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Published in:
Advanced Functional Materials

DOI:
[10.1002/adfm.201601612](https://doi.org/10.1002/adfm.201601612)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Liu, Y., Stuart, M. C. A., Buhler, E., Lehn, J.-M., & Hirsch, A. K. H. (2016). Proteoid Dynamers with Tunable Properties. *Advanced Functional Materials*, 26(34), 6297-6305. <https://doi.org/10.1002/adfm.201601612>

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Proteoid Dynamers with Tunable Properties

Yun Liu, Marc C. A. Stuart, Eric Buhler,* Jean-Marie Lehn,* and Anna K. H. Hirsch*

A range of doubly dynamic proteoid biodynamers based on the polycondensation of various categories of amino acid hydrazides with a dialdehyde have been generated through formation of two types of reversible C=N bonds (imine and acylhydrazone). Their structures and properties (rates of polymerization and dynamic character) have been characterized by NMR, small-angle neutron scattering, dynamic light scattering, and cryo-transmission-electron microscopy. Three types of structures (nanorods, globular nano-objects, oligomers) are obtained at different rates after polycondensation. Competitive polymerization shows that electrostatic effects markedly influence polymerizations when two oppositely charged monomers are used, and an exchange experiment demonstrates the preferential incorporation of a specific monomer in the biodymer chain. Taken together, all our results show that the side chains of the amino acid hydrazides have a strong influence on the rates of polymerization, structures, and dynamic properties of the resulting biodynamers. The present study provides a basis for the rational design and synthesis of various types of well-ordered structures and adaptive materials, offering great potential for utilization in the field of biomaterials science.

1. Introduction

Constitutionally dynamic chemistry (CDC)^[1] is characterized by the formation of reversible linkages, either reversible covalent bonds or noncovalent interactions as connections between subunits of a molecular or supramolecular entity, and provides

a new way to rationally designed, functional materials. As a consequence of the dynamic character, the resulting constitutionally dynamic materials (CDMs)^[2] can present advantages over constitutionally “static” materials, such as self-healing, tunable mechanical and optical properties, bio- or environmental degradability, and stimulus-responsiveness.

Applying CDC to the field of polymer chemistry leads to dynamic polymers, dynamers,^[2,3] in which monomers are connected by either noncovalent or reversible covalent connections.^[4] These adaptive materials are formed by polycondensation of ditopic monomers that bear complementary functional groups. Various types of reversible covalent bonds such as thermally activated alkoxyamines,^[5] Diels–Alder linkages,^[6] imines,^[7] boronate esters,^[8] and disulfides^[9] can be used as the linkage of monomers. Such dynamers are able to undergo changes in response to external stimuli even after polymerization,

enabling adaptations in their constitution, length and sequence. As a consequence, their mechanical^[10] and optical properties^[11] are controllable and may also be adaptable. These modifications can be induced by physical or chemical stimuli, such as light,^[12] temperature,^[13] pH,^[14] electrical field,^[15] sound,^[16] and metal cations.^[17]

The incorporation of biologically relevant molecules leads to the generation of biodynamers,^[18] in which the functional properties (biocompatibility, recognition, catalysis) of naturally occurring biopolymers and dynamic character of CDMs are combined. Dynamic nucleic acid analogues (DyNAs)^[18b] are biodynamers bearing nucleobase residues; they are of great interest for complementary binding to nucleic acid strands, making them good candidate vectors for nonviral gene delivery. Dynamic analogues of polysaccharides^[18c,d] (glycodynamers) have attracted our attention given the involvement of polysaccharides in a wide range of biological or pathological events such as immune response, inflammation, cancer, cell adhesion or cell–cell recognition.^[19] All these biodynamers have great potential as “smart” biomaterials, which may be utilized in biomedical and bioengineering fields.

In addition to DyNAs and glycodynamers, we have recently reported dynamic proteoid polymers prepared via the condensation of a water-soluble dialdehyde (nonbiological component) with amino acid hydrazides (biological component), featuring two different types of reversible C=N bonds (imine and acylhydrazone), thus being doubly dynamic.^[20] We found that the reversible polycondensation is driven by the

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DOI: 10.1002/adfm.201601612

self-organization/folding of the polymeric material formed, reminiscent of the hydrophobic effect, the main driving force for protein folding. Proteoid biodynamers are of particular interest in relation to the issue of how far protein folding has been instrumental in the evolution of the primary sequence toward a well-defined 3D structure.

Based on these considerations, we have performed a comprehensive study on the influence of side chains of amino acid hydrazides on the properties of biodynamers. We report here the design and synthesis of a range of proteoid dynamers featuring a variety of amino acid hydrazides and characterize them in terms of structure, rate of polymerization, and dynamic character using a variety of physical techniques.

2. Results and Discussion

2.1. Design and Generation of Dynamic Proteoids

Reversible imine-type bonds have been implemented in biomedical and material sciences to generate self-healing gels, pH-responsive micelles as drug-delivery vehicles and bioactive substances.^[21] By linking the monomers with reversible imine bonds, the resulting biodynamers are endowed with a pH-responsive character due to the basicity of the reactive amines. Owing to the different reactivities of the amino and carbonyl groups used, the resulting imine- or acylhydrazone-bearing biodynamers can have different stabilities. The presence of two types of C=N bonds (true imines and acylhydrazones) in one biodnamer affords doubly covalent biodynamers, potentially displaying a third form of dynamic behavior through structure-formation processes (conformational dynamics).^[22]

Doubly dynamic proteoids are formed by the polycondensation of a water-soluble dialdehyde and bifunctional amino acid hydrazides in a nucleation–elongation (N–E) manner with component selection.^[20,23] In order to systematically investigate the influence of the amino acid side chain, we chose to use the amphiphilic dialdehyde **1** (see Figure 1), featuring a tricyclic aromatic core and a hexaglyme chain.^[24] For reactions performed in an aqueous environment, the hexaglyme moiety endows the

biodynamers with water-solubility, as biodnamer formation is driven by hydrophobic interactions involving the tricyclic core and stabilization by the hydrophilic hexaglyme chains, which are solvent-exposed. In addition to π – π -stacking interactions between the dialdehyde core and the side chains of aromatic amino acid hydrazides, the polarity and charge of the amino acid side chain may also play a role. Furthermore, the proteoid biodynamers may be affected by the presence of cations in the polymerization buffer due to the numerous metal-binding sites and the potential for cation– π interactions. Along these lines, we chose amino acid hydrazides with various types of side chains such as **2–4** with aromatic rings, **5–8** with charged side chains, and **10–11** with different polarity (see Figure 1) as the other component of dynamic proteoids to evaluate various factors that might influence the dynamic character and structure of the resulting doubly dynamic proteoids (Scheme 1).

In our previous study,^[20] we found the mechanism of polymerization to be N–E. The main feature and crucial step of N–E polymerization is nucleation, that is, the formation of a critical size of polymer chain (nucleus), after which elongation of the existing polymer becomes more favorable than initiation of a new chain. At pD 5, acylhydrazone formation proceeded readily and went to completion, whereas the amine did not afford the corresponding imine. However, the driving force of polycondensation, which lies in the enhanced stability of dynamic proteoids resulting from folding of the main chain through hydrophobic and π – π -stacking interactions, favored the nucleation and led to the formation of biodynamers. It is still unclear whether the polymer chain necessarily consists of alternating imine and acylhydrazone bonds or of scrambled connections. We also observed that the side chain of the amino acid hydrazide has a direct influence on the structure and physicochemical properties of the resulting biodynamers. This can be ascribed to different degrees of stabilization of the resulting polymer chains depending on the side chain of the building block. Based on our previous experience, we prepared doubly dynamic proteoids by polycondensation of dialdehyde **1** and amino acid hydrazides **2–11** (see Figure 1) in aqueous solution at pD 5 (5×10^{-3} or 10×10^{-3} M, NaCl, CsCl solution or d_3 -acetate buffer). Under these conditions, both imines and

Figure 1. Structures of the dialdehyde **1** and of amino acid hydrazides used.

Scheme 1. Generation of doubly dynamic proteoids using one equivalent of dialdehyde **1** and one equivalent of amino acid hydrazides. Poly(1-3) was synthesized at a concentration of 5.0×10^{-3} M in an aqueous solution of 0.1 M NaCl and 0.1 M CsCl at pD 5. Poly(1-5) was synthesized at a concentration of 10.0×10^{-3} M in aqueous d_3 -acetate buffer at pD 5. The other dynamic proteoids were synthesized at a concentration of 5.0×10^{-3} M in aqueous d_3 -acetate buffer at pD 5. The monomers **1** and **5** and an unfolded heptamer are shown as space filling models (hexaglyme groups in monomer **1** omitted for clarity).

acylhydrazones are efficiently formed to generate the desired dynamic proteoids. The polycondensation was monitored by ^1H -NMR spectroscopy, and the resulting dynamic proteoids were characterized by small-angle neutron scattering (SANS),^[25] dynamic light scattering (DLS),^[26] and cryo-transmission-electron microscopy (cryo-TEM) to provide further information on the shape and size of the biodynamers obtained.

2.2. Structural Characterization of the Dynamic Proteoids

We investigated solution-state polymer morphologies by SANS, DLS, and cryo-TEM, given that mass spectrometry does not provide information on the length of the intact polymers due to the inherent lability of the imine linkages.

2.2.1. Effect of Side Chains

Figure 2 shows variations in scattered neutron intensity as a function of the scattering wave vector q for each of the studied polymers in D_2O in aqueous d_3 -acetate buffer at pD 5. The solutions were homogeneous, clear, transparent, and stable over time. We observed three strikingly different characteristic behavior patterns, which are represented in Figure 2a–c, respectively, as described hereafter.

Nanorod Behavior: Large Objects with High Scattered Intensity: Figure 2a displays the scattering profile of samples poly(1-5) and poly(1-6). The two spectra are markedly similar, suggesting that morphologically related structures are present in

solution. Each scattering profile exhibits a Guinier regime at low q associated with the finite size of the scattering objects, one intermediate regime in which the q -dependence of the scattered intensity can be described by a power law with an exponent close to -1 , a second Guinier regime at the upper q range corresponding to the cross section of the scattered objects, and eventually a dip, which is the initial part of the oscillating term of the shape-dependent form factor of the particle cross section (see Figure 2a dashed lines). The intermediate q^{-1} dependence, which is most apparent for poly(1-5), is characteristic of objects of rigid-rod structure; whereas the Guinier regimes at high and low q values can be used to extract information regarding the cross section of the rods and the weight-averaged molecular weight (M_w)/radius of gyration (R_g) of the polymer, respectively. Each of the two spectra fit well to a Guinier model at low q values and to rigid-rod and cross section models at larger q , which yield measurements describing both the global (M_w and R_g) and local (linear mass density (μ), cross-sectional area (a_c), and cross-sectional radius of gyration (r_c)) structural features of the rod-like objects (see Table 1).

The data obtained at low- q values corresponding to relatively large spatial scales can be fitted by the Guinier law shown in Equation (1)

$$\frac{1}{I(q)} = \frac{1}{I(0)} \left(1 + q^2 \frac{R_g^2}{3} \right) \quad (1)$$

The extrapolation to zero- q of the scattered intensity, $I(q^2 = 0)$, provides a measure of the weight-averaged molecular weight (M_w) of the particle. The inset of Figure 2a shows the plot of

Figure 2. SANS profiles collected at 20 °C. Each polymer was prepared by allowing the appropriate monomers to equilibrate and the spectra did not change anymore after 2 d in aqueous d_3 -acetate buffer at ambient temperature and pD 5 prior to data collection. For clarity, the spectra have been shifted by one log unit along the y-axis with respect to each other. The inset shows the plot of $1/I$ versus q^2 and the best linear fit to the data (see Equation (1)). a) poly(1-6) (5×10^{-3} M) and poly(1-5) (10×10^{-3} M) samples; b) poly(1-4) (5×10^{-3} M), poly(1-10) (5×10^{-3} M), and poly(1-11) (5×10^{-3} M) samples; c) poly(1-8) (10×10^{-3} M), poly(1-9) (5×10^{-3} M), and poly(1-7) (5×10^{-3} M) samples; d) poly(1-3) (5×10^{-3} M) in the presence of NaCl or CsCl at 0.1 M and pD 5.

$1/I$ versus q^2 for both samples. From the best linear fit to the data, (R_g) = 97 ± 5 and 57 ± 5 Å for poly(1-5) (10×10^{-3} M) and poly(1-6) (5×10^{-3} M), respectively, are obtained. The extrapolation of $I(q)$ to $q = 0$ is relatively high indicating the presence of large objects, and provides $M_w = 138 \pm 14$ and 72 ± 7 kDa, which corresponds to a degree of polymerization (DP) of about 214 and 107 for poly(1-5) and poly(1-6), respectively.

In the intermediate q regime, the scattering curves can be fitted satisfactorily by a rigid-rod model. Figure 2a shows

the fits realized for both polymers by means of the form factor^[18d,24d,25,27] derived for rigid-rod particles and given by

$$P(q) = \frac{\pi}{qL} \quad (2)$$

where L is the contour length of the rod. In the dilute range, the R_g of rod-like particles with large aspect ratio is given by $R_g^2 = L^2/12$. For poly(1-5) and poly(1-6) we obtain $L = 336$

Table 1. Structural parameters obtained from fitting of data presented in Figure 2a to a low- q Guinier law, a rigid-rod model, and a high- q Guinier expression for the form factor of the section. (M_{dimer} = dimer molecular weight, ϕ = monomer volume fraction, $\Delta\rho^2$ = contrast per unit volume, $I(q^2 = 0)$ = scattered intensity extrapolated at $q = 0$, M_w = weight-averaged molecular weight, DP = averaged degree of polymerization, R_g = radius of gyration, R_h = apparent hydrodynamic radius, $\rho = R_g/R_h$ obtained from combining SANS and DLS, μ = linear mass density, r_c = cross-sectional radius of gyration, a_c = cross-sectional area.)

Sample	M_{dimer} [g mol ⁻¹]	M_w [g mol ⁻¹]	DP	R_g^a [Å]	R_h^b [Å]	R^c [Å]	ρ ratio	$\phi \times 10^{-3}$	$\Delta\rho^2$ [cm ⁻⁴] $\times 10^{21}$	μ [g mol ⁻¹ Å ⁻¹]	r_c [Å]	a_c [Å ²]	$I(q^2 = 0)$ [cm ⁻¹]
Poly(1-5)	643.8	138 000	214.4	97	75	60 ± 30	1.29	4.41	2.137	374	14.95	493	1.48
Poly(1-6)	672.8	71 738	106.6	57	52	45 ± 15	1.10	2.3	2.27	330	12.7	435	0.426

^a) Obtained from SANS experiments; ^b) Obtained from DLS measurements; ^c) Obtained from cryo-TEM experiments. Error bar is 10% for all values except R (Å).

and 197 Å, respectively, in agreement with the model derived for equilibrium polymers that predict a $C^{1/2}$ dependence of the average length of the self-assembled polymers. Thus, the contour-length difference between the two polymers as determined by SANS could also be due to the concentration of the samples, with polymers derived from 10×10^{-3} M Lys-containing monomers giving measurably longer chains than those derived from 5×10^{-3} M Arg-containing monomers. The high q data can be fitted by a Guinier expression for the form factor of the section^[18d,24d,25,27]

$$V_{\text{chain}}P(q) = \frac{\pi a_c}{q} \exp\left(\frac{-q^2 r_c^2}{2}\right) \quad (3)$$

where r_c is the radius of gyration of the cross section. By fitting the two equations above to the experimental data, the linear mass density (μ , mass per unit length) of the polymers can be determined, the cross-sectional area, (a_c) and r_c . From the fits of Figure 2a, we obtain $\mu = 374 \pm 40$ Da Å⁻¹, $a_c = 493 \pm 50$ Å² and $r_c = 15 \pm 1.5$ Å and $\mu = 330 \pm 40$ Da Å⁻¹, $a_c = 435 \pm 50$ Å², and $r_c = 13 \pm 1.5$ Å for poly(1-5) and poly(1-6), respectively. From the values of M_w and μ , the contour length of the polymers is obtained: $L = M_w/\mu = 369$ and 218 Å for poly(1-5) and poly(1-6), respectively, values in excellent agreement with those of 336 and 197 Å obtained using the rod-like model above and indicating the formation of well-defined rods.

This last point is also confirmed by the so-called ρ -ratio derived here from combining the particle size characteristics determined from static (SANS) and dynamic light scattering (DLS) measurements.^[24a] The analysis of the diffusive single relaxation of the scattered electric field correlation function (Figure 3), neglecting Virial effects, allows the determination of the hydrodynamic radius (R_h) of the nanorods. The ρ -ratio is simply defined as $\rho = R_g/R_h$ and provides also an important indication of the particle topology. Values of the apparent R_h and ρ -ratio (1.29 and 1.10 for poly(1-5) and poly(1-6), respectively) are collected in Table 1.

Figure 3. Scattered electric field autocorrelation function, $g^{(1)}(q, t)$, at $\theta = 90^\circ$, for solutions of 5×10^{-3} M poly(1-6) (nanorods), 5×10^{-3} M poly(1-10) (globular nano-objects), and 10×10^{-3} M poly(1-8) (oligomers) in acetate buffer at pD 5 and $T = 20^\circ\text{C}$. The inset shows the normalized distribution of scattered intensity as a function of the size obtained with the Contin method.

These experimental values are in very good agreement with the theoretically calculated values for cylinders of length L and diameter D given by the following expression:

$$\rho = \frac{R_g}{R_h} = \frac{1}{\sqrt{3}} \ln\left(\frac{L}{D} - 0.5\right) \quad (4)$$

Indeed, calculation gives $\rho = 1.36$ and 1.13 for poly(1-5) ($L = 336$ Å and $D = 30$ Å) and poly(1-6) ($L = 197$ Å and $D = 26$ Å), respectively.

Although cylindrical nanorods formed by poly(1-5) seem a little more compact than those of poly(1-6), the fitting results presented in Table 1 suggest that within the error bars, the local structures of the various rods are rather similar (μ , a_c , and r_c). Assuming full disk-shaped cross sections with $a_c = \pi R^2$, leads to incompatible results as the obtained geometrical radius of the disk $R = 12.2$ Å is smaller than r_c . A possible explanation is the formation of hollow cylinders with a cross-sectional area given by $a_c = \pi(R^2 - R_i^2)$ and $r_c = \sqrt{2/2} \times \sqrt{(R^2 + R_i^2)}$, where R and R_i are the external and internal radii of the section of hollow cylinders, respectively. Using experimental values of a_c and r_c , we find ($R = 17.4$ Å, $R_i = 12.04$ Å) and ($R = 15.2$ Å, $R_i = 9.6$ Å) for poly(1-5) and poly(1-6), respectively. The thickness of the section given by $R - R_i$ is equal to ≈ 5.5 Å for both polymers. The formation of cylindrical nanorods presumably can be explained by cisoid conformations of the N–N bonds (acylhydrazones), which allows the winding of the polymer backbone into a regular helical conformation to form the walls of cylinders, leaving the hexaglyme substituents solvent-exposed to stabilize the cylinder.^[24d] Poly(1-5) is presumably packed in a more compact manner than poly(1-6) owing to the weaker repulsion between cationic sites in the former polymer.

Globular Nano-Objects: Figure 2b displays the neutron scattering patterns obtained for poly(1-4), poly(1-10), and poly(1-11). For these polymers, there is only one Guinier regime associated with the mass and R_g of the nano-objects. An oscillation associated with the shape-dependent form factor of the particle cross section (indicated by an arrow) is slightly visible for sample poly(1-4) that also exhibits the highest scattering signal. The absence of the q^{-1} -dependence of $I(q)$ in the intermediate q -range indicates that these polymers may not be considered as rigid rods anymore. Values of M_w and R_g obtained using a Guinier analysis (see Equation (1)) and neglecting the excluded volume interactions, are collected in Table 2. The difference in M_w is in line with the difference in rates of polymerization and may be attributed to the difference of hydrophobicity or concentration for poly(1-11) (5×10^{-3} M).^[20]

For poly(1-11), the Guinier plateau is followed by a slight upturn at low- q associated with the presence of few larger aggregates of ≈ 100 nm (less than 0.01% in g cm^{-3}) as confirmed by DLS measurements. These aggregates display a characteristic slow mode that also prevents any size determination in the short time range with a very good accuracy. The formation of these minority aggregates may result from the presence of impurities. We can thus neglect the presence of this minute aggregated matter and consider the system as a quasi-mono-disperse solution (single population) of globular nano-objects. Nevertheless, the electric field correlation function is mainly characterized by a fast diffusive relaxation (see Figure 3) with

Table 2. Structural parameters obtained from fitting of data presented in Figure 2b–d to a low- q Guinier law.

Sample	M_{dimer} [g mol ⁻¹]	M_w [g mol ⁻¹]	DP	$R_g^{a)}$ [Å]	$R_h^{b)}$ [Å]	$R^{c)}$ [Å]	ρ ratio	$\phi \times 10^{-3}$	$\Delta\rho^2$ [cm ⁻⁴] $\times 10^{21}$	$I(q^2 = 0)$ [cm ⁻¹]
Poly(1–3) NaCl 678.8		39 233	57.8	28.5	40	— ^{d)}	0.7125	2.3	1.9	0.195
Poly(1–3) CsCl 678.8		18 912	27.9	16.7	25 ^{e)}	— ^{d)}	0.67 ^{e)}	2.3	1.9	0.094
Poly(1–4)	652.74	31 995	49	36.42	45	30	0.81	2.24	1.823	0.1486
Poly(1–7)	630.7	6277	10	9	— ^{f)}	14	—	2.2	1.91	0.03
Poly(1–8)	644.7	3000	4.7	9.32	15 ^{e)}	14	0.62 ^{e)}	4.41	1.883	0.02834
Poly(1–9)	614.73	4141	6.7	9.31	16 ^{e)}	12	0.58 ^{e)}	2.1	2.123	0.021
Poly(1–10)	602.7	21 447	35.6	30	36	19	0.83	2.06	1.99	0.1
Poly(1–11)	616.7	18 489	30	25.2	40 ^{e)}	18	0.63 ^{e)}	1	2.04	0.0429

^{a)}Obtained from SANS experiments; ^{b)}Obtained from DLS measurements; ^{c)}Obtained from cryo-TEM experiments. Error bar is 10% for all values; ^{d)}Cryo-TEM experiments were not performed; ^{e)}The presence of a slow mode due to a few large aggregates prevents any R_h , and thus ρ -ratio, determination with a very good accuracy. In this case, R_h is obtained by applying the Contin method to our data; ^{f)}The amplitude of the slow mode is too large and prevents any analysis of the correlation functions in the short time range and thus determination of the nano-objects R_h .

characteristic time inversely proportioned to q^2 , giving R_h for poly(1–4), poly(1–10), and poly(1–11) (see Table 2). Indication on the structure and degree of compactness of the nano-objects is provided here by the ρ ratio. Neglecting the Virial effects, the value of the ratio is lower than 1 for the three samples (comprised between 0.63 and 0.83 that can be calculated from SANS and DLS) and close to 0.775, a value calculated for homogeneous hard spheres and thus indicates the formation of dense and globular structures, reminiscent of folded protein assemblies. Protein-like copolymers are known to afford spherical objects, if both hydrophilic and hydrophobic monomers are used, as is the case with the globular objects we observed earlier.^[20,28] The distribution width obtained using the cumulant method (see Supporting Information) is about 1.5 nm, a value characteristic of systems with a low dispersity.

Oligomers: For polymers poly(1–7), poly(1–8), and poly(1–9), there is again only one Guinier regime but with a much lower scattering level (see Figure 2c). Similar SANS analyses were conducted and gave a degree of polymerization ranging between 5 and 10 depending on the sample and corresponding to oligomers of about 9 Å R_g (see Table 2).

Analysis by DLS of the systems provides information on the composition of the homogeneous solutions at larger scales.^[26,29] Despite filtering through 0.2 µm membranes, oligomer solutions contain also minute amounts of aggregates. The scattered field autocorrelation functions are bimodal (see Figure 3). There are clearly two distinct cooperative relaxation processes with characteristic times inversely proportional to q^2 . The fast relaxation mechanism is associated to the diffusion of oligomers with an apparent R_h of ≈ 15 Å (see Table 2), whereas the slower relaxation indicates the presence of large biodynamer aggregates (≥ 150 nm depending on the samples). As the ratio between the size of aggregates and oligomers is large, the contribution of few large aggregates (less than 0.01%) to the DLS signal is significant as shown by the height of the amplitude of the slow mode. These aggregates are not visible in the SANS q -ranges. The morphology of the oligomer assemblies may be globular with a ρ ratio smaller than 1. However, the presence of this slow mode prevents a determination of R_h with a good accuracy.

Cryo-TEM Studies: The cryo-TEM images (Figure 4 and Figure S1, Supporting Information) give a direct visualization of the structures of polymers and are in good agreement with DLS and SANS results. All the physicochemical and structural features of these polymers are summarized in Table 1. The above results and analysis indicate that three important factors have great influence on the morphology and DP of the biodyn timers: (1) Aromatic rings, should enhance the stability of the resulting polymers presumably through π - π -stacking interactions, so poly(1–2), poly(1–3),^[20] and poly(1–4) form globular nano-objects and display medium DPs. (2) Charge to some degree hinders the folding of polymers (poly(1–5), poly(1–6), poly(1–7), and poly(1–8)) by electrostatic force, so that the resulting polymer cannot form globular nano-objects. However, positively charged polymers (poly(1–5) and poly(1–6))

Figure 4. Cryo-TEM images of (a) poly(1–4), b) poly(1–6), c) poly(1–7), d) poly(1–10). No stain was used and image acquisition was achieved at a 2 µm defocus. Scale bar = 50 nm.

have high DP above 100 and are rod-shaped, whereas negatively charged biodynamers (poly(1–7) and poly(1–8)) feature a DP of less than 10. It appears that interaction between the cations and π cloud of the monomers, and possibly also with the oxygen atoms of the hexaglyme chains, facilitates the formation of the biodymer while the repulsion between anions and the π cloud (as well as with the hexaglyme chains) may hinder polymerization. (3) Polarity. The stabilities of poly(1–10) and poly(1–11) appear to be enhanced, which may be ascribed to hydrogen bonds (OH–O, OH–N, and NH–O) and/or OH– π interactions including the hydroxyl groups in the side chains, therefore, the resulting polymers undergo folding to form globular nano-objects and have medium DP, while poly(1–9) forms oligomers and has a low DP. It indicates that a hydroxyl group in the side chain of an amino acid hydrazide is important for globular nanostructures.

2.2.2. Effect of Metal Cations

The effect of cations in the aqueous solution on formation of the exemplary Tyr-containing biodynamers at 5×10^{-3} M and pD 5.2 is shown in Figure 2d. Interestingly, while the structure of both biodynamers is globular with a ρ -ratio lower than 1 and close to the value predicted for hard spheres (see Table 2), the size is much larger for poly(1–3) in NaCl than for poly(1–3) in CsCl. Whereas the DP is equal to 28 in the presence of 0.1 M CsCl, it reaches a value of 58 in the presence of 0.1 M NaCl. This difference can presumably be ascribed to complexation of Na^+ cations by the hexaglyme chains, which is reflected in a modification in chemical shift between the O–CH₂ signals in D₂O and in 0.1 M NaCl aqueous solution. In contrast, no such change is observed in the case of 0.1 M CsCl, indicating that complexation does not occur as a result of weaker interactions with the oxygen atoms of the hexaglyme chain (see Figure S2, Supporting Information). Na^+ cations may stabilize poly(1–3) through binding to the oxygen atoms and by cation– π interaction with monomer 1. Taken together, these interactions may play a significant role in the generation of all biodynamers.

2.3. Rates of Polymerization, Dynamic Character, and Selectivity

2.3.1. Rates of Polymerization

We investigated the influence of the side chains on the rate of polymerization by monitoring the consumption of dialdehyde 1 in polycondensation under acidic conditions (pD 5). Each of the amino acid hydrazides 4–11 was added to an equimolar amount of dialdehyde 1 to generate the corresponding polymer (see Figure S3–S11, Supporting Information), while (poly(1–2) and poly(1–3) were synthesized as previously described,^[20] and the consumption of dialdehyde 1 was monitored by ¹H-NMR spectroscopy until it was fully consumed and until the spectra no longer changed after 2 d (see Figure S3–S7, Supporting Information). We found that the factors that affect the structure of the polymers also influence the rates of polymerization.

Figure 5. Formation of poly(1–3), poly(1–4), poly(1–5), and poly(1–6). Percentage of unreacted dialdehyde 1 versus time. Poly(1–4) (5×10^{-3} M), poly(1–5) (10×10^{-3} M), and poly(1–6) (5×10^{-3} M) were formed in aqueous d_3 -acetate buffer at pD 5. And poly(1–3) (5×10^{-3} M) in 0.1 M NaCl and CsCl aqueous solution at pD 5. For poly(1–3), due to the extreme line broadening observed, it is impossible to integrate the NMR spectra accurately between $t = 300$ min and $t = 930$ min due to the overlapping signals. After polymerization has reached completion ($t = 2$ d), the signal corresponding to dialdehyde 1 was no longer visible and not affected by overlapping signals.

The generation of poly(1–4) was completed in 2 h (see Figure 5). It corresponds to the fastest polymerization and suggests that simple aromatic rings may greatly accelerate the process of polymerization by stabilizing the resulting polymer through π – π -stacking interactions. In the formation of positively charged rod-like poly(1–5) and poly(1–6) (see Figure 5), dialdehyde 1 was entirely consumed in 1 d, but the negatively charged poly(1–8) and poly(1–7) that yield only oligomers were hard to generate. Even after weeks, signals from dialdehyde 1 were still visible, which is in agreement with the conclusions from the structural characterization and illustrates that positive charge facilitates polymerization, for instance, through cation–O,N and cation– π interactions. On the contrary, negative charge hinders it owing to electrostatic repulsion with these sites. Meanwhile, polymers poly(1–9), poly(1–11), and poly(1–10) underwent medium or slow polymerization, especially for poly(1–9) and poly(1–10), signals of dialdehyde 1 were still present even after 20 d. It indicates that polarity does not appear to play an important role for the rate of polymerization. Taken together, our results show that, on the one hand, the aromatic hydrazides undergo the fastest polymerization, while the positively charged hydrazides are also fast but slower than aromatic ones. On the other hand, the negatively charged and other amino acid hydrazides form the resulting polymers at medium or slow rates. All these results also indicate that there is no correlation between the rate of polymerization and size of the resulting biodymer.

In order to investigate the effect of cations on the rate of polymerization, poly(1–3) was formed in 0.1 M NaCl and CsCl aqueous solution at pD 5 (see Figure 5). After 2 d, the polymerization reached equilibrium. When compared with

poly(1–4), polymerization of poly(1–5) and poly(1–6) in aqueous d_3 -acetate buffer, proceeded fast in the first 300 min, but then slowed down and did not reach completion after 16 h. It suggests that in the first 300 min, the rate may be dominated by interactions between the aromatic rings, after which, the polymerization slows down even if stable and medium-sized polymers are formed and the pD needs to be readjusted to reach completion. Comparing the formation of poly(1–3) in aqueous NaCl and CsCl solution, it is faster with NaCl, presumably complexation of Na^+ by hexaglyme helps to accelerate the reaction, but it slows down in the absence of complexation in CsCl solution.

2.3.2. Monomer Selection in Competitive Polymerization

Based on the above analysis, it is clear that the side chains (aromatic ring, charge, and polarity) have a strong influence on the rates of polymerization and on the structures of the resulting polymers. We were therefore interested to determine whether these factors may lead to preferential polymerization with monomer component selection, as observed in the condensation of dialdehyde 1 with dihydrazides of different types.^[22] To this end, we performed two three-component competition experiments. In the first case, dialdehyde 1 was left to react with monomers 2 and 9 at a concentration of 5×10^{-3} M for each component at pD 5. We chose monomers 2 and 9 based on their different rates of polymerization (fast and slow, respectively) to investigate whether they display similar relative rates in competition experiments. In the second case dialdehyde 1 was reacted with monomers 5 and 8, displaying fast and slow polymerization, respectively, and featuring opposite charge. This system was designed to illustrate whether oppositely charged building blocks might affect the rates in competitive polymerization experiments. We monitored both competition experiments by 1H -NMR spectroscopy.

In the first case (see Figure S13, Supporting Information), dialdehyde 1 was entirely consumed after 1 d, and the system had reached equilibrium. Signals of both monomers 2 and 9 decreased, and monomer 2 was preferentially incorporated into the polymeric chain. Integration showed that 85% of monomer 2 was consumed while only 15% of monomer 9 was incorporated into the biodynamer. This observation is in line with the conclusions from the two-component systems in which building block 2 rapidly afforded true polymers.

In the second case, we observed that the system reached completion after 1 d (see Figure S14, Supporting Information). However, rather than affording poly(1–5) rapidly, a novel three-component polymer was generated preferentially. Based on integration, 40% of positively charged monomer 5 and 60% of negatively charged monomer 8 were incorporated into the resulting polymer. This behavior may be attributed to the fact that the resulting polymer is stabilized through electrostatic interaction between monomers of opposite charge. Thus, electrostatic effects markedly influence competitive polymerizations when two oppositely charged monomers are used.

For neutral monomers, the influence of the side chains on the rates of polymerizations is in line with the individual polymerization rates.

2.3.3. Dynamic Character

The dynamic character of the biodyn timers was demonstrated in a monomer exchange experiment (see Figure S15, Supporting Information). Adding an equimolar amount of monomer 9 (slow polymerization) to the polymer poly(1–3) (fast polymerization) at pD 5, and monitoring by 1H -NMR spectroscopy under acidic conditions showed that the system reached equilibrium on a certain timescale. Integration showed that only 10% of monomer 9 was incorporated into poly(1–3) at the expense of monomer 3. The reverse experiment, addition of an equimolar amount of monomer 3 to poly(1–9) gave similar results (see Figure S16, Supporting Information). Integration of the 1H -NMR spectrum showed that 87% of monomer 9 was substituted by monomer 3 from poly(1–3). Taken together, these results show that the systems indeed reached thermodynamic equilibrium. This preferential incorporation of monomer 3 is probably due to the more hydrophobic character and π - π -stacking interactions that stabilize the resulting polymer. The preferential incorporation of 3 over 9 in the biodynamer chain is in line with the polymeric structures and DPs mentioned above, as well as with the relative rates of formation.

3. Conclusions

We have reported herein a range of doubly dynamic proteoid biodyn timers based on the polycondensation of different types of amino acid hydrazides with the nonbiological dialdehyde 1, through formation of two types of reversible C=N bonds (imine and acylhrazone). We have characterized their structures, rates of polymerization and dynamic character. The polymerization reaction is driven by the self-organization/folding of the resulting polymers. We have demonstrated that the side chains of the amino acid hydrazides have a strong influence on the rates of polymerization, structures, and dynamic properties of the resulting biodyn timers. The aromatic rings in the side chains can greatly accelerate the polymerization and stabilize the resulting polymers presumably by π - π -stacking interactions affording globular nano-objects. Positively charged side chains favor the polymerization and yield rod-shaped polymers, while negatively charged groups hinder polymerization leading to the formation of only oligomers. Hydroxyl groups in the side chains can stabilize the resulting polymers and generate globular nano-objects held together presumably by hydrogen bonds; the rates of polymerization, however, are not markedly increased. Finally, the side chain of leucine slows polymerization down, affording oligomers. Interestingly, we found that cations in aqueous solution affect the properties of the polymer and substantially slow down the rate of polymerization. Furthermore, the selective incorporation of the most suitable component from a pool of two monomers into the polymer chain optimizes the properties of the polymer and the results are in line with the relative individual rates of polymerization. In addition, when two oppositely charged monomers are used, electrostatic effects play a major role, favoring the incorporation of both monomers and generating largely neutral polymers. Taken together, the present results provide a basis for the rational design and synthesis of different types of well-ordered structures and adaptive

materials offering great potential for utilization in the field of biomaterials science. The resulting responsive materials combine the properties of each monomer, especially the biocompatibility stemming from the adaptability provided by the dynamic character resulting from the reversible covalent bonds. Such dynamic biomaterials might thus find application in both biomedical and bioengineering fields.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

Y.L. was supported by a Ph.D. fellowship from the Chinese Scholarship Council. A.K.H.H. received funding from the Dutch Ministry of Education, Culture and Science (Gravitation Program 024.001.035) and gratefully acknowledges the Human Frontiers Science Program for a research fellowship. Financial support was also provided by the European contract DYNAMIC (MRTN-CT-2005-019561), as well as by the University of Strasbourg and the CNRS (UMR 7006).

Received: March 30, 2016

Revised: June 9, 2016

Published online: July 4, 2016

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