, or hyaluronic acid is challenging due to difficulties in tailoring material properties and biopolymer's inherent variability between production batches.[3] Here, we explored the potential of side chain functionalization of gelatin with the aim to develop defined polymer systems with tailorable properties by enabling specific non-covalent interactions.[4] These physical netpoints shall form the basis for supramolecular polymer networks.[5-7] The side chain functionalization shall inhibit typically observed trimerization of gelatin-chains to collagen-type triple helices,[8,9] which in unfunctionalized gelatin is thermodynamically driven,[10] by steric hinderance and altered properties of groups engaged in hydrogen bonding. The challenge to identify suitable functional groups and specific attachment sites was addressed by molecular modeling investigations of (functionalized) gelatin bulk materials. We selected as functional groups to be introduced as side chains to gelatin amino acid derived desaminotyrosine (DAT) and desaminotyrosyl tyrosine (DATT) as these moieties can interact by two different mechanisms:, aromatic π–π interactions and hydrogen bonds via the phenolic group (as H-bond donor or acceptor). DAT and DATT[11,12] analogues are biocompatible and have been used to trigger physical interactions in synthetic polymer networks.[12,13] The amino groups on lysine residues (3 mol-% of the amino acids of gelatin)[14] and protein chain ends can be selectively targeted via the free carboxylic acid group of DAT and DATT. The nucleophilic reactivity of amino groups is much higher when compared to hydroxyl groups, thereby

making this process chemoselective. DAT-functionalized gelatin (GA1) possessed one tyrosine derivative on each free amino group, while DATT functionalized gelatin (GA2) had two of these functionalities on each amino group. In this way, a systematic increase in potentially interacting side chain functional groups was realized forming a key parameter in the resulting polymer network system.

Molecular models of gelatin (G) and functionalized gelatins GA1 and GA2 containing 25 wt.-% water were generated. Chain organization and contacts were analyzed to validate that the desired interactions were observable leading to a knowledge-driven approach to tailorable materials. The corresponding materials were synthesized, processed into films, and experimentally studied by wide angle X-Ray spectroscopy (WAXS, on dried films), temperature modulated DSC (TM-DSC) and tensile tests (at 25 wt.-% water content, corresponding to the modeling). Finally, their swelling properties in water were investigated.

Experimental Part

Materials:

Gelatin type A and β-mercaptoethanol were purchased from Fluka. Desaminotyrosine (DAT), *N*-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC), 2,4,6-trinitro-benzensulfonic acid (TNBS), and *N*,*N*-Diisopropylethylamine (DIPEA) were purchased from Sigma Aldrich. DMSO, NMP, Trifluoroacetic acid (TFA), and ethyl acetate (EtOAc) were purchased from Merck. IRIS Biotech GmbH was the provider of DCM, L-Tyrosine *tert*-butyl ester (H-L-Tyr-OtBu), and Triisopropylsilane (TIPS).

Simulation details

The Amorphous cell and Discover interfaces from Accelrys Software Inc., with the Material Studio Modeling Environment, Release 4.3 (San Diego: Accelrys Software Inc., 2007) were used to construct and equilibrate three independent atomistic bulk models for G, GA1 and GA2 using the CFF91 force field.[15] The amino acid (aa) sequence of gelatin selected for this

study is a 276 aa portion of a human collagen type I (GenBankTM accession No. NP_000079). DAT and DATT were attached through an amide bond to the amino functions of each lysine residue of the gelatin molecule. The basic technique for packing and equilibration of the cells are described elsewhere.[16] A final 5 ns NPT-MD simulation at 30 °C, with a time step of 1 fs is run for data collection. The calculated elastic constants of the gelatin models were obtained utilizing a constant-strain minimization method (static method) applied to the equilibrated system.[17] The X-ray scattering intensity I(Q) of the models was calculated from the projections of each of the interatomic vectors, $\mathbf{r}_{jk}$, on the respective scattering vector Q.[18] Aromatic interactions were calculated from the spatial distance of centroids atom, defined for every aromatic ring, along the trajectory when the geometric distance between two of them is below 7.5 Å. Hydrogen bond interactions involving the phenolic group were calculated along the trajectory using the following criteria ($1 < d_{D\text{-}H\text{-}A} < 2.5$ Å, D-H-A > 90.0°).

Synthesis of Desaminotyrosyl-tyrosine (DATT)(4)

Desaminotyrosine (1) (76 mmol) was activated with EDC (91 mmol), and DIPEA (215 mmol) in NMP (80 mL) and further reacted with H-L-Tyr-OtBu (63 mmol) in NMP (30 mL) at -5 °C for 1h and 17 h at room temperature. The mixture was precipitated in water, and extracted with ethyl acetate, the organic phases washed with 0.1 M aq. HCl, 0.1 M aq. $NaHCO_3$, and conc. NaCl solution, and dried over $MgSO_4$, to give 3 (14.35 g, 69 %) as white powder. ^{1}H NMR (DMSO-d_6) δ = 9.18, 9.10 (s, OH), 8.09 (d, NH), 6.95 (t, Ph), 6.63 (t, Ph), 4.27 (m, C_αH), 2.79 (1H, m, C_βH) , 2.73 (1H, m, C_βH) , 2.63 (2H, m, C_βH), 2.31 (t, C_γH), 2.33 (t, C_δH), 1.32 (s, CH3) ppm; IR (υmax/cm^{-1}): 3343 (O-H), 2978, 2957 (C-H alkane), 1712 (s, C=O), 1645 (s, N-H), 1614 (N-H), 1600 (N-H), 1514 (vs, C-N), 1368 (C-O-), 1152 (C-O). ($C_{22}H_{27}NO_5$) m/z (ESI): Calcd. 385.19; Found 386.19 (M+H+), m.p.: 140.3 °C.

Desaminotyrosyl-tyrosine-OtBu (3) (23 mmol) was deprotected by reaction with TFA (76 mL) in DCM (153 ml) at 0 °C (1h) first and 12 h at room temperature. The solvents were evaporated under reduced pressure, the residue was redissolved in 0.1 M HCl and extracted with ethyl acetate. The organic phase was washed with conc. NaCl solution, dried over anhydrous $MgSO_4$, filtered, and evaporated to yield 4 as a white powder (7.1 g, 93%). ^1H NMR (DMSO-d_6) δ = 12.57 (s, COOH), 9.18 (s, OH), 8.06 (d, NH), 6.96 (ad, Ph), 6.63 (ad, Ph), 4.34 (m, $C_\alpha H$), 2.89 (m, $C_\beta H$), 2.72 (m, $C_\beta H$), 2.61 (t, $C_\gamma H$), 2.3 (t, $C_\delta H$) ppm; IR (υmax/cm^{-1}): 3247 (OH), 2957-2919 (C-H aliphatic), 1713 (vs, C=O), 1651 (s, N-C), 1614 (N-H), 1600 (N-H), 1514 (vs, C-N), 1550 (COO^-), 1333 (COOH), 682 (COOH). ($C_{18}H_{19}NO_5$): m/z (ESI) Calcd. 329.13; Found: 330.13 ($M+H^+$); m.p. 161.2.

Functionalization of gelatin

DAT or DATT (29 mmol) was activated by reaction with EDC (32 mmol.) and NHS (43 mmol) in 110 mL of DMSO at 37 °C. After 3 h, β-Mercaptoethanol (43 mmol) was added. A gelatin solution (15 g in 150 mL DMSO) was then added to the mixture and stirred at 37 °C for 5 h. The functionalized product was then precipitated in ethanol, filtered, washed with ethanol and acetone, and dried under vacuum. The degree of substitution was determined by ^1H NMR spectroscopy and a TNBS colorimetric assay.[13]

Material characterization

Films (thickness = 380 ± 30 μm) were prepared by casting a 5 wt.-% aq. gelatin solution into polystyrene Petri dishes followed by drying at a temperature of 40 °C and 80% r.h. Shortly after drying, the films were statically hydrated in a controlled r.h. chamber at 80% r.h. and 30 °C for 1 week. *Thermal analysis* was performed using a Phönix DSC 204 F1 (Netzsch) differential scanning calorimeter. *Wide angle X-ray scattering* (WAXS) measurements were carried out using the X-ray diffraction system Bruker D8 Discover with a two-dimensional detector from Bruker AXS (Karlsruhe, Germany) equipped with a copper tube operating at 40 kV and 40 mA producing Cu K_α-radiation (λ = 0.154 nm). *Tensile tests* were performed on a

Zwick Z005 (Zwick GmbH, Germany) using standard test specimen (ISO 527-2/1BB) punched from the gelatin films for a minimum of 5 probes. The *degree of swelling* Q in water was calculated at equilibrium at 23 °C.

Results and Discussion

Molecular Modeling Investigations

Molecular models of pure gelatin G and functionalized gelatins GA1 and GA2 with 25 wt.-% water content were realized as in this state not only a monomolecular layer of water molecules around the peptide backbone is present, but there are tightly bound water molecules and freely moveable water molecules as well that allow chain movement.[19]

The kinetic and potential energies were plotted as a function of simulation time, verifying that they fluctuate randomly about constant mean values, which proved that the models were in equilibrium (data not shown). Then, the generated models were validated against experimental data. The dihedral angles of the peptide backbone ϕ, ψ, and ω, for the three models were analyzed (data not shown). The ω values were close to 0° and ± 180°, which correctly shows the *cis* and *trans* amide bonds in peptide chains and reflect the partial double bond character of amide bonds. This result confirmed the suitability of the used force field to model peptidic structures. The torsional angles φ (N-C-Cα-C) and ψ (C-Cα-C-N) showed a more statistical organization along the simulation time that correlated with the simulated amorphous structure. Nevertheless, certain features of the typical helical collagen-like conformation were retained as indicated by the peak around -77° for φ and around 160° for ψ. The calculated WAXS spectra (data not shown) corresponded to amorphous materials without regular helical chain organization.

The most important point in the introduction of aromatic groups into gelatin were the potential interactions between aromatic rings at different functionalization sites, which would lead to additional intra- and interchain contacts. The formation of aromatic interaction clusters

requires a certain distance and steric arrangement of the participating aromatic groups, where typical interactions are π–π stacking arrangements and edge-to-face CH/π-contacts. The distance of aromatic group centroids in both cases should not have a distance larger than 7.5 Å to allow effective interaction.[20,21] The collagen-like triple helix of gelatin and the introduced netpoints are schematically shown in **Figure 1**a and visualized for the different models in Figure 1b-d. The frequency of such contacts between pairs of phenyl rings, not considering internal interactions between the two phenyl rings of one DATT functionalization site, were averaged over 3 independent packing models. An increased number of phenyl-phenyl cluster interactions was clearly observed for the models with an increasing number of phenyl groups in the modifier (gelatin: 0.3 ±0.6, GA1 1.7 ± 1.2, GA2 6 ± 1.7 contacts). Hydrogen bonds from or to the phenolic groups to the polymer backbone, functional side groups, and other phenols were identified as well, representing 16% (GA1) to 20% (GA2) of all hydrogen bonds in the models. This means that the desired physical netpoints are actually represented in the models and, as they increase with increasing number of tyrosine-derived moieties, thermomechanical material properties were likely to change depending on the functionalization. The calculated values for Young's modulus E, compressive modulus K and shear modulus G, which were determined by a static method,[22] were similar for all models (**Table 1**). The models furthermore gave insight in the free volume accessible to water, which was quite similar in all materials. However, due to the higher hydrophobicity of GA1 and GA2 to G, a lower water binding capacity for the functionalized gelatins was expected.

Syntheses

Desaminotyrosyl tyrosine DATT (4) was synthesized as shown in **Scheme 1**. The reaction of either phenol group in the coupling or deprotection step was not observed. The degree of functionalization of GA1 and GA2 was determined by two independent methods giving similar values: integration of ^{1}H NMR signals and a colorimetric assay based on the reaction

of free amino groups with TNBS. Functionalization with DAT gave 80 ± 10 mol-% yield (with respect to gelatin lysine residues) while functionalization with DATT gave 91 ± 3 mol-% yield, both of which are good yields for polymer analogous reactions. As the degree of functionalization based on NMR (giving the total amount of introduced aromatic rings) and reaction of amino functions gave similar results, it could be demonstrated that under the chosen reaction conditions only the amino functional groups were reactive. The hydroxyl functions on serine and threonine were nonreactive, which permitted a systematic and controlled functionalization of gelatin, albeit with a limited concentration of aromatic modifiers. Gelatin films were prepared by drying a 5 wt.-% aqueous solution at 40 °C at a r.h. of 80%.

Material Characterization

Material properties are summarized in **Table 2**. The degree of helicalization (X_c) of the gelatin chains in dry films was directly studied with WAXS. In addition to the scattering peak at $2\Theta = 21°$, which represents the amorphous region of the material, unmodified gelatin showed peaks at $2\Theta = 8°$ and 32° corresponding to triple helical regions and α-helical individual segments, respectively.[23] The latter peaks were also observed for GA1, though much weaker than in unmodified gelatin. GA2 displayed only one peak at 21°. The tendency to adopt typical helical collageneous features was therefore reduced by the introduction of the bulky aromatic groups in GA1 and GA2 though not totally suppressed.

Equilibration in a thermochamber at 30 °C and 80% r.h. for seven days was then performed in order to set the water content for all sample films to 25 wt.-%, which corresponded to the molecular models. The melting temperatures T_m were taken from the first heating run in TM-DSC experiments, while the glass transition temperatures T_g were taken from the second heating run.[24] The functionalized materials showed a reduction of T_m (from 144 °C to 123-127 °C) with a simultaneous reduction of the melting enthalpy comparing gelatin and the

functionalized gelatins, whereas the transition was very broad (~50 °C). The reduction of T_m can be related to a reduction in helices length, while the reduction of ΔH_m is likely due to a reduction of overall level of order (crystallinity or helicity). The low values of ΔH_m of 14-22 $J \cdot g^{-1}$ reflect a low overall crystallinity of the materials. T_g differs only slightly between gelatin and functionalized gelatins (110 °C compared to 103-105 °C) and might be related to an increase in dangling chain ends. The thermal transitions are well below thermal decomposition (> 250 °C as measured by TGA).

The influence of the degree of aromatic functionalization and of the related helical suppression can also be seen in the mechanical properties of the films as determined using tensile tests. The introduction of aromatic substituents on gelatin led to a more brittle behaviour of the respective materials. Although the Young's moduli were changed only slightly, a pronounced effect was observed in the reduction of maximum tensile strength σ_{max} and elongation at break ε_b, which is most likely related to the decreasing contents of crosslinking triple helical domains.

All experimental results showed a clear reduction of helicity of the functionalized gelatins (< 1%). The introduction of additional bulky side chains on free amino groups, e.g. on the lysines, were likely to sterically hinder the helicalization and block hydrogen bonds to these groups needed for the association of different chains or the binding of water, which would increase chain mobility. The additional $\pi-\pi$ interactions and hydrogen bonds from the tyrosine derivatives might increase chain rigidity. However, as there is only a limited number of free amino groups present in gelatin, still some helicalization was observed. Physical crosslinks formed by small aromatic clusters are likely to be less stable against deformations than large triple helical domains. Together with the observed reduction in helicity, this is the reason for the experimentally observed reduction in Young's modulus of GA2, which was functionalized with molecules bearing two aromatic groups (DATT).

The most prominent effect of gelatin functionalization was observed when samples were immersed in an aqueous environment. A clear decrease in the degree of swelling (Q) with increasing number of aromatic moieties introduced per amino group was detected (Gelatin showed a degree of swelling of 2800 Vol.-%, while the introduction of one aromatic moiety per lysine residue led to a decrease in Q (1730 ± 130 Vol.-%) and GA2 showed an even more drastical reduction of Q (265 ± 55 Vol.-% in absolute values). This behaviour is likely to be of interest for potential biomedical applications of gelatine-based materials, e.g. in Regenerative Therapies.[25-27]

Conclusion

The strategy to systematically change the properties of gelatin-based materials by selective functionalization was successful. For this purpose, molecular models of (functionalized) gelatins as amorphous bulk materials were developed and analyzed, which predicted an increasing number of specific π–π interactions and hydrogen bonds of gelatins functionalized with tyrosine derived compounds when increasing the number of the phenol moieties. Experimental data on synthesized compounds showed variation of macroscopic properties according to the number of introduced aromatic groups. The influence of already a relatively small number of these novel functional groups led to a dramatic reduction of swelling capacity and noticeable influence on other properties such as the elastic modulus as well as chain organization. These are key properties whose control will broaden the scope of applications for gelatin-based materials. The modeling gave results in the same order of magnitude (e.g. Young's modulus) and trends as in the experimental data. Differences between the models and synthesized materials are likely because the models were idealized structures. Future modeling studies could be used to predict the effects of even higher degrees of functionalization (e.g. on alternative attachment points or by utilizing modifiers with more

than two aromatic units) or other functional groups for a knowledge-based decision on synthetic targets.

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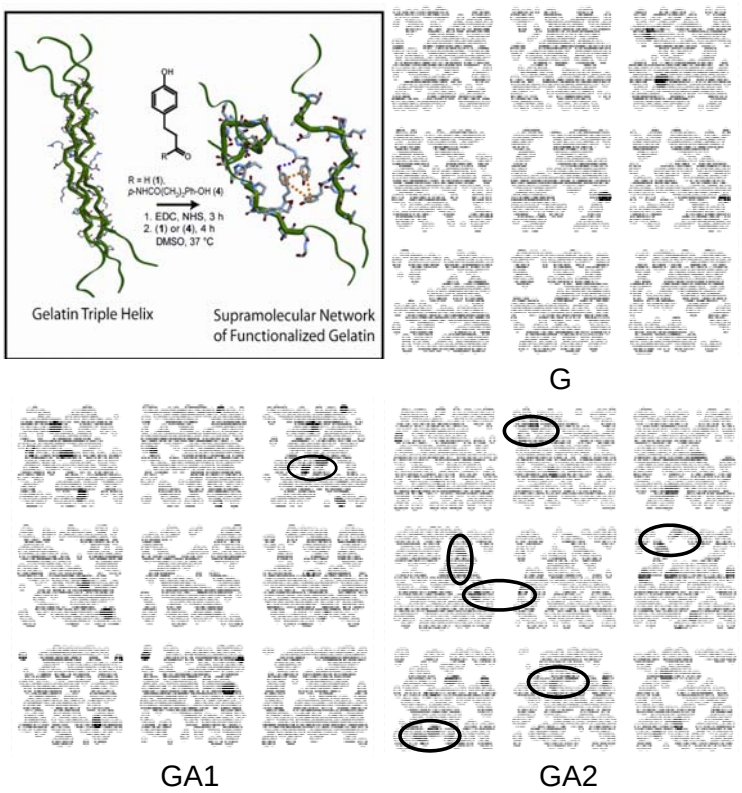
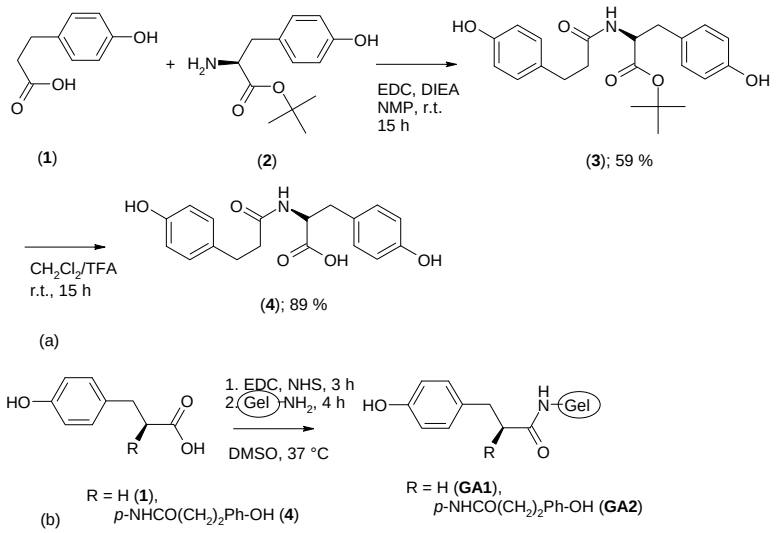


Figure 1. a) While pure gelatin forms physical netpoints by triple helicalization, functionalization with tyrosine-derived molecules reduces helicalization and leads to specific interchain contacts by π–π interactions ('aromatic clusters') and hydrogen bonds. b-d) Representation of the chain organization and aromatic clusters (circled) of the equilibrated models of G (b), GA1 (c), and GA2 (d) as a series of 9 about 4.0 Å thick slices cut perpendicularly to the x-axis for each model. The aromatic rings are highlighted in dark.

Table 1 Summary of calculated Young's modulus *E*, compressive modulus *K*, and shear modulus *G* for G, GA1, and GA2 at 25 wt.-% water content.

Mechanical property	**G**	**GA1**	**GA2**
E [GPa]	6.67 ± 0.44	6.43 ± 0.26	6.65 ± 0.06
K [GPa]	5.82 ± 0.08	5.89 ± 0.27	5.71 ± 0.11
G [GPa]	2.55 ± 0.19	2.44 ± 0.03	2.54 ± 0.03



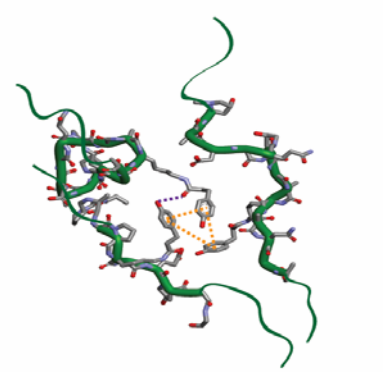
Scheme 1. Synthetic route to (a) desaminotyrosyl-tyrosine and (b) gelatin functionalization.

Table 2: Thermal transitions, degree of helicity, mechanical properties, and degree of swelling of gelatin and the functionalized gelatins.

	T_m [1] [°C]	ΔH_m [1] [J·g^{-1}]	T_g [2] [°C]	ΔC_p [2] [J·(g·K)$^{-1}$]	X_c [%]	E [Gpa]	σ_{max} [MPa]	ε_b [%]	Q [vol.-%]
G	144	22	110	0.35	2.3	2.26 ± 0.27	69 ± 18	6 ± 1.2	2800 ± 330
GA1	123	17	103	0.35	0.9	2.03 ± 0.24	51 ± 27	3 ± 0.8	1730 ± 130
GA2	127	14	105	0.36	0.8	2.08 ± 0.46	39 ± 10	3 ± 0.9	265 ± 55

T_m: melting temperature, ΔH_m: melting entropy; T_g glass transition temperature, ΔC_p: change of heat capacity at T_g, X_c: degree of helix content, E: Young's modulus, σ_{max}: maximum tensile strength, ε_b: elongation at break, Q; degree of swelling, [1] determined in the 1st heating run, [2] determined in the 2nd heating run.

The functionalization of gelatin with tyrosine derivatives specifically at the lysine residues leads to reduction of Young's modulus and degree of swelling. The netpoints of the physical networks are aromatic clusters rather than triple helical regions, as could be shown by WAXS and molecular modeling, and therefore depend on the degree of functionalization and not the thermomechanical treatment of the materials.



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