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Engineered Narrow Size Distribution High Molecular Weight Proteinoids, Proteinoid-Poly(L-Lactic Acid) Copolymers and Nano/Micro-Hollow Particles for Biomedical Applications

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Abstract

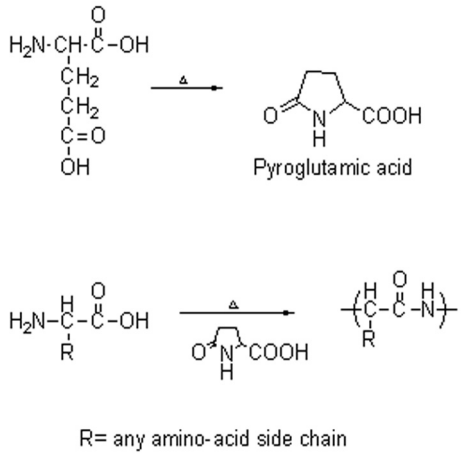
Proteinoids are unusual polymers formed by thermal condensation of amino acids. Several types of proteinoids made of one to three different amino acids, in absence or presence, of low molecular weight poly(L-lactic acid) (PLLA), were synthesized. The polymerization kinetics, molecular weights and physical and mechanical properties of these proteinoids were elucidated. The ability to obtain several high-MW durable proteinoids, by using different amino acids as building blocks, along with incorporating PLLA in their structure, yields a new perspective of biodegradable polymers and polymer particles. Under suitable gentle conditions, the proteinoids can self-assemble to form nano- and micron-sized hollow particles of relatively narrow size distribution. This self-assembly process was used for encapsulation of different molecules within the produced proteinoid particles. One of the encapsulated materials used was indocyanine green (ICG), a known and FDA-approved near-IR dye used for medical cancer diagnosis. The ICG-encapsulated proteinoid particles were tested for biodistribution in mice. The proteinoid particles are non-toxic and stable; hence, they may be excellent candidates for various biomedical applications, e.g., cell labeling and separation, controlled release, drug targeting, etc.

Keywords: Proteinoid; Self-assembly; Amino acids; Thermal condensation; Microwave polymerization; Encapsulation

Introduction

Biodegradable polymers have gained great interest in the last years due to their potential use in a wide area of biomedical applications, including the field of drug delivery [1-6]. When designed for use as drug delivery agents, the polymers should meet several required features such as non-toxicity of the polymer itself and its degradation products and the ability to control hydrolysis rate and mechanical properties by simple manipulations [7]. Since the polymers are prepared for medical use, it is apparent that the starting materials and the optional hydrolytic segments of such biodegradable polymers should be non-toxic and water-soluble [8].

In this work, polymers made of natural amino acids are prepared and discussed. When intended for medical use, the biodegradable polymer is chosen according to its features serving the designated need. In general, such synthetic polymers may offer greater advantages over natural ones since they can be designed according to the desired use, providing a wide range of desired properties and predictable characteristics. Furthermore, synthetic polymers represent a reliable source of raw materials, free from concerns of infection or immunogenicity [9-11].



Scheme 1: Thermal polymerization of amino acids through pyroglutamic acid catalysis.

Proteinoids, polymers made of amino acids by thermal condensation polymerization, are unique synthetic polymers studied by Fox et al. [12-18]. The amino acids, either natural or synthetic, are polymerized by step-growth polymerization in a special procedure discovered by Fox and Harada [19-23]. The procedure involves heating amino acids until they melt at first, and then the polymerization is initiated to give the proteinoid. The polymerization takes place at a relatively high temperature (e.g., 180°C), in absence of a solvent or a catalyst, and in an inert atmosphere. The accepted explanation of this polymerization process is that glutamic acid is used as a solvent for the other monomers, as it is condensed into pyroglutamic acid. The pyroglutamic acid initiates the polymerization with the rest of the present amino acids [22-25]. A brief description of the polymerization process is shown in Scheme 1.

An important advantage in using biodegradable polymers bearing one or more stereogenic centers- optically active polymers- is the

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high variety of features they offer, when used as carriers for drug delivery systems [26]. This study is intended to broaden the scope of biodegradable polymers by synthesizing new proteinoids carrying various stereogenic centers. The main goal is to provide a large choice of biodegradable proteinoids with pre-determined characteristics, while using amino acids as building blocks. Hence, the new selection is based on chiral building blocks carrying positive/negative charge, hydrophilic/hydrophobic nature or any desired combination of the above [27]. Homopolymers, random copolymers and block-copolymers of two amino acids or more can be designed and synthesized, presenting thereby new proteinoid materials with specific, desired nature. The large variety of amino acids, either natural or synthetic, makes it possible to obtain a large library of different proteinoids, by simply changing the amino acids ratios. One proteinoid is completely different from another, by the fact that they are made of different amino acid monomers. This fact provides each proteinoid special features, and possibly influences the character of particles made from it [28-31]. Up until now, most, if not all, of the reported proteinoids in the literature were synthesized from at least seven amino acids and possessed relatively low molecular weights. Thus, high molecular weight proteinoids made of two to three natural amino acids, along with proteinoids containing poly(L-lactic acid) (PLLA) segments are presented for the first time. The high molecular weights of polymers in general, improve the chemical and physical properties, since the intermolecular forces are enhanced between the polymeric chains.

In the present article, several types of new proteinoids were synthesized, using different building blocks. The natural amino acids glutamic acid, aspartic acid, phenylalanine and lysine were used in different proportions in order to obtain proteinoids bearing different characteristics. Also, PLLA was incorporated within the proteinoids in order to affect the physical and chemical properties of the resulted proteinoid and proteinoid particles.

Microwave-assisted polymerization

The use of microwave for chemical synthesis is gaining growing interest in the last few years [32,33]. Using microwave heating, certain reactions are complete within seconds or minutes. In the field of synthetic polymer chemistry, microwave energy has been used for the polymerization of vinyl monomers, ring opening polymerization of caprolactam and caprolactone [34-36], condensation polymerization of polyesters [11,37,38], polyanhydrides [39], polyamides and polyimides [40]. This study tested this approach as an additional way for the polymerization of amino acids. A comparison between the polymers obtained by the thermal polymerization and the microwave-assisted polymerization was accomplished as well.

Preparation of micro- and nano-sized particles

After preparation, the crude proteinoids can self-assemble to form micro- and nano-sized particles [41,42]. As opposed to polystyrene microparticles, for example, which are formed during the polymerization process of styrene in the presence of a surfactant [43], the proteinoid particles are formed through a self-assembly process. This process is completed only after the polymerization itself. The procedure involves either dissolving the dried crude proteinoid in water by heating and then cooling slowely [44], or by pH changes of the proteinoid solution in water [45]. This way, particles of the size range of several nanometers to 10 μm may be formed [46]. When the self-assembly procedure is done in the presence of a suitable molecule such as drug or dye, a proteinoid particle containing the molecule is formed [47]. In this work, we encapsulated several molecules within one of the proteinoids: toluene, perfluorohexane, fluorescein, solvent blue 59 and the NIR dye indocyanine green (ICG). The NIR proteinoid particles were tested for biodistribution in a mouse model, showing their ability to penetrate different parts of the body. This way, the proteinoid particles can be used as delivery systems, depending on the proteinoid special features [48-53].

Experimental Section

Materials

The following analytical-grade chemicals were purchased from commercial sources and were used without further purification: (L) glutamic acid, (L)aspartic acid, (L)lysine and (L)phenylalanine, sodium chloride, sodium hydroxide (NaOH 1 N), hydrochloric acid (HCl 1 N), human serum albumin (HSA), bovine plasma fibrinogen, toluene, perfluorohexane (PFH), solvent blue 59 and fluorescein from Sigma (Rehovot, Israel); Poly(L-lactic acid) (PLLA) MW 2,000 Da from Polysciences (Warrington, PA, USA); Minimum Essential Medium (MEM) eagle, Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), 1 % glutamine, 1 % penicillin/streptomycin and mycoplasma detection kit from Biological Industries (Bet Haemek, Israel); Cytotoxicity detection kit from Roche Diagnostics, USA; Human colon adenocarcinoma LS174t cell line from American Type Culture Collection (ATCC). Water was purified by passing deionized water through an Elgastat Spectrum reverse osmosis system (Elga Ltd., High Wycombe, UK).

Preparation of proteinoids by thermal condensation polymerization

(L)glutamic acid was heated to the molten state (180°C) in an oil bath, under nitrogen atmosphere. The molten mass was stirred at 180°C for 30 min. To this, different contents of additional (L)amino acids were added to give a total monomer weight of 5-5.01 g, as specified in Table 1, and kept at 180°C under nitrogen. The mixture was mechanically stirred at 150 rpm for 3 h. The product is a highly viscous orange-brown paste, which hardens to give a glassy mass when cooled to room temperature. Then, water (10 mL) was added to the crude product, and the mixture was stirred for 20 min. The solution was then intensively dialyzed through a cellulose membrane (3500 Da MWCO) against distilled water. The content of the dialysis tube was then lyophilized to obtain a yellow-white proteinoid powder.

Polymerization kinetics was studied by collecting proteinoid samples from the reaction vessel at different time periods of the polymerization at 180, 190 and 200°C. The samples were then analyzed by both ninhydrin test for the determination of the primary amine

Polymer	Amino acid content (g)[a]				
	(L)Glu	(L)Asp	(L)Lys	(L)Phe	PLLA
Prot1	5	-	-	-	-
Prot2	2.5	2.5	-	-	-
Prot2[b]	0.15	0.15	-	-	-
Prot3	2.5	-	-	2.5	-
Prot4	1.25	2.5	-	1.25	-
Prot5	1.67	1.67	1.67	-	-
Prot6	1.67	-	1.67	1.67	-
Prot7	1.25	-	2.5	1.25	-
Prot8	2.25	-	-	2.25	0.5

[a]In all proteinoids made by thermal condensation polymerization the total monomer content was 5-5.01 g; [b]made by microwave-assisted polymerization.

Table 1: Amino acid content of the different proteinoids.

groups content and Biuret test for the determination of the amide groups content of the various proteinoids [54].

Proteinoid analysis and characterization

Amino acid analysis was carried out using Waters AccQTag method [55]. Before analysis, the material was hydrolyzed by standard acid hydrolysis, using 6 N HCl at 110°C for 22 h. The molecular weights and polydispersity index of the dried crude proteinoids were determined using Gel Permeation Chromatography (GPC) consisting of a Waters Spectra Series P100 isocratic HPLC pump with an ERMA ERC-7510 refractive index detector and a Rheodyne (Coatati, CA) injection valve with a 20 µL loop (Waters, MA). The samples were eluted with super-pure HPLC water through a linear BioSep SEC-s3000 column (Phenomenex) at a flow rate of 1 mL/min. The molecular weights were determined relative to poly(ethylene glycol) standards (Polymer Standards Service-USA, Silver Spring, MD) with a molecular weight range of 100-450000 Da, Human Serum Albumin (HSA, 67 kDa) and bovine plasma fibrinogen (340 kDa), using Clarity chromatography software. The optical activities of the proteinoids were determined using a PE 343 polarimeter (PerkinElmer). All of the measurements were done in water, at 589 nm at 25°C. Fourier Transform InfraRed (FTIR) measurements of the crude proteinoids were done by the Attenuated Total Reflectance (ATR) technique, using Bruker ALPHA-FTIR QuickSnap™ sampling module equipped with Platinum ATR diamond module. The thermal behavior of the proteinoids was determined using Differential Scanning Calorimetry (DSC) and Thermo Gravimetric Analysis (TGA) with a TGA/DSC 1 STARe system (Mettler Toledo, Switzerland). The samples were heated between 25 - 400°C at a rate of 10°C/min under nitrogen atmosphere.

Carboxyl group analysis

The content of free carboxyl groups in the synthesized proteinoids is an essential factor in determining their solubility in different media, thus helping to understand their stability at different sites in the human body with different pHs. In order to determine the free carboxyl groups in the synthesized proteinoids, a titrimetric method was carried out [56]. Briefly, to a known quantity of dry proteinoid, a known excess of 0.05 N NaOH was added, followed by the addition of 37% formaldehyde solution. The unreacted NaOH was back-titrated with standard 0.05 N HCl. A blank titration was also performed. In addition, human serum albumin (HSA) was titrated for comparison.

Preparation of proteinoids by microwave-assisted polymerization

Microwave polymerization method was conducted by a Biotage microwave synthesizer (Biotage Initiator™). Briefly, a mixture of (L) glutamic acid and (L)aspartic acid (150 mg each) was sealed in a 2.0-5.0 mL microwave glass vial with a magnetic stirrer. The reaction was irradiated for 60 min at 120°C, at a pressure of 0-1 bar and a power of 200 W [11,57]. The microwave-made Prot2 was cleaned, dried and characterized by the same techniques as mentioned above.

Incorporation of poly(L-lactic acid) into the proteinoids

In order to effect the chemical and physical properties of the product, a thermal polymerization of (L)glutamic acid and (L)phenylalanine was carried out in the presence of low molecular weight poly(L-lactic acid) (PLLA, 2000 Da). The proteinoid-PLLA (Prot8) consists of 2.25 g of each amino acid and 0.5 g of PLLA. After polymerization, it was washed, dried and characterized as described earlier.

Preparation and characterization of proteinoid nano/micro-particles

Proteinoid particles were prepared by a self-assembly mechanism. Briefly, 100 mg of the dried proteinoid were added to 10 mL double-distilled water. The mixture was then heated to 80°C until the crude proteinoid dissolves completely. Proteinoid particles were then formed by removal of the heating and leaving the mixture to cool to room temperature. The size and size distribution of the formed proteinoid particles were controlled by changing various parameters of the self-assembly precipitation process, e.g., salt concentration or cooling rate [46]. The obtained particles were dialyzed as previously described to wash off excess reagents. This effect of salt concentration and cooling rate on the size of the proteinoid particles was studied with Prot3.

Hydrodynamic diameter and size distribution of the particles dispersed in double distilled (DD) water were measured at room temperature with a particle DLS analyzer model Nanophox (SympatecGmbH, Germany). Dried particle size and size distribution were measured with a Scanning Electron Microscope (SEM). SEM pictures were obtained with a JEOL, JSM-840 Model, Japan. For this purpose, a drop of dilute particle dispersion in distilled water was spread on a glass surface, and then dried at room temperature. The dried sample was coated with carbon in vacuum before viewing under SEM. The average particle size and distribution were determined by the measurement of the diameter of more than 200 particles with image analysis software (Analysis Auto, Soft Imaging System GmbH, Germany).

The density of the particles was determined by pycnometry [58]. Briefly, dry pre-weighed particles were put in a calibrated pycnometer, which was then filled with water. The density of the sample can then be calculated from the known density of the water, the weight of the pycnometer filled only with water, the weight of the pycnometer containing both the sample and water, and the weight of the sample, as described in the literature [58].

Cytotoxicity of the proteinoid particles

In vitro cytotoxicity of the proteinoid particles was tested by using human colon adenocarcinoma LS174T cancer cell line. The tests were done on Prot2, Prot4, Prot5, Prot7 and Prot8. The cell line is adherent to the used culture dishes. LS174T cells were grown in MEM that was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin. Cells were screened to ensure they remained mycoplasma-free using Mycoplasma Detection Kit [59].

Cell cytotoxicity was assessed by measuring the release of cytoplasmic lactate dehydrogenase (LDH) into cell culture supernatants. LDH activity was assayed using the Cytotoxicity Detection Kit according to the manufacturer's instructions [60]. Cells (3×10^5 cells per well) were seeded and grown to 90–95% confluency in 24 well plates before treatment with the proteinoid particles. Cell cultures that were not exposed to the particles were included in all assays as negative controls. Cell cultures that were treated with 1% Triton-x-100 were used as positive controls. To test if the particles can interact with LDH kit compounds, cell cultures were exposed to a mixture containing maximal nano/micro-particles concentration dispersed in PBS and 1% Triton-x-100.

The proteinoid particles were freshly dispersed in PBS (1.25 and 2.5 mg/mL) and then added to the 95% confluent cell culture in culture medium. The cell cultures were further incubated at 37°C in a

humidified 5% CO_2 incubator and then checked for cellular cytotoxicity at intervals of 24 h. The percentage of cell cytotoxicity was calculated using the formula shown in the manufacturer's protocol [60]. All samples were tested in tetraplicates.

Particle stability

Proteinoid particles aqueous dispersions (1 mg/mL) were put in a refrigerator at 4°C for 6 months. Samples were taken at different time periods, filtered through a centrifugation tube (Vivaspin 3000 Da MWCO) and the filtrate was checked by UV at 200-210 nm, to find aqueous soluble proteinoid. Also, the particle aqueous dispersions were checked by Nanophox for their size and size distribution. In order to check the particle stability after drying, the particles were lyophilized to dryness and then dispersed in an aqueous phase to their original concentration. The samples size and size distribution were then rechecked by Nanophox.

Encapsulation of different materials within the particles

Prot8 was used to encapsulate several materials. To the heated proteinoid mixture (10 mg in 9.8 mL NaCl 10^{-5} N, at 80 °C), 1% w/w of the wanted material (dissolved in 200 µL NaCl solution) was added. The heating was stopped, the mixture was mechanically stirred at 250 rpm and left to cool to room temperature in order to form the proteinoid particles containing the encapsulated material. The encapsulated materials were toluene, perfluorohexane (PFH), fluorescein, solvent blue 59 dissolved in toluene and indocyanine green (ICG). The particles were washed and characterized as mentioned before for their size and size distribution. Also, the fluorescein and ICG-containing proteinoid particles were characterized by UV and fluorescence. Absorbance spectra were obtained using a Cary 100 UV–Visible spectrophotometer (Agilent Technologies Inc.). Excitation and emission spectra were measured by a Cary eclipse spectrofluorometer (Agilent Technologies, Inc.).

Biodistribution in a mouse model

Male BALB/C mice (Harlan Laboratories, Israel) were utilized in this study under a protocol approved by the Institutional Animal Care and Use Committee at Bar-Ilan University. The biodistribution of the NIR fluorescent Prot8 nanoparticles was studied in normal 8-weeks-old mice, weighing 20-25 g at the time of experiment. Prior to the experiment, mice were anesthetized by intraperitoneal injection of Ketamine (40-80 mg/kg body weight) and Xylazine (5-10 mg/kg body weight), and the mice's skin was shaved with an electric animal clipper.

100 µL of either nanoparticle dispersion or free ICG solution (0.01 mg/kg body weight, dissolved in PBS) were administered to the mice through tail vein injection at a concentration of 2 mg/mL. During image acquisition, mice remained anesthetized by the intraperitoneal injection of Ketamine/Xylazine. Image cubes were obtained from the mice at several time points up to 24 h after injection. Each treatment group includes 3 mice for each time point (5 min, 20 min, 1 h and 24 h); 2 uninjected mice served as negative control. The experiment was repeated twice, testing a total of 52 mice. At the end of the experiment, the mice were euthanized by cervical dislocation, and organs were taken for imaging (liver, spleen, kidney, duodenum, colon, brain, heart, tibia bone and blood).

Fluorescence images were acquired using a Maestro II *in vivo* fluorescence imaging system (Cambridge Research &Instrumentation, Inc., Woburn, MA). The system is equipped with a fiber-delivered 300 W xenon excitation lamp, and images can be acquired from λ=500-950 nm by a 1.3 megapixel CCD camera (Sony ICX285 CCD chip). Each pixel within the image cube therefore has an associated fluorescence spectrum. The software for the Maestro system (Maestro 2.10.0) contains several algorithms to process the spectral data cubes to remove undesired auto-fluorescence signal and generate overlaid images for multiple fluorophores. A deep red excitation/emission filter set was used for our experiments (λex: 700-770 nm, λem>780 nm). The liquid crystal tunable filter (LCTF) was programmed to acquire image cubes from λ=780 nm-860 nm with an increment of 10 nm per image. The camera was set to 150 ms (whole body image), 15 ms (liver), 500 ms (spleen), 7000 ms (kidney), 10 ms (duodenum), 500 ms (colon), 1000 ms (brain), 1000 ms (tibia bones), 200 ms (heart) and 1000 ms (blood) exposure times. Fluorescence intensity measurements were performed using ImageJ NIH (National Institutes of Health) software.

Results and Discussion

Synthesis and characterization of the proteinoids

As described above, different proteinoids were prepared by thermal condensation of different monomer contents. Table 2 exhibits the different synthesized proteinoids and their characteristic molecular weights, polydispersity and optical activity.

Table 2 indicates a relatively low PDI values for the obtained proteinoids. This is quite unexpected since the polycondensation of the various amino acids is random. The highest PDI (2.32) was observed for Prot1, composed of the single amino acid (L) glutamic acid, while the PDIs of the other proteinoids composed of at least 2 amino acids range between 1.01 and 1.27. All of the thermally-made proteinoids have relatively high molecular masses of 26-195 kDa. This indicates that

Proteinoid[a]	Mw (Da)[b]	Mn (Da)[b]	Mp (Da)[b]	PDI[c]	Optical Activity $[\alpha]_D^{25°C}$ (°)[d]
Prot1	26250	11300	11320	2.32	+6.5
Prot2	181540	144940	195300	1.25	-4.4
Prot2[e]	500240	497280	503070	1.01	+8.1
Prot3	164930	138250	158740	1.19	-9.0
Prot4	87660	84410	85250	1.04	-3.3
Prot5	195080	165870	191440	1.17	-7.4
Prot6	190390	163290	204050	1.16	-15.1
Prot7	72260	56880	42870	1.27	+2.8
Prot8	168300	156600	136800	1.07	-4.6

[a]The proteinoids were prepared at 180°C according to the experimental section; [b]molecular masses were measured by GPC, Mp is the molecular mass at the peak; [c]PDI is the polydispersity index, given by Mw/Mn; [d]specific optical rotation (c=1, in H_2O, at 25°C); [e]made by microwave-assisted polymerization. Each experiment was performed 3 times, with an error of 0.5-1.7%.

Table 2: Mw, Mn, Mp, PDI and optical activity of the various proteinoids.

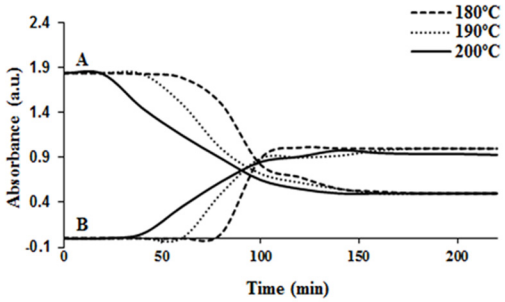

Figure 1: Thermal polycondensation kinetics of (L)glutamic acid and (L) phenylalanine to yield Prot3 at 180, 190 and 200°C by two tests: ninhydrin test (A) and Biuret test (B).

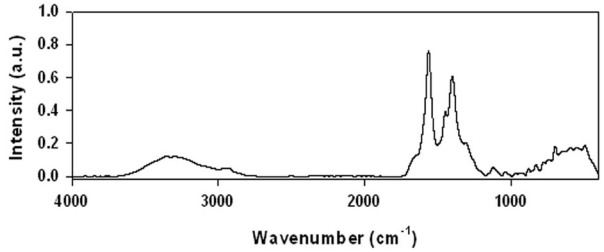

Figure 2: FTIR spectrum of Prot3. Characteristic peaks of NH stretching at 3360 and 2990 cm^{-1}, amide CO stretching at 1565 cm^{-1}, an amide NH bending band at 1450 cm^{-1} and CO bending at 500-700 cm^{-1} are shown.

Polypeptide	[Carboxyl groups] (mmol/g)
Albumin	56
Prot1	150
Prot2	155
Prot3	90
Prot4	122
Prot5	88
Prot6	87
Prot7	80
Prot8	102

Each experiment was performed 4 times, with an error of 0.5-2%.

Table 3: Carboxyl group content in the proteinoids and albumin.

the polymerization procedure by thermal heating used here provides relatively long polymer chains. This fact can be an advantage for different uses later, since polymers with such high molecular weights are usually mechanically stronger and resemble natural proteins. Table 2 indicates that the lowest molecular weight was observed for the proteinoid composed of the single amino acid (L)glutamic acid (Prot1) and the highest one for the proteinoid composed of (L)glutamic acid, (L)aspartic acid and (L)lysine (Prot 5). Prot2, which was synthesized by microwave-assisted polymerization, reached an abundantly higher molecular weight. In this procedure, a 500 kDa proteinoid chain was made, about twice the size of the thermal proteinoid. This kind of procedure gives us better yield over 60 min, compared to the 3 h needed usually. It can be used further for higher molecular weights and more rigid proteinoids. However, unfortunately, this kind of proteinoid does not self-assemble into spherical-shaped particles.

All of the proteinoids exhibit optical activity, although the monomers are known to racemize during the thermal process [61]. This fact can become a benefit later in the design of a stereospecific drug carrier, for example.

The polymerization kinetics was examined over 220 min at three different temperatures (180, 190 and 200°C) as illustrated in Figure 1. The results shown refer to the synthesis of Prot3, consisting of (L) glutamic acid and (L)phenylalanine. Similar results were observed for the other systems, Prot1-8, as well. It can be seen that at all temperatures, the polymerization takes place mainly over the first 100 min. After that, both ninhydrin (A) and Biuret (B) tests show no significant drop of the free amines (ninhydrin signal) or rise of the peptide bonds (Biuret signal). This figure also shows that as the temperature of the reaction was raised, the rate of the reaction increased. However, the preferred reaction temperature is 180°C, as the yield of the reaction is higher since the amino acids decompose faster at the higher temperatures.

The proteinoids were also characterized by infrared spectroscopy. All proteinoids showed characteristic peaks of NH stretching at 3360 and 2990 cm^{-1}, amide CO stretching at 1565 cm^{-1}, an amide NH bending band at 1450 cm^{-1} and CO bending at 500-700 cm^{-1}. A representative spectrum of Prot3 is showed in Figure 2.

Carboxyl group analysis

The presence of excess dicarboxylic amino acids in the synthesized proteinoids, aspartic acid and glutamic acid, provides acidic nature to the resulted polymers. This is also evident from carboxyl group content of the synthesized proteinoids where it shows higher values of 80-155 mmol/g compared with albumin (Table 3). This is true also in Prot5-7, where lysine is also a part of the polymer. Moreover, aspartic and glutamic acid moieties in the proteinoids, along with lysine, impart the hydrophilic nature of the whole proteinoid. The biodegradability rate of various amino acid polymers increases with their hydrophilicity [56]. Therefore, it is more appropriate to choose these proteinoids as ideal biomaterials for drug delivery applications.

Thermal properties of the proteinoids

The thermal properties of the proteinoids were investigated by DSC and TGA, as presented in Table 4.

The melting temperatures of the different proteinoids range between 78-246°C. The wide range of temperatures derives from the difference in the monomeric units used in each proteinoid. When using phenylalanine, as in Prot3, 4, 6 and 7, the resulted proteinoid gains significant rigidity in the overall structure, due to the aromatic rings which allow pi-stacking. Hence, these proteinoids melt at higher temperatures. When PLLA is incorporated into the proteinoid, as

Proteinoid	Tm(°C)[a]	ΔHm (J/g)[a]	Tdec (°C)[b]	Weight loss (%)
Prot1	102	-300.7	300	55
Prot2	89	-428.7	297	57
Prot3	103	-174.9	341	54
Prot4	217	-183.4	330	25
Prot5	78	-251.5	339	57
Prot6	241	-90.1	373	47
Prot7	246	-139.4	385	64
Prot8	117	-420.5	268	47
PLLA[c]	150	-57.2	349	90

[a]Tm and ΔHm were measured by DSC; [b]Tdec (temperature of decomposition) was measured by TGA/DSC and refer to the exothermal peak in DSC; [c]commercial PLLA 2000 Da parameters were measured similar to the made proteinoids. Each experiment was performed 3 times, with an error of 1-1.5%.

Table 4: Thermal properties of proteinoids produced by thermal polymerization.

Solution	Particle size (nm)[c]	Standard deviation (%)
Water	Over 3000	200
NaCl 10^{-5}N	196.2 ± 23.9	12.2
NaCl 10^{-3}N	486.2 ± 54.3	11.2
NaCl 0.1N	573.4 ± 47.9	17.5
NaCl 1N	1035.5 ± 325.1	31.4
PBS	2151.0 ± 204.3	9.5

[a]Particles were made of Prot3; [b]particles made by heating to 80°C with mechanical stirring and then slow cooling to room temperature; [c]the particle size and size distribution were measured by DLS.

Table 5: Effect of self-assembly conditions on proteinoid particle size and size distribution.[a,b]

Heating time (min)	Stirring[a]	Cooling method[b]	Particle size (nm)[c]	Standard deviation (%)
30	_	slow	722.5 ± 84.8	11.7
60	_	slow	2293.7 ± 277.8	12.1
30	+	slow	811.4 ± 118.5	14.6
30	_	fast	1898.1 ± 213.2	11.2

[a]Stirring by a magnetic stirrer; [b]slow cooling by removal of the heating and leaving the sample to cool to room temperature, fast cooling by refrigerating the sample; [c]the particle size was measured by DLS.

Table 6: Effect of self-assembly reaction conditions on proteinoid particle size and size distribution.

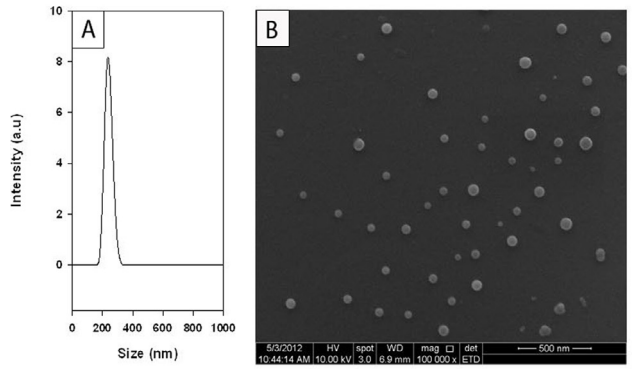

Figure 3: Hydrodynamic size histogram (A) and SEM image (B) of Prot8 particles.

in Prot8 compared to Prot3, the Tm rises mildly (103 and 117°C, respectively), due to the presence of 2000 Da rigid polymer chains in the overall proteinoid structure.

The TGA/DSC measurements of the proteinoids show decomposition temperatures of 268-385°C. Most proteinoids lose at this temperature range around 50% of their weight. The decomposition measured at 400°C of most proteinoids is between 47-64%, except Prot4 (25%). Pure PLLA decomposes at 349°C almost completely (90% weight loss). Prot8, composed of PLLA segments (10%), has the lowest decomposition temperature of all proteinoids (268°C). This can be explained by the non-uniformity of the structure of the whole proteinoid due to the inserted segments of 2000 Da PLLA within the random segments of polymerized amino acids.

Preparation and characterization of the proteinoid particles

The crude proteinoids were self-assembled in a variety of environments, as mentioned before, in order to find the best conditions for forming the nano/micro particles. The desired particles, spherically-shaped, have to be small and uniform in size through the whole sample. Each self-assembly method gave particles of different properties, e.g., different size and size distribution. In order to compare between the different conditions, Prot3 was examined. First, different salt solutions were investigated: double-distilled water, 10^{-5}-1 N NaCl solutions and PBS at the same proteinoid concentration (10 mg/mL), heating for 30 min at 80°C and cooling slowly to room temperature, simply by removing the heat source and leaving the mixture to cool. As seen in Table 5, the smallest particle size was achieved using 10^{-5} N NaCl solution, with a relatively good standard deviation of 12%. In general, as the ionic strength of the salt solution decreases, the particle size and size distribution decreases [46]. When the self-assembly process takes place in water, in the absence of the salt, the particles formed are large, over 3 μm, and have a very wide size distribution. In PBS, the particles formed are around 2 μm with a relatively narrow size distribution. This way, a large variety of proteinoid particles of different size and size distribution can be made, by changing their outside environment only.

In addition to the outside environment control, the effect of the reaction conditions on the size was checked by changing the heating, stirring and cooling parameters. Four proteinoid samples in water (10 mg/mL) were introduced to different conditions in terms of heating time after dissolving the proteinoid polymer, stirring and cooling method. The results are shown in Table 6. As seen, the smallest particles were produced by the first procedure: heating for 30 min and cooling slowly to room temperature. Overall, the different procedures gave particles of narrow size distributions.

Self-assembly of Prot8 was carried out in the best conditions found until now: 10^{-5} N NaCl solution, heating at 80°C for 30 min, followed by slow cooling to room temperature. The procedure produced spherical proteinoid particles of 103 ± 11 nm hydrodynamic diameter and 70 ± 15 nm dry diameter, as shown in Figure 3. The hydrodynamic diameter of these particles dispersed in water is illustrated by the typical light scattering measurement shown in Figure 3A. The dry diameter of the proteinoid particles is illustrated by the typical SEM photomicrograph shown in Figure 3B. The difference in the particle size between the SEM and the light scattering measurements is due to the fact that SEM measurements determine the dry diameter, whereas light scattering measurements takes into account the hydrated water layers adsorbed onto the particle's surface. As mentioned above, Prot8 has small PLLA segments incorporated within the random polymerized amino acid segments. This fact grants the proteinoid a more hydrophobic backbone. These hydrophobic segments are packed better in the interior part of the spherical particles during the self-assembly precipitation process. The addition of the PLLA segments also leads to the formation of smaller size particles. For example, under similar preparation conditions Prot3, composed of (L)glutamic acid and (L)phenylalanine, provided particles of 196.2 ± 23.9 nm while Prot8 composed of (L)glutamic acid, (L) phenylalanine and PLLA provided particles of 103 ± 11 nm diameter.

It should also be noted that overall, the proteinoid particles maintain their size and size distribution upon change of the outside environment, given that the change is not by drastic pH values. This way, for instance, Prot8 particles preserve their size when transferred from water to PBS.

Density measurements indicated that all proteinoid particles possess a very low density, ranging from 0.001 to 0.014 g/mL indicating that the particles formed are hollow, as already indicated for the proteinoids prepared by Fox et al. [17]. The hollow nature of the particles is significantly important for applications such as ultrasound imaging agents, drugs and dyes encapsulation, controlled released, etc.

As suggested in our and previous studies, the proteinoid forms

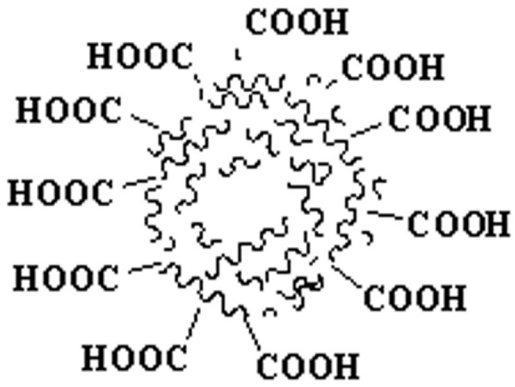

Figure 4: Schematic representation of the self-assembled proteinoid particles; hydrophobic moieties are represented by scribbled lines. When lysine is also a part of the proteinoid, as in Prot5-7, some carboxyl groups are exchanged with amine groups.

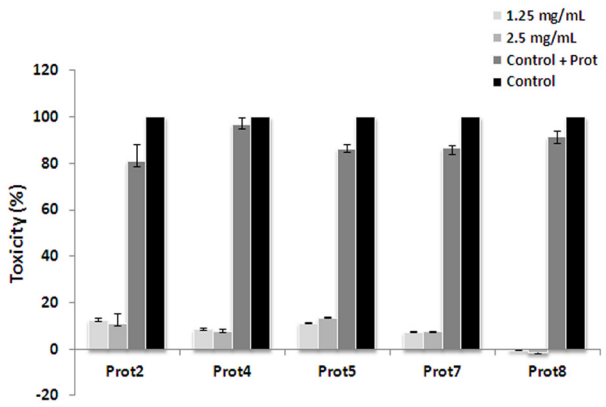

Figure 5: Cytotoxic effect of the proteinoid particles on colon adenocarcinoma LS174T cells measured by the LDH assay. Cells (3×105) were incubated with the proteinoid particles dispersed in PBS (1.25 mg/mL and 2.5 mg/mL) according to the experimental section. Cells were incubated with Triton-x-100 1% as positive control (100% toxicity). In addition, cells were incubated with Triton-x-100 1% and each one of the proteinoids to revoke any interaction. Untreated cells (negative control) were similarly incubated. Each bar represents mean ± standard deviations of 4 separate samples.

particles of different sizes according to the nature of its surrounding. The hydrophobic portions of the crude proteinoid are assembled within the particle matrix, while the polar hydrophilic groups (carboxylic acids and amines) are exposed to the aqueous environment, as illustrated in Figure 4 [56].

Cellular cytotoxicity of the proteinoid particles by LDH assay

In order to revoke cell toxicity of the new proteinoid particles, in vitro cytotoxicity of the particles was assessed by measuring the release of cytoplasmic lactate dehydrogenase (LDH) into the cell culture supernatants. LDH is an intracellular enzyme that catalyzes the reversible oxidation of lactate to pyruvate. Since LDH is mainly present in the cytosol, it is released into the supernatant only upon cell damage or lysis [60]. When tested by the LDH quantitative assay, Prot2, 4, 5, 7 and 8 particles dispersed in PBS at concentrations of 1.25 and 2.5 mg/mL had none, or minor cytotoxic effect on the human colon adenocarcinoma cell line LS174T (Figure 5). Treatment of the cells with Prot2 and Prot5 particles at both concentrations produced the highest LDH levels (up to 13% toxicity), when compared to untreated (blank) cells, indicating minor toxicity of these proteinoids to this cell line. Prot8 had the lowest, or no cytotoxic effect on the cells treated with both concentrations. This proteinoid is therefore the most suitable for treating cells, considering its low toxicity.

Particle stability

The stability against agglomeration of the proteinoid particles dispersed in an aqueous phase (1 mg/mL) and kept in the refrigerator was examined at different time periods over 6 months as described in the experimental section. Overall, the proteinoid particles remain in the same size after 6 months in storage at 4°C. Also, the degradation and /or dissolution of the proteinoid particles in the aqueous continuous phase was tested by the filtration centrifugation method and resulted in negative results in the filtrate, meaning no degradation or dissolution occurs at this temperature over 6 months. When lyophilized to dryness, the proteinoid particles can be redispersed in water completely while the particle size and size distribution remains the same. This means that the particles can be stored as a freeze-dried powder as well, and redispersed when needed, without the need to add cryoprotectants as mentioned in the literature [62].

Encapsulation of different materials within the proteinoid particles

Different materials were encapsulated within Prot8 according to the description in the experimental part. Fluorescein encapsulation was successful, as no fluorescein was detected in the aqueous filtrate. The fluorescein encapsulation process yields 425.2 ± 21 nm particles, which were found stable for at least 6 months. Figure 6 exhibits the absorbance and emission spectra of free fluorescein compared to encapsulated fluorescein particles. A 14 nm red-shift of the absorbance spectrum of the fluorescent proteinoid particles, compared to free fluorescein in solution, was observed. This indicates that the physical binding to the proteinoid, which places the dye in a more hydrophobic environment, affects the dipole moment of the dye. The fluorescence and absorbance of the fluorescent proteinoid particles dispersed in PBS and stored at 4°C remain the same after 6 months.

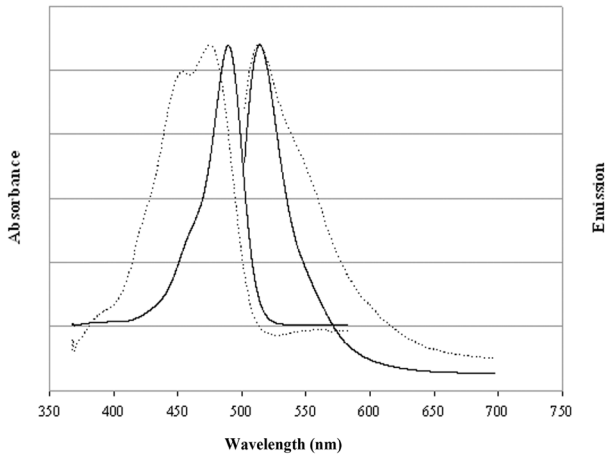

Figure 6: Absorbance and emission spectra of free fluorescein (dotted lines) and fluorescein proteinoid particles dispersed in water (solid lines). The maximum absorbance of free fluorescein and fluorescein encapsulated proteinoid particles occurs at approximately 475 nm and 489 nm, respectively. The fluorescence emission maxima occur at approximately 513 nm and 514 nm, respectively.

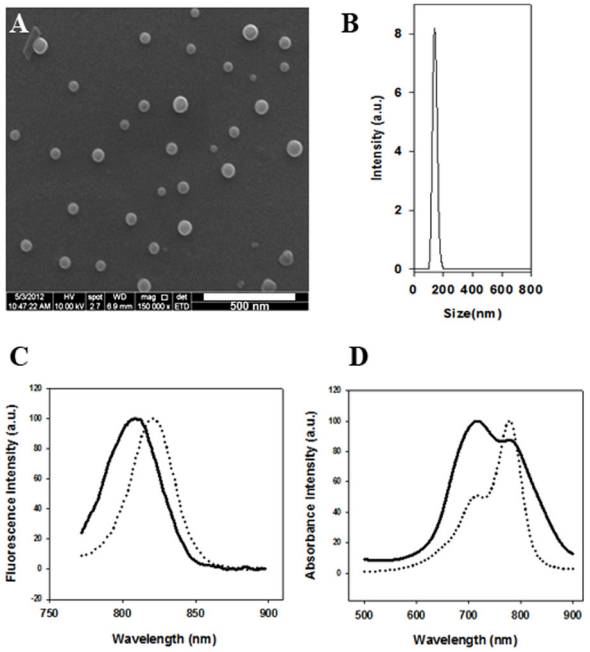

Figure 7: Characterization of the NIR fluorescent P(EF-PLLA) nanoparticles. A, B: SEM image and hydrodynamic size histogram of the NIR fluorescent P(EF-PLLA) nanoparticles, respectively; C,D: emission and absorbance spectra of free ICG (dotted lines) and nanoparticles containing ICG (solid lines), respectively.

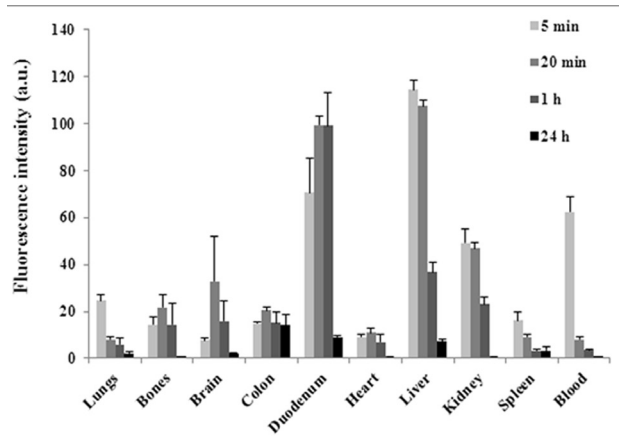

Figure 8: Fluorescence intensities of different organs taken at 5 min, 20 min, 1 h and 24 h post i.v. injection into mice tail veins. 12 mice (each experiment group contained 3 mice) were anesthetized and treated with NIR fluorescent P(EF-PLLA) nanoparticles (2 mg/mL, 0.01 mg/kg body weight per mouse). Blood was drawn and organs were harvested at each time point. 2 uninjected mice served as negative control. The experiment was repeated twice with similar results.

Similarly, the encapsulation of toluene was successful, as no organic separate phase was present at the end of the process. In this case, the proteinoid serves as a surfactant for dispersing the organic toluene droplets in water. No phase separation was observed within 6 months following the encapsulation. Particles containing toluene reached a size of 411.9 ± 59.1 nm. The encapsulation of solvent blue 59 dissolved in toluene resulted in slightly larger particles, 442.5 ± 66.6 nm. In this case, as in the encapsulation of toluene, no phase separation is observed over 6 months. When PFH is present during the particle formation, encapsulation yields 546.9 ± 52.4 nm particles. Examination the PFH particles over 24 h, shows a clear phase separation. Hence, the PFH particles can only serve immediate needs, such as ultrasound contrast agent [63].

Likewise, the NIR FDA-approved ICG was encapsulated successfully in the Prot8 nanoparticles. Figures 7A and 7B show that the dry (A) and hydrodynamic (B) diameters of the NIR fluorescent Prot8 nanoparticles are 70 ± 15 nm and 145 ± 20 nm, respectively. Figure 7C and 7D exhibit the fluorescence and absorbance spectra of the NIR fluorescent Prot8 nanoparticles compared to those of the free dye in solution. The absorbance spectra show no shift in the absorbance. However, due to the dye encapsulation process the maximal absorbance peak of the free ICG became minimal and the minimal peak of the free dye became maximal after encapsulation, since the ICG molecules get close to each other inside the nanoparticle interior and aggregation may occur and cause this change in absorbance [64,65]. Moreover, a blue-shift of 12 nm in the emission spectrum of the NIR fluorescent nanoparticles compared to the free ICG in solution was also observed, due to the dye molecule aggregation inside the particle.

Biodistribution in a mouse model

NIR fluorescent Prot8 nanoparticles (2 mg/mL, 0.01 mg/kg body weight per mouse) were injected into mice through the tail vein. Organs from mice were harvested and blood was drawn 5 min, 20 min, 1 h and 24 h post injection of the nanoparticles into the tail vein. The calculated fluorescence intensities of the lungs, bones, brain, colon, duodenum, heart, liver, kidney, spleen and blood screening are shown in Figure 8. Evidently, the results show that the nanoparticles penetrated and were found in all organs. It is shown clearly that by 20 min the majority of the inserted quantity of the fluorescent nanoparticles is cleared from the blood. The nanoparticles concentrate mostly at the liver and are possibly evacuated from the body. It is also apparent that the nanoparticles pass the blood-brain barrier (BBB), since they are found in the brain at 20 min post injection. This may open up a scope of drug targeting to the brain for drug molecules which are usually blocked. Overall, it was demonstrated that following a single i.v. injection of the nanoparticles, fluorescence intensity at all organs decreased over time, and only traces of fluorescence could be seen after 24 h.

Conclusions

In the present study, 9 new proteinoids were prepared using (L) glutamic acid, (L)aspartic acid, (L)phenylalanine, (L)lysine and PLLA. The polymerization was carried out by a simple straight-forward condensation polymerization in heat. The optimal conditions for polymerization were tested, including changing the temperature and using microwave radiation. The proteinoids made are of high molecular weights and possess optical activity, which can be later used in specific drug delivery using chirality. The proteinoids are thermally stable and each proteinoid has enough carboxylic acid and/or amine functional groups, which can be later used to bind desired molecules, such as drugs and dyes. The incorporation of 2000 Da PLLA into the proteinoid backbone presented a stable proteinoid as well. Proteinoids were manipulated in several ways to give proteinoid sphere-shaped nano/micro-particles, and to optimize this process. Specific conditions were found for the production nano hollow particles of narrow size distribution. The incorporation of PLLA segments into the proteinoids increased the hydrophobic interior part and resulted in smaller size hollow particles. The particles were found to be non-toxic and stable over time. Also, encapsulation of different materials was carried

out, giving organic-filled particles and fluorescent particles. Most importantly, ICG, a NIR fluorescent FDA-approved dye for imaging, was successfully encapsulated and the particles were found in many organs after injection to mice.

In summary, proteinoid formation and cytotoxicity tests indicate that these particles are suitable for further *in vivo* testing. Our future plans include further optimization of the particles and *in vivo* imaging in various animal models, to test the extent of targeting. We also plan to encapsulate within the optimal proteinoid particles other fluorescent dyes and anticancer drugs such as paclitaxel and/or doxorubicin, so that the particles may be used for combined imaging and therapy applications.

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