CHAPTER V

CONTROLLED HYDROPHOBIC/HYDROPHILIC CHITOSAN: COLLOIDAL PHENOMENA AND NANOSPHERE FORMATION

Abstract

The N-phthaloylchitosan grafted poly(ethylene glycol)methyl ether (mPEG) gives milky solution when dispersing in water and a series of solvents. The appearance of turbidity is depending on the types of solvents, i.e., protic and aprotic solvents. The N-phthaloylchitosan grafted mPEG shows an aggregation of sphere-like particles as observed from scanning electron microscope (SEM). The information from transmission electron microscope (TEM) declares that the spheres are at nano level. When the chain length of mPEG is as high as 5×10^3 Dalton, the sphere size becomes as small as 80-100 nm in average as observed from TEM. By simply adjusting the hydrophobic/hydrophilicity on chitosan chain, a stable nanosphere can be obtained directly.

Introduction

For the past decades, the rapid progress in analytical instrument has brought us to an understanding about the performance of polymer chain at molecular level [1]. Up to now, there have been several reports about a well-defined colloidal structure at micro- and/or nano-scale where the novel properties are discovered [2]. In some cases, the colloid is induced by the self-aggregated polymer [2] to form micro and/or nanospheres under specific conditions, such as the hydrophobic/hydrophilicity [3], ionic strength [2], and hydrogen-bonding network [4]. Recently, nanospheres have received much attention as a material for advanced applications, espectially, catalysts [5] and drug delivery systems (DDS) [3]. For DDS, the nanospheres are attractive since the drug incorporation can be achieved without destroying the active sites from harsh reactions in conjugation steps, at the same time the self-aggregation is eventually formed without crosslinker. Akashi et al. [6] proposed a polystyrene core-corona sphere which showed the ability to immobilize peptide drugs and antibodies. Here, it can be expected that micro and/or nanospheres obtained from a biopolymer backbone have the advantages about the biodegradability [7], biocompatibility [8], bioactivity [9], and non-toxicity [10].

Chitosan is an aminopolysaccharide in a deacetylated form of chitin which exists as the second most abundant natural polymer. Recently, the production of chitosan spheres for DDS was challenged via some specific processing techniques such as suspension crosslinking [11], spray drying coagulation [12], emulsification/solvent evaporation [13]. The spheres were achieved in the range of 10–700 μm. Up to now, there has been no report about chitosan spheres at micro and/or nano level obtained from the chemical modification. The functionalized chitosan studied by Ouchi et al. [14] might be the foremost approach showing the initiation of self-aggregation of chitosan.

For the past few years, we have concentrated on the modification of chitosan based on the balancing of polarity on the chain [15] in order to obtain novel derivatives. Through a specific molecular structure combining with the interaction induced from chitosan and solvent molecules, we expect to achieve the spheres in a controllable size with high stability in solvents, especially water. The present work is, thus, focused on a controlled structured chitosan by simply introducing

hydrophobic/hydrophilic groups to induce the spheres at the nano-size, which hardly obtained from general processing techniques.

Experimental

Materials

Chitosan with 90% deacetylation (M_v = 1.7 × 10^5 Dalton) was supported by the Seafresh Chitosan (Lab), Co., Ltd., Thailand. Phthalic anhydride and succinic anhydride were purchased from Fluka Chemika, Switzerland. Poly(ethylene glycol)methyl ethers (mPEG, M_n 550 and 5000 Dalton) were obtained from Aldrich Chemical Company, Inc., USA. 1-Ethyl-3-(3'-dimethylaminopropyl)carbodiimide, hereinafter abbreviated as water soluble carbodiimide (WSCI), was purchased from TCI, Japan. 1-Hydroxy-1H-benzotriazole, monohydrate (HOBt) was obtained from BDH Laboratory Supplies, England. All chemicals were used without further purification.

Sample Preparation

An amount of mPEG (M_n = 5000 Dalton, 6 × 10^{-3} moles) was reacted with succinic anhydride (1 mole equiv to mPEG) in *N,N*-dimethylformamide (DMF) at 60 °C for overnight. The mixture was reprecipitated in diethyl ether to obtain mPEG-COOH, **2a**. Compound **2a** (0.40 moles equiv to N-phthaloylchitosan, **1** [16]) was stirred with **1** (3.71 × 10^{-3} moles) at room temperature for overnight in DMF containing HOBt (3 moles equiv to **2a**) and WSCI (3 moles equiv to **2a**). The mixture was dialyzed in water and washed thoroughly with methanol to obtain white particles, **3a**. Compound **2b** and **3b** was prepared as similar to **2a** and **3a**, respectively but using mPEG with M_n = 550 Dalton. The N-phthalimido group was removed as reported [16] to yield **4a**.

Characterization Part

Compound **1**: Elemental Analysis, Anal. Calcd. for $(C_{14}H_{13}O_6N)_{0.8}$ $(C_6H_{11}O_4N)_{0.1}$ $(C_8H_{13}O_5N)_{0.1}$: (%) C, 56.17; H, 4.75; and N, 5.20. Found: (%) C, 56.18; H, 4.45; and N, 4.35: FT-IR (KBr, cm^{-1}) 3472 (OH), 1776 and 1714 (C=O

anhydride), and 721 (aromatic ring): ^{13}C CP/MAS NMR (δ, ppm) 23.3 (CH_3), 57.0 (C-2), 64.7 (C-6), 73.2 (C-3, C-5), 80.5 (C-4), 100.4 (C-1), 131.1 (aromatic ring), and 169.1 (C=O): ^{1}H-NMR (δ, ppm) 1.7 (CH_3 in acetamide), 3.4-5.0 (pyranose ring), and 7.6-7.7 (aromatic ring).

Compound **2a**: FT-IR (KBr, cm^{-1}) 3472 (OH), 2875 (C-H stretching), 1736 (C=O), and 1105 (C-O-C): ^{1}H-NMR (δ, ppm) 2.4 (CH_2 in succinic anhydride), 3.2 ($O-CH_3$), and 3.5 (CH_2 in PEG).

Compound **3a**: Elemental Analysis, Anal. Calcd. For $(C_{14}H_{13}O_6N)_{0.509}(C_{245}H_{471}O_{122}N)_{0.291}(C_6H_{11}O_4N)_{0.027}(C_{468}H_{927}O_{236}N)_{0.073}$ $(C_8H_{13}O_5N)_{0.064}(C_{239}H_{471}O_{12}N)_{0.036}$: (%) C, 54.67; H, 8.58; and N, 0.52. Found: (%) C, 56.22; H, 4.82; and N, 6.82: FT-IR (KBr, cm^{-1}) 3464 (OH), 2882 (C-H stretching), 1776 and 1714 (C=O anhydride), 1714 (C=O ester), and 721 (aromatic ring): ^{1}H-NMR (δ, ppm) 2.4 (CH_2 in succinic anhydride), 3.2 ($O-CH_3$), 3.5 (CH_2 in PEG), 2.8-4.7 (pyranose ring), and 7.6-7.8 (aromatic ring).

Compound **4a**: FT-IR (KBr, cm^{-1}) 3412 (OH), 2882 (C-H stretching), 1714 (C=O ester), 1654 (amide I), 1549 (amide II), and 895 (pyranose ring): ^{1}H-NMR (δ, ppm) 1.8 (CH_3 in acetamide), 2.4 (CH_2 in succinic anhydride), 2.9 ($O-CH_3$), 3.3 (CH_2 in PEG), 3.2-5.0 (pyranose ring), and 7.6-7.7 (aromatic ring).

Results and Discussion

In the first step, N-phthaloylchitosan, **1** (phthalimido group substitution = 80 %) was prepared in order to have homogeneous reaction system. At the same time, to achieve the controlled hydrophilic/hydrophobic chitosan, we consider phthalimido group as a hydrophobic segment and mPEG as a hydrophilic one. However, the mPEG introduction onto chitosan chain requires conjugating or coupling agent. Here, WSCI was selected as a conjugating agent. The mPEG was modified to be mPEG-COOH, **2** with succinic anhydride to enhance the reactivity. Comparing Fig. 1(a) to Fig. 1(b), the peak at 1736 cm^{-1} confirms the conjugating structure of **2a** via ester bonds.

In the next step, **2a** was introduced onto **1** to obtain white particles, **3a**. The amount of mPEG incorporation was determined by EA. Although the molar ratio of mPEG-COOH has been varied, the amount of mPEG attached onto chitosan was saturated at a certain level. For example, the conjugation of **2a** onto **3a** was limited at 0.03 moles equiv to pyranose rings. At present, we are studying the factor to control the mPEG conjugation.

In the final step, the amino groups of chitosan were recovered to obtain **4a**. By comparing the performance of **3a** and **4a**, we can clarify the effect of hydrophobic/hydrophilic chain involved in colloidal phenomena and sphere formation (see *Effect of Amino Group*).

Colloidal Phenomena and the Effect of Solvents

Chitosan and its most derivatives are insoluble in organic solvents and water. It is important to note that although the N-phthalimido group has not yet been removed, soon after the mPEG was conjugated onto **1** to obtain **3a**, the compound gave a milky solution in water and the turbidity was maintained even after 4-5 days as shown in Fig. 2(a). This appearance might come from the fact that there are some hydrations by hydrogen bonds between water and **3a** (at oxygen atoms of mPEG) (Scheme 2(a)). The dispersion stability of **3a** also reflects the function of polyethylene glycol chains about the steric hindrance to obstruct chitosan chain packing.

The colloidal phenomena were observed from the turbidity in a series of solvents as shown in Fig. 2(a). It was found that the turbidity level was largely depending on the types of solvents, i.e., protic and aprotic ones. In order to understand how the turbidity is related to the interaction between **3a** and solvent molecules, we tabulate the values of dielectric constant and dipole moment as well as the appearance of the turbidity. Here, the effects are focused on the hydrogen bonding and polar-polar interaction.

In the cases of protic solvents (1% acetic acid, methanol, ethanol, and iso-propanol), the turbidity was clearly observed (Fig. 2(a)). This might come from the hydrogen bond formation as detailed in the case of water. When it comes to iso-propanol, the hydrophobicity of the solvent might reduce the hydration; as a result,

the turbidity becomes insignificant. For aprotic solvents, **3a** is completely dissolved in DMF and dimethyl sulfoxide (DMSO), however, it is insoluble in toluene and n-hexane. The reason why the turbidity could not be seen in the cases of DMF and DMSO may be due to both high dielectric constant and high dipole moment (Table 1). Scheme 2(b) illustrates the complete dissolvation of **3a** in DMSO when a polar-polar interaction occurs between solvent molecules (δ^+ at sulfur atom) and chitosan at not only mPEG chains but also phthalimido groups (δ^- at oxygen atoms). In the cases of n-hexane and toluene, the precipitation of **3a** might come from the lack of both hydrogen bonds and polar-polar interactions.

It is important to clarify the dispersion stability of **3a** in chloroform. The low dielectric constant of chloroform implies the difficulties in hydrogen bond formation with **3a**. On the other hand, a certain value of dipole moment (μ=1.04) suggests a charge-separated structure of chloroform to favor the interaction with **3a**. As a result, the turbidity in chloroform was less than those in aprotic solvents.

Formation of Spheres of Nano-Sizes

Fig. 3(a) shows that chitosan starting material is irregular flakes. However, after phthaloylation, the compound obtained (**1**) appears as partially round shape (Fig. 3(b)), implying the sphere initiation by the hydrophobic phthaloimido groups (see *Effect of Amino Group*). The spherical particles are significant after mPEG incorporation as seen in the cases of **3a** and **3b** (Fig. 3(c)-(e)). The sizes are averagely observed to be 200 nm for **3a** (Fig. 3(c) and (d)) and 400 nm for **3b** (Fig. 3(e)) as determined by SEM.

The observation from TEM gives us the information about individual particle to determine the precise structure and size (Fig. 4). Compounds **3a** and **3b** are identified to be spheres at the average sizes of 80 nm and 400 nm, respectively. The different sphere size might relate to the chain length of mPEG. Serizawa et al. [17] reported polystyrene-mPEG core-corona nanospheres where the longer mPEG chain gave the smaller spheres. Here, we also found that the higher the molecular weight of mPEG is, the smaller the sphere size will be as characterized by TEM.

Although the drying process in sample preparation for either SEM or TEM may bring the physical structure deformation, the results obtained declared the regular size of spherical particles. We speculated that the sphere formation might occur mainly from the aggregation of chitosan chains under the hydrophobic and hydrophilic interaction as shown in Scheme 2(a) rather than the swelling effects, and thus the spherical structures are maintained. As a result, in the case of protic solvents, the strong interaction induced by the solvent enhances the sphere formation as evidenced in Fig. 3(c)-(e) and Fig. 4.

Effect of Amino Group

It should be noted that **1** also gives milky solution in water (Fig. 2(c)). This might be due to the tendency to form self-aggregation based on the hydrophobic phthalimido groups on chitosan chain. The stable dispersion under nanosphere formation is satisfied when the chitosan chains have both the phthalimido groups and polyethylene glycol chains (see *Formation of Spheres of Nano-Sizes*). When phthalimido groups were removed from **3a** to obtain **4a**, the turbidity could not be observed anymore and the compound precipitated (Fig. 2(b)). In addition, compound **4a** dissolves very well in acetic acid. This might be related to the protonation of amino groups and the hydrogen bonds between water molecules and mPEG chains. Here, SEM photograph also confirmed that **4a** does not show the sphere-like structure (Fig. 3(f)).

Conclusion

N-phthaloylchitosan grafted mPEG was a good model to fulfill the conditions for colloidal phenomena where the nanospheres were induced. The stability of the appearance of milky solution was enhanced in protic solvents where the hydrogen bonds were accomplished. The mPEG with M_n = 5000 Dalton gives the spheres with the sizes about 80-100 nm, whereas mPEG with M_n = 550 Dalton provides the sphere with the average size of 400-500 nm as declared by TEM. At present, we are studying the related derivatives, the effects of mPEG chain length and content as well as the potential applications.

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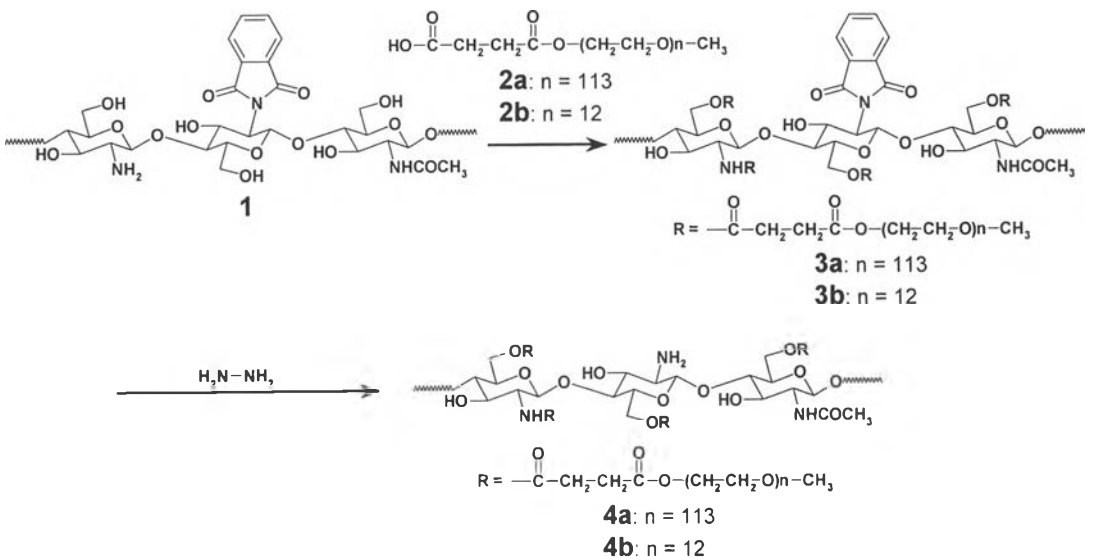
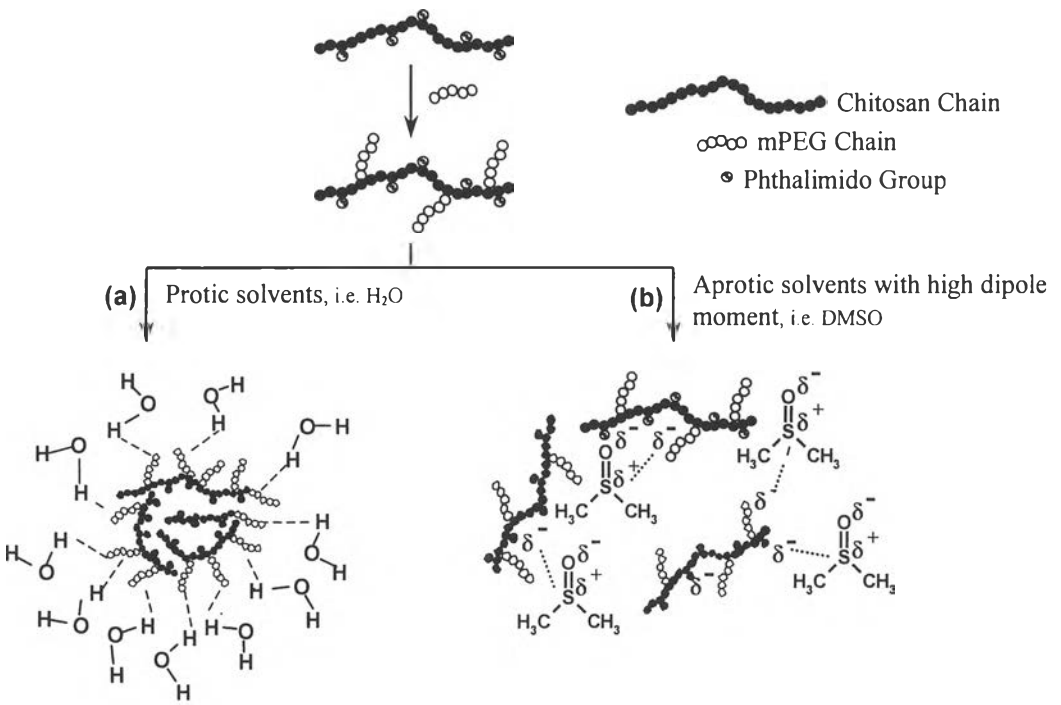


Scheme 1 (Yoksan et al.)



Scheme 2 (Yoksan et al.)

Figure Captions

Fig. 1 FTIR spectra of (a) mPEG, (b) **2a**, (c) chitosan, (d) **1**, (e) **3a**, and (f) **4a**

Fig. 2 Appearance of (a) **3a,** (b) **4a** dispersed in various solvents: **a** water, **b** 1% acetic acid, **c** methanol, **d** ethanol, **e** iso-propanol, **f** chloroform, **g** DMSO, **h** DMF, **i** toluene, and **j** n-hexane, and (c) **1** dispersed in water

Fig. 3 Scanning electron microscopy photographs at 25 kV of (a) chitosan (15,000×), (b) **1** (20,000×), (c) **3a** (20,000×), (d) **3a** (50,000×), (e) **3b** (20,000×), and (f) **4a** (20,000×)

Fig. 4 Transmission electron microscopy photographs at 30,000× of (a) **3a** and (b) **3b**

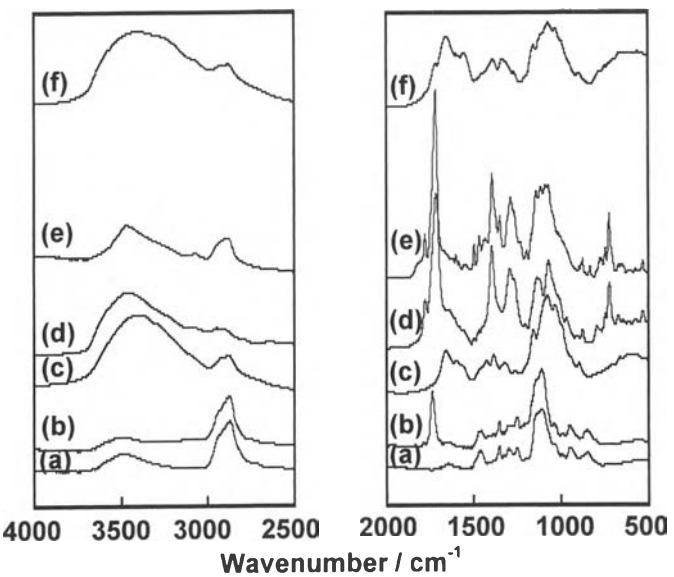


Fig. 1 (Yoksan et al.)

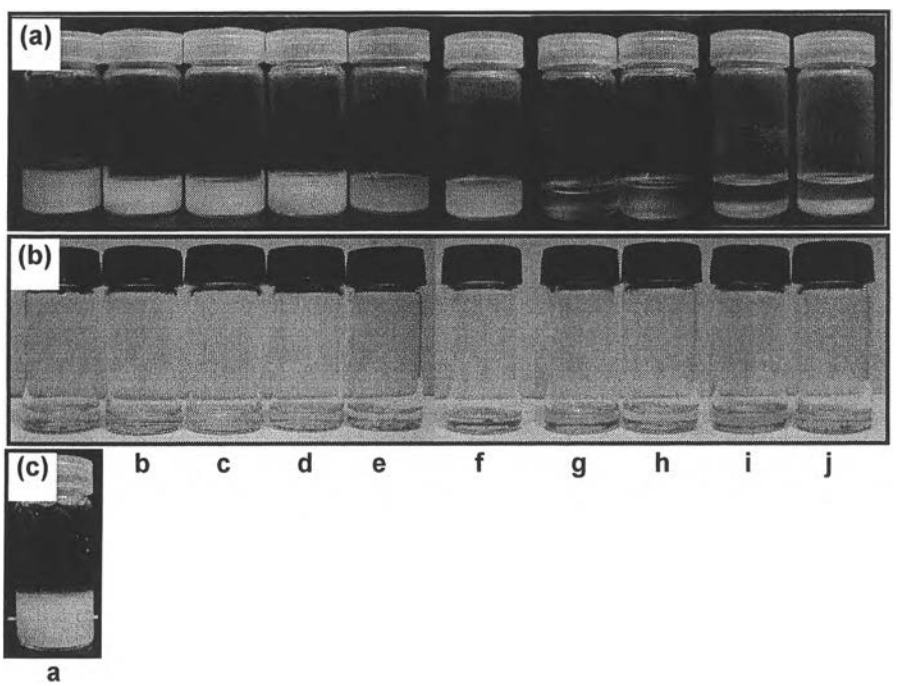


Fig. 2 (Yoksan et al.)

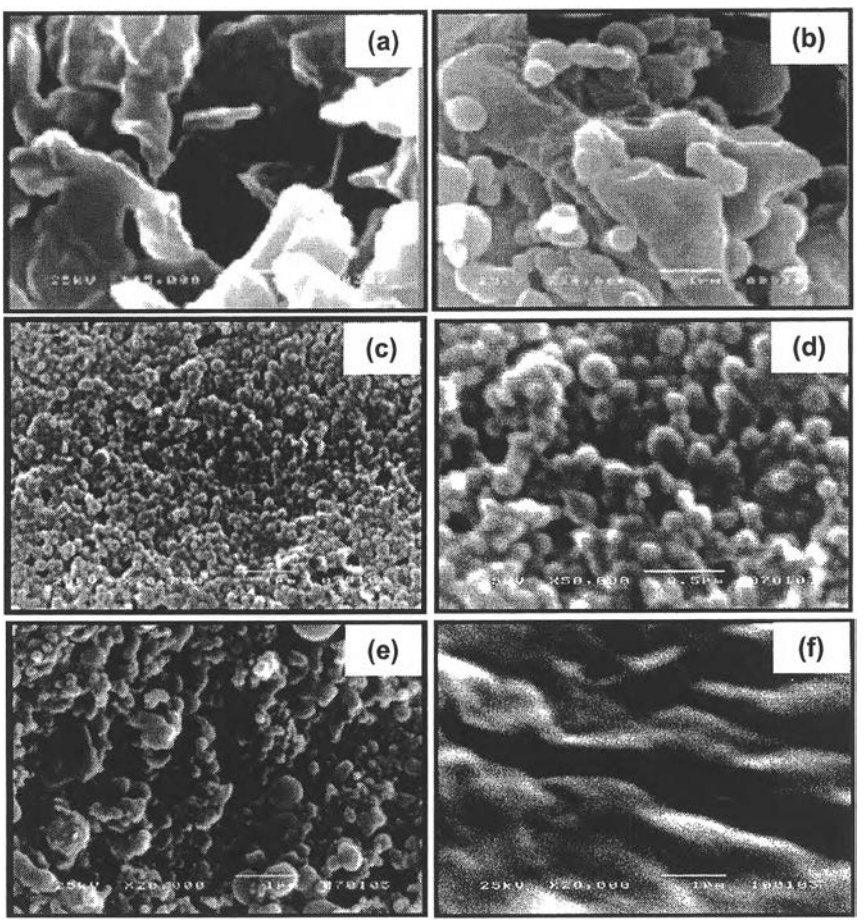


Fig. 3 (Yoksan et al.)

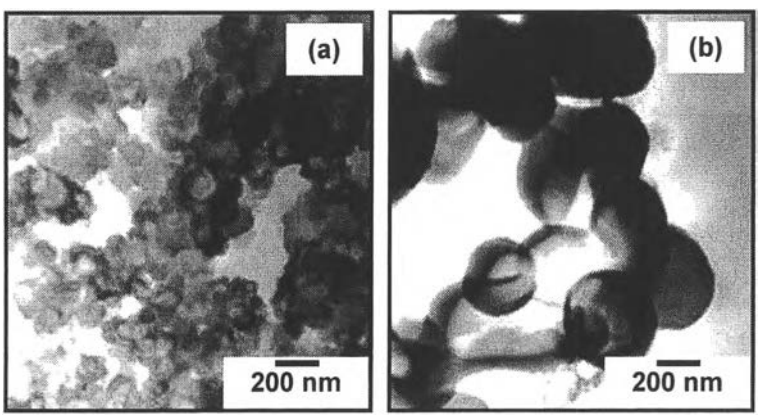


Fig. 4 (Yoksan et al.)

Table Caption

Table 1 Evaluation of colloidal formation of **3a** in various solvents relating to dielectric constant and dipole moment

Solvents	Dielectric constant[a] at 25 °C / Debye	Dipole moment[a] / Debye	Appearance[b]
Water	78.54	1.85	±
Methanol	32.63	1.70	±
Ethanol	24.30	1.69	±
Iso-propanol	15.80	1.58	±
Chloroform	4.81	1.04	±
DMSO	47.00	3.96	+
DMF	36.70	3.82	+
Toluene	2.38	0.38	-
n-Hexane	1.89	0.08	-

[a] Lide DR (eds) Handbook of Chemistry and Physics, 72nd edn. CRC Press, USA, pp 8-51, 8-49, and 9-18 - 9-26

[b] ±, Colloidal formation; +, Solvation; –, Precipitation

Table 1 (Yoksan et al.)