Colloid Polym Sci (2004) 282: 1097–1104
DOI 10.1007/s00396-003-1040-9

ORIGINAL CONTRIBUTION

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A new approach for the characterization of insoluble amphiphilic copolymers based on their emulsifying properties

Received: 31 May 2003
Accepted: 11 November 2003
Published online: 16 January 2004

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Abstract The purpose of the present study was to propose a new approach for the characterization of insoluble amphiphilic copolymers using their emulsifying properties. The capacity of four newly-synthesized block copolymers to stabilize a model emulsion formed by mixing equal volumes of water and ethyl acetate was investigated. The copolymers were composed of dextran or heparin and poly(isobutylcyanoacrylate) and were obtained either by anionic or by radical polymerization of isobutylcyanoacrylate in emulsion, initiated on dextran or heparin. A quantitative analysis of the stability of the emulsion obtained with the different copolymers was evaluated using a Turbiscan MA 2000. The results suggested that this approach provides a new methodology for investigating emulsifying properties of polymers that are insoluble in pure solvents, allowing quantitative comparisons to be made between them.

Keywords Poly(isobutylcyanoacrylate)-polysaccharide copolymer · Turbiscan · Emulsion

Introduction

Amphiphilic copolymers are generally composed of hydrophilic and lipophilic moieties. Different structures can be created among these types, depending on the method of synthesis; they can be linear or branched, and the number of hydrophilic and hydrophobic chains can also vary [1]. A well-known group of such copolymers contains poly(ethylene glycol) (PEG) chains as the hydrophilic moiety [1]. In pharmacy, the growing interest in the development of the so-called ''stealth'' nanoparticulate drug carriers has led to the synthesis of a large number of new block copolymers, including PEG chains combined with hydrophobic biodegradable polymers [2, 3, 4, 5, 6, 7, 8, 9]. The PEG chains are exposed on the nanoparticle surface, exerting efficient protein repulsive effects and therefore reducing opsonization by macrophages of the mononuclear phagocyte system [10, 11, 12, 13, 14].

Recently, new drug delivery system developments considered nanoparticles coated with oligo- or polysaccharides [15, 16, 17, 18, 19, 20, 21, 22, 23]. This strategy, based on a biomimetic approach, has great potential to improve site-specific targeting and tissue permeation by mimicking the natural tissue addressing and transport mechanism. These mechanisms are very often mediated by oligosaccharides and carbohydrates [24]. Indeed these systems are obtained from amphiphilic block copolymers containing oligo or polysaccharides. One problem with these copolymers resides in the fact that they are insoluble in both organic and aqueous solvents and cannot be characterized easily. Indeed, methods of characterization requiring the full solubilization of the copolymers are not applicable, and the copolymers can only be analyzed by methods based on the characterization of solid powders which are generally less sensitive. In addition, none of the usual techniques applied for the characterization of the surface active property of amphiphilic compounds can be used, since solutions of the molecule must again be prepared [25].

Therefore, the purpose of the present study was to investigate whether these insoluble amphiphilic copoly-

mers could be characterized by the mean of their emulsifying properties, according to an original approach involving the formation of an emulsion, the stability of which could be measured using the recently developed apparatus, Turbiscan MA 2000. This apparatus scans the turbidity profile of an emulsion along the height of a glass tube filled with the emulsion, following the fate of the turbidity profile over time. The analysis of the turbidity profiles leads to quantitative data on the stability of the studied emulsions and allows us to make objective comparisons between different emulsions.

Experimental

Materials

Isobutylcyanoacrylate (IBCA) was kindly provided by Loctite (Dublin, Ireland). Dextran (M_w 71,000 g/mol) and heparin (M_w 19,000 g/mol) were purchased from Sigma (Saint Quentin Fallavier, France). Ethyl acetate and tetrahydrofuran (THF) were obtained from Carlo Erba (Val de Reuil, France), and dimethyl sulfoxide was supplied by Aldrich (Saint Quentin Fallavier, France). Cerium IV ammonium nitrate was purchased from Fluka (Saint Quentin Fallavier, France). All chemicals were reagent grade and used as purchased. A Turbiscan MA 2000, lent to us by Formulaction (Toulouse, France), was used to monitor the emulsion stability.

Methods

Preparation of poly(isobutylcyanoacrylate)

0.5 ml of IBCA was added drop-wise to 10 ml of MilliQ water under vigorous stirring at room temperature. The polymerization was allowed to proceed for 1 h. The poly(isobutylcyanoacrylate) (PIBCA) formed was recovered by lyophilization. The sample was then frozen at −18 °C and freeze-dried for 48 h in a Christ alpha 1-4 freeze dryer (Bioblock Scientific, Illkirch, France) without a cryoprotecting agent.

Preparation of PIBCA-polysaccharide copolymers by radical polymerization

Dextran or heparin (0.1375 g) was dissolved in nitric acid (8 ml, 0.2 M) at 40 °C and placed under gentle stirring and argon bubbling for 10 min. Then 2 ml of a solution of cerium (IV) ammonium nitrate (8.10^{-2} M in 0.2 M nitric acid), and 0.5 ml of IBCA were added under vigorous stirring. Argon bubbling was maintained for additional 10 min. The reaction was allowed to continue at 40 °C under gentle stirring for 40 min. After cooling to room temperature, 1.25 ml of 1.02 M trisodium citrate was added, and the pH was adjusted to 7.0 with NaOH. The copolymer prepared with heparin was named PHR and the copolymer obtained with dextran was named PDR. The heparin and dextran content of the PIBCA-copolymers were respectively 11.08% in PHR and 21.11% in PDR, as determined by elemental analysis [22, 23].

Preparation of PIBCA-polysaccharide copolymers by anionic polymerization

Dextran or heparin (0.1375 g) was dissolved in 10 ml of 0.2 M nitric acid at 40 °C, under gentle stirring and argon bubbling for 10 min. Then, 0.5 ml of IBCA was added under vigorous magnetic stirring. Argon bubbling was maintained for additional 10 min and the reaction was allowed to continue for 40 min at 40 °C under gentle stirring. After cooling to room temperature, the pH was raised to 7.0 as described above. The copolymers prepared with heparin and dextran by anionic polymerization were named PHA and PDA respectively. The heparin and dextran content of the PIBCA-copolymers were respectively 10.14% in PHA and 27.07% in PDA, as determined by elemental analysis [22, 23].

Purification and lyophilization of the copolymers

After polymerization, the copolymer-containing suspensions (12 ml) were dialyzed three times against 1 liter of distilled water at room temperature for 90 min, and one time overnight (Dialysis membranes MWCO 100,000 Da, Spectra/Por, Biovalley, Marne la Vallée, France). The purified copolymers were frozen at −18 °C and lyophilized as explained before.

Solubility of the polymers

5 mg of polymer – either dextran, heparin, PIBCA, PHA, PHR, PDA or PDR – was introduced into a glass tube with 500 µl of a solvent including ethyl acetate, DMSO, THF and MilliQ water. Samples were kept at room temperature (19 °C) for 3 days. After this period of time, if all of the polymer powder was dissolved, the polymer was declared soluble in the corresponding solvent. On the contrary, if the polymer powder remained undissolved, the polymer was declared insoluble.

Preparation of the emulsions and study of their stability

12 mg of a polymer (dextran, heparin, PIBCA, PHA, PHR, PDA or PDR) and a blend of 6 mg PIBCA and 6 mg dextran or heparin were introduced into a glass tube together with 3 ml of MilliQ water and 3 ml of ethyl acetate. A control emulsion was prepared without polymer, with 3 ml of MilliQ water and 3 ml of ethyl acetate. The samples were shaken at 28 °C according to the following sequential protocol: vortex 30 s, tube turned upside down twice, vortex 30 s, tube turned upside down 20 times, vortex 30 s. Immediately afterwards the agitation was stopped, and very quickly 5 ml of the sample was introduced into the measurement cell of the Turbiscan MA 2000 (Formulaction, Toulouse, France). The transmission of the light across the sample ($\lambda = 850$ nm) and the backscattering of the incident beam given by the emulsion were simultaneously and automatically recorded every minute for 90 min.

Principle of the emulsion destabilization measurements

The destabilization of the emulsion prepared with or without the polymer was evaluated using a Turbiscan MA 2000 (Formulaction, Toulouse, France), which allows the optical characterization of any type of dispersion [26, 27]. In this apparatus, the dispersion is placed in a flat-bottomed cylindrical glass cell and scanned from the bottom to the top by a light source and detector devices, in order to monitor the optical properties of the dispersion along the height of the sample placed in the cell (Fig. 1). A pulsed near infra-red light

source working at 850 nm is used as the incident light, and two synchronized detectors are used to collect the optical characteristics of the dispersion. One measures the light going through the sample, giving a vertical scan of the transmission pattern of the sample. The second detector measures the light scattered by the sample at an angle of 135° from the incident beam, and provides a backscattering profile of the dispersion in the cell [26]. These measurements are made simultaneously every 40 μm along the vertical length of the cell. Therefore, the scan of a 50 mm height sample provides transmission and backscattering patterns, including 1,250 points of measurement, in less than 20 s. Each scan provides a curve representing the signal received by both detectors as a function of the position in the cell, giving a kind of macroscopic fingerprint of the sample at a given time (Fig. 1). By repeating the scan of a sample at different time intervals, one can study the stability or the instability of a dispersion in detail (Fig. 2). Calculations of either creaming, sedimentation or phase separation rates can be evaluated, and the mechanism making the dispersion unstable can be deduced from the transmission or the backscattering data [26, 27].

Results

The solubilities of the different homopolymers and copolymers are reported in Table 1. None of the tested solvents could dissolve the copolymers, while the homopolymers could be dissolved either in water or in one of the organic solvents. Figure 3 shows the turbidity profiles of a series of emulsions containing different copolymers at times 0, 2, 10 and 30 min. The reference consisted of the model emulsion without the addition of any homopolymer or copolymer. The turbidity profile showing the transmission of the light across the sample displayed two distinct clear domains (transmission ~100%) separated in the middle by a thin turbid zone (transmission ~0%). This reference emulsion separated almost immediately and the turbid zone appearing in the middle of the sample corresponded to the interface between water (clear domain at the bottom part of the sample), and ethyl acetate (clear domain at the top part of the sample). The behavior of the other emulsions were modified by the introduction of the copolymers: the appearance of clear domains occured slowly, although for some of the emulsions no clear domain could be observed either in the top part or in the bottom part of the sample.

The turbidity profiles monitored for the emulsions prepared with the copolymers obtained by anionic polymerization (PHA and PDA) showed a faster appearance of the clear domain compared to emulsions prepared with the corresponding copolymers obtained by radical polymerization (PHR and PDR). PIBCA-dextran copolymers, including both PDA and PDR, lead to a rather rapid appearance of a clear domain in the top part of the emulsions, whereas PIBCA-heparin copolymers (PHA and PHR) showed a slow appearance of a clear domain in the bottom part of the emulsions. Photographs of the emulsions taken 24 hours after preparation were in agreement with the turbidity profiles monitored with the Turbiscan. The emulsions prepared with the copolymers obtained by radical polymerization (PHR and PDR) still showed a part of the sample

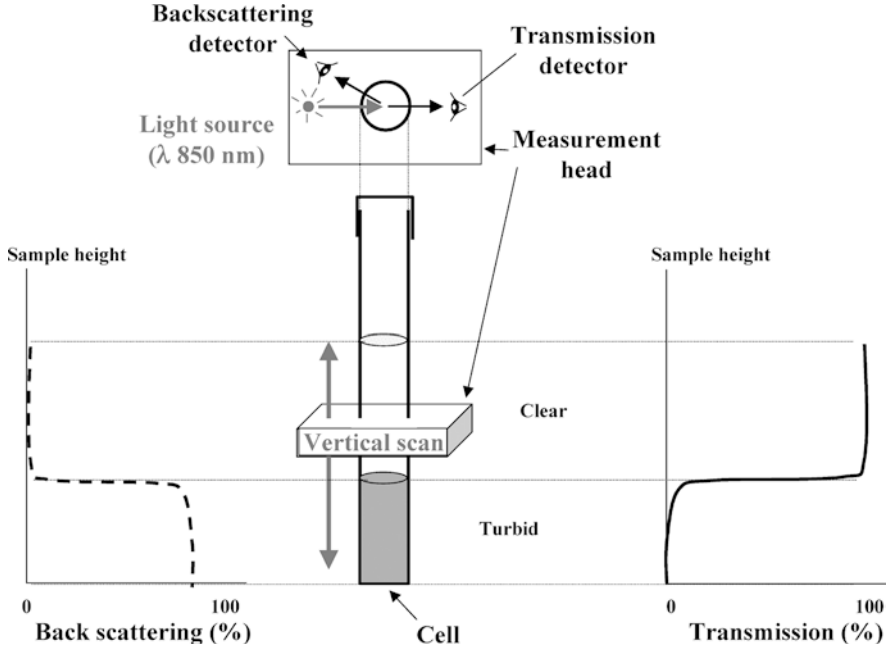

Fig. 1 Measurement principle of Turbiscan MA 2000

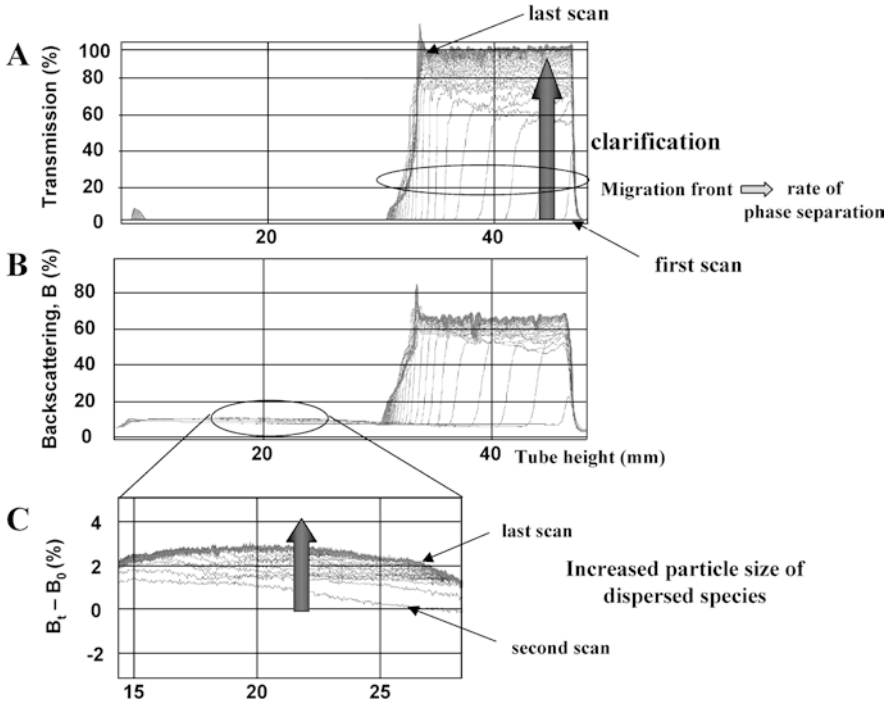

Fig. 2 Example of raw data obtained by following the evolution with time of the destabilization of the emulsion formed with the copolymer PDR. The sample was scanned every minute for 90 min. A: transmission data, B: backscattering data, C: enlargement of the backscattering data, plotted as the variation from the first scan value (in other words, B recorded at time t minus B recorded at time 0) in the part of the sample which remained totally turbid (0% transmission) over the 90 min of emulsion monitoring

Table 1 Solubility of the polymers studied in various solvents

Solvent	Ethyl acetate	DMSO	THF	Water
Dextran	I	S	I	S
Heparin	I	I	I	S
PIBCA	S	I	S	I
PHA	I	I	I	I
PHR	I	I	I	I
PDA	I	I	I	I
PDR	I	I	I	I

I: insoluble; S: soluble

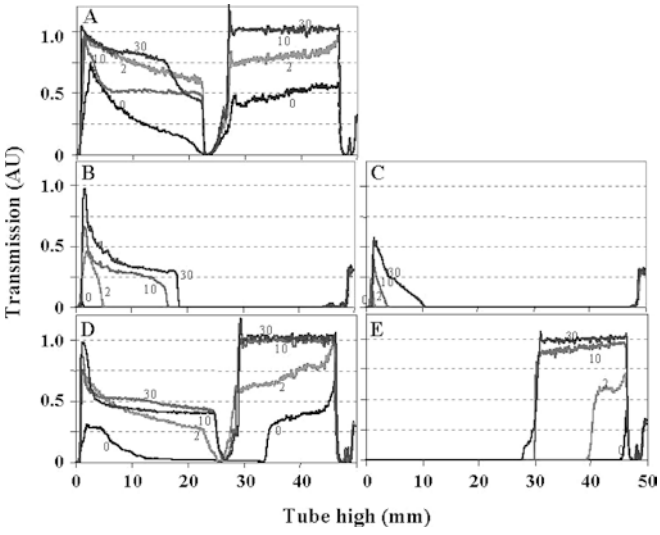

Fig. 3 Turbidity profiles for the emulsions, as monitored by the turbiscan at times 0, 2, 10 and 30 min during the analysis of the model emulsion without copolymers (A) and with copolymers PHA (B), PHR (C), PDA (D), and PDR (E)

remaining turbid, corresponding to an emulsion (Figs. 4 and 5).

The appearance of a clear domain in the emulsion was highlighted by the increase of the light transmission in the corresponding part of the tube as monitored by the Turbiscan. This leads to the appearance of a transmission peak that becomes larger as the clear domain extends in the sample (Fig. 3). By measuring the peak thickness at different times, it was possible to follow the rate of migration of the moving front between the clear and turbid domains in the sample. Figure 6 reports the evolution of the peak thickness, evaluated from the turbidity profiles monitored over the first 20 min of the experiment at a transmission level of 20%. This measurement was made in two zones starting from the middle of the sample and considering the top and the bottom parts. The initial slopes of these curves were calculated and reported in Table 2. They represent the migration rate of the moving front for the top and the bottom parts of the sample. For the control emulsion, the blend of homopolymers, and the emulsions prepared with either PIBCA, dextran and PDA, the maximum thicknesses of the clear domains at both ends of the samples were already reached after 1 or 2 min (plateau in Fig. 6). In these cases, the rate of migration of the moving front was too fast to be evaluated. Different

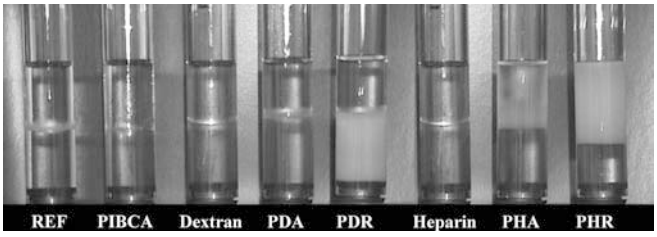

Fig. 4 Photograph of the different emulsions taken 24 hours after preparation

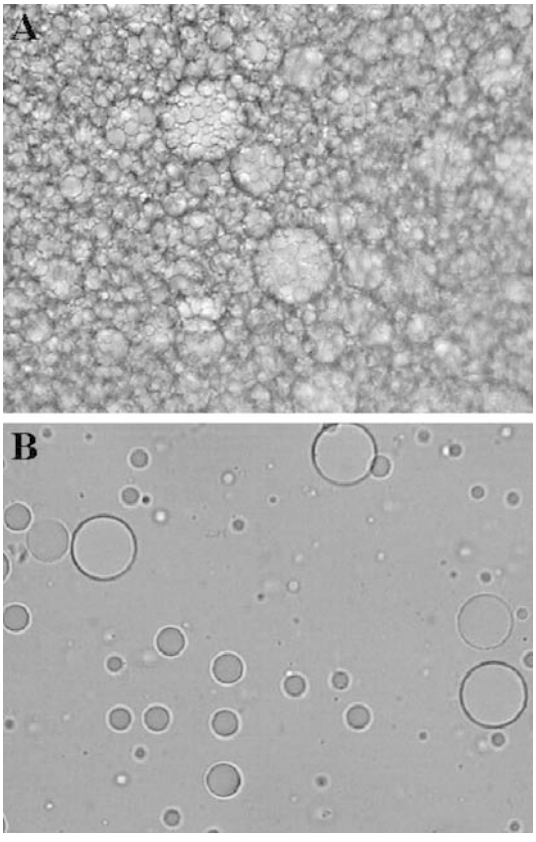

Fig. 5 Micrographs of the turbid domain in the emulsions prepared with PHR (A) and PDR (B)

migration rates could be measured for the emulsions prepared with heparin, PHA, PDR and PHR. The results were quite different according to the part of the sample taken into account. Emulsions prepared with PDR showed no moving front in the bottom of the sample. The emulsion remained turbid in this part of the sample over the period of the experiment, and even up to 24 hours after the start of it, as shown in Fig. 4. The emulsions prepared with PHA and PHR showed no moving front in the upper part of the samples, which remained turbid for at least 20 min. After 24 h, only the emulsion prepared with PHR remained fully turbid in its upper part, as shown in Fig. 4. The rate of the moving front in the bottom of the emulsion obtained with PHR was slower than that obtained with PHA.

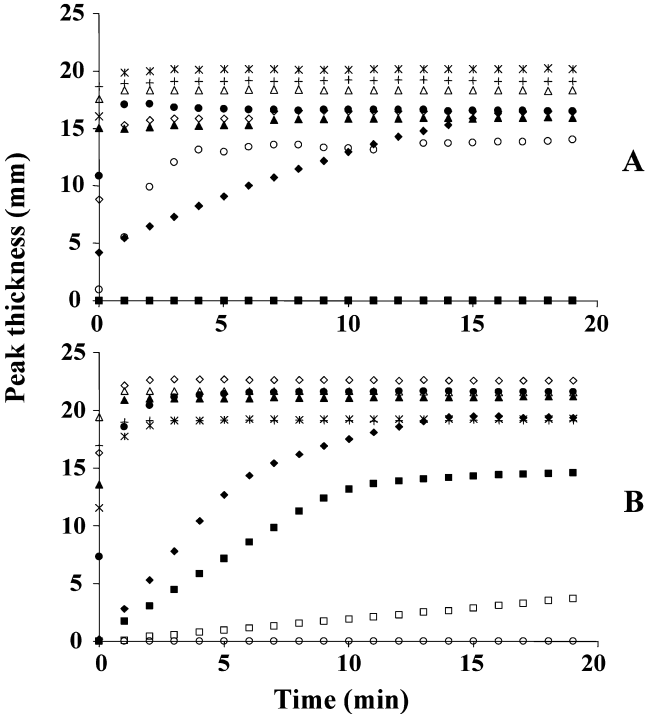

Fig. 6 Peak thickness of the clear domains appearing in the top (A) and in the bottom (B) part of the samples. Model emulsion (+); model emulsion with heparin (filled diamonds); dextran (filled triangles); PIBCA (*); PIBCA and heparin (unfilled diamonds); PIBCA and dextran (unfilled triangles); PHA (filled squares); PHR (unfilled squares); PDA (filled circles); PDR (unfilled circles)

Table 2 Rate of displacement of the moving front separating turbid and clear domains within the emulsions, evaluated from the slope of the curves showing the variation of the peak thickness with time in the top and bottom parts of the emulsion samples, as represented in Fig. 4

Polymer added in the emulsion	Rate of displacement of the moving front separating turbid and clear domains (mm/min)	
	Bottom part of the sample	Top part of the sample
None	F	F
PIBCA	F	F
Dextran	F	F
Heparin	2.5	0.9[a]
PIBCA + dextran	F	F
PIBCA + heparin	F	F
PHA	1.4	0
PHR	0.2	0
PDA	F	F
PDR	0	4.4

F: Plateau was reached after 1 or 2 min, indicating a very fast moving front; [a]at time 0 a small clear domain was already detected by the turbiscan

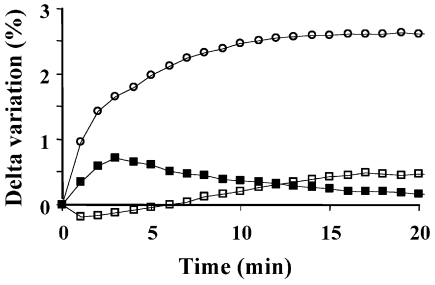

Fig. 7 Variation in the backscattering, monitored over 20 min, for the turbid domain of the emulsions prepared with PHA (filled squares); PHR (unfilled squares); PDR (unfilled circles)

The variations in the backscattering in the parts of the emulsions which remained turbid over the period of investigation are represented in Fig. 7. Whereas almost no variation in the backscattering was found for the upper part of the samples prepared with PHA and PHR (change in backscattering from time zero $< 0.5\%$), a very small increase of 2.5% could be measured for the lower part of the sample prepared with PDR during the first 10 min of the experiment. Afterwards, it reached a plateau that remained constant up to 90 mins (Fig. 2).

Discussion

The purpose of this study was to investigate whether Turbiscan could be an helpful methodology for characterizing the newly-developed block copolymers. It is based on the determination of their capacity to stabilize a model emulsion, deliberately chosen as unstable. According to Table 1, the copolymers were insoluble in the current solvents used for polymer characterization, while the corresponding homopolymers constituting the blocks were either soluble in water (for the polysaccharide part) or in one of the tested organic solvents (for the PIBCA part). The copolymers were also found to be insoluble in ethyl acetate and in water, which were the solvents chosen for the model emulsion used in this study.

Interestingly, despite the insolubility of the copolymers in both water and ethyl acetate, the copolymers dissolved quite easily in the emulsion obtained with these two solvents. This could be achieved because of the presence of an interface between water and ethyl acetate where the copolymers could find suitable environments to solubilize (the polysaccharide moiety can dissolve in water and the PIBCA part can dissolve in ethyl acetate). In the same way, it was found that the copolymers act as a surfactant, since they improved the stability of the model unstable emulsion prepared with water and ethyl acetate. This was highlighted by the monitoring of the optical properties of the emulsions using the Turbiscan (Fig. 3) and by the photographs taken 24 h after the preparation of the emulsion (Fig. 4). On the contrary, the homopolymers constituting the different blocks of the copolymers, and their corresponding blend, were not able to stabilize the emulsion, which separated into two phases in under 2 min (Table 2).

The monitoring of the stability of the emulsion using the Tubiscan, and the analysis of the transmission profiles showed that the different copolymers were not equivalent in stabilizing the model emulsion. The copolymers prepared by radical polymerization, PHR and PDR, were found to provide much better stabilization of the emulsion than the corresponding copolymers prepared by anionic polymerization, especially PDA. The copolymers PHR and PHA, containing heparin, a negatively-charged polysaccharide, appeared to provide better stabilization of the emulsion than the corresponding copolymers PDR and PDA, containing dextran, which is a neutral polysaccharide. This was clearly shown by the comparison between the different emulsions concerning the appearance of at least one clear domain. This domain resulted from the expulsion of either water or ethyl acetate from the initial emulsion, depending of the copolymer considered. Indeed, a clear domain appeared at the bottom of the sample prepared with PHR and PHA, suggesting that these emulsions were expelling water. While the bottoms of these samples were clearing, the tops remained turbid. On the contrary, in the emulsion prepared with PDR, a clear domain appeared at the top of the sample, suggesting that it corresponded to ethyl acetate (due to the lower density of this solvent compared to the density of water). In this case, the bottom of the sample remained fully turbid. In the emulsion containing PDA, clear domains appeared rapidly at both ends of the sample, indicating that this copolymer was only poorly able to stabilize the emulsion.

For the more stable emulsions, the appearance of the clear domain could result from two phenomena: coalescence, or creaming and sedimentation of the dispersed droplets, as illustrated in Fig. 8 [28]. Coalescence results from the fusion of emulsion droplets that are growing in size. Creaming and sedimentation are simple phenomena of flotation or sedimentation (depending on the density of droplets of constant size). To distinguish between these two phenomena that may be occurring in the emulsions, an analysis of the variation in backscattering for the remaining turbid part of the sample was performed. Indeed, the backscattering given by a turbid system can be correlated to the size of the dispersed droplets [26]. Almost no variation was found, indicating that the droplet size remained constant within the turbid parts of the samples over the period of investigation. Therefore, the appearance of the clear domain resulted from a creaming or sedimentation of the dispersed droplets, depending on the copolymer used to prepare the emulsion.

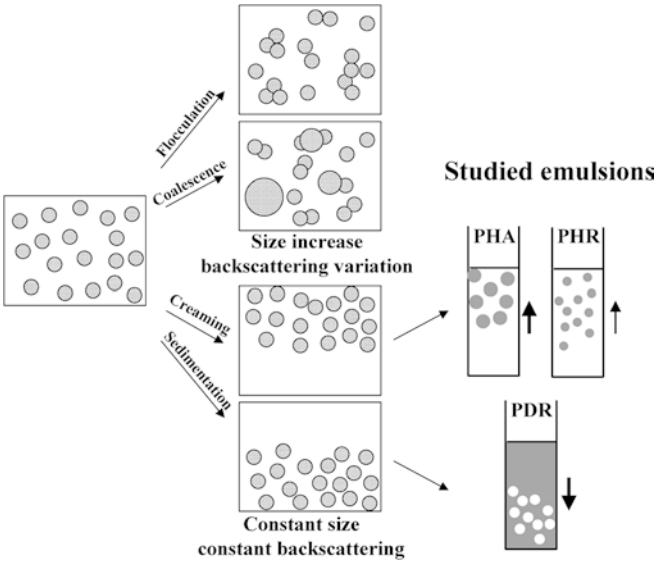

Fig. 8 Schematic representation of the evolution of the droplets in the emulsions, and of the interpretation of the data obtained with the emulsions prepared with polysaccharide containing PIBCA copolymers. In the studied samples gray represents ethyl acetate, and white represents water

It can be concluded that three copolymers, PHR, PHA and PDR, possess sufficient surface active properties to stabilize the emulsion formed by mixing ethyl acetate with water. Based on the respective densities of water and ethyl acetate, the type of the emulsion formed with these copolymers could be identified. Emulsions showing creaming phenomena contained dispersed droplets of ethyl acetate whereas emulsions displaying sedimentation phenomena contained dispersed droplets of water (Fig. 8). Therefore, PHR and PHA allowed the formation of oil-in-water emulsions, whereas PDR promoted the formation of a water-in-oil emulsion. This suggested that the heparin-containing copolymers (PHR and PHA) were better hydrophilic surfactants than PDR since they promoted the formation of oil-in-water emulsions. This property could also be related to the charge status of the polysaccharide. Indeed, heparin-containing copolymers were negatively-charged and were the most hydrophilic surface active copolymers, whereas the dextran-containing copolymer which was neutral appeared to be the more lipophilic one.

The rate of creaming or of sedimentation occurring in the emulsion depends on the droplet size [29]. This could explain the difference observed between the emulsions stabilized with the PIBCA-heparin copolymers obtained by anionic (PHA) and radical (PHR) polymerization.

Although the compositions of PDA and PDR were very similar, their capacities to stabilize the water/ethyl acetate emulsion were found to be completely different. This could be explained by the fact that the two copolymers resulted from different mechanisms of polymerization, which involved different initiation mechanisms. In the case of anionic polymerization, the initiation of the alkylcyanoacrylate polymerization occurs through the hydroxyl groups of the polysaccharide. Therefore, the polymerization can start at several places on the same dextran chain, leading to the formation of a grafted copolymer [30]. On the other hand, in the radical polymerization, the dextran chain is split into two smaller daughter chains during the initiation of the polymerization, and the radical responsible for the initiation of the polymerization is created at one chain end of the daughter chain [31]. Therefore, the PIBCA chain growing from the radical initiation point produces a linear block copolymer. This shows that the capacity to stabilize the emulsion could also be related to the structure of the block copolymer.

Conclusions

The data presented in this study showed that the Turbiscan MA 2000 could be a suitable tool for investigating the surface properties of block copolymers composed of hydrophilic and hydrophobic moieties, even if these copolymers are insoluble in the individual solvents selected to prepare the emulsion. This apparatus allowed a quantitative comparison of the emulsification properties of different insoluble amphiphilic block copolymers. For instance, it was shown that the charge of the copolymer could influence the type of emulsion formed and that the copolymer structure could influence the emulsion stability. This technique provides an original alternative method for the characterization of insoluble amphiphilic copolymers based on the investigation of their surface active properties. Further work in this area should investigate series of such copolymers and perform comparisons with series of known systems, in order to propose a new quantitative approach for the characterization of the surface active properties of insoluble amphiphilic copolymers, and to set up comparative scales.

Acknowledgements The authors are very grateful to Dr. A. De Sainte Marie, Formulaction (Toulouse, France) for lending us the Tubiscan MA 2000, and to Dr. K. Broadley, Loctite (Dublin, Ireland) for providing us with isobutylcyanoacrylate. The authors would also like to thank Dr. N. Mignard, Formulaction (Toulouse, France) for his careful reading of the part of the manuscript explaining the principle of the measurements. Cédric Chauvierre was a fellow of the Ministry of Research, France.

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