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Brief Report

A Facile Method for Morphological Characterization at Nano Scale

Ali Dinari[1], Seyedeh Sahar Mortazavi Farsani[2], Soheila Mohammadi[3], Farhood Najafi[4], Mahdi Abdollahi[1*]

[1] Polymer Reaction Engineering Department, Faculty of Chemical Engineering, Tarbiat Modares University, Tehran, Iran.
[2] Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran.
[3] Nano Drug Delivery Research Center, Health Technology Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran
[4] Department of Resin and Additives, Institute for Color Science and Technology, Tehran, Iran.

**Corresponding author:* Mahdi Abdollahi , Polymer Reaction Engineering Department, Faculty of Chemical Engineering, Tarbiat Modares University, Tehran, Iran. Tel: 02182884959: E-mail: abdollahim@modares.ac.ir

Background: Dynamic light scattering (DLS) and electron microscopy (EM) are the most practical techniques for nanoparticles (NPs) characterization. However, the impediments which involved the sample preparation method lead to failure in provided results of mentioned device analysis. These problems will be intensifying, if the examined samples are the soft nanocarriers such as organic ones or biological samples.

Objectives: In order to achieve the appropriate results from DLS and EM analysis, an optimized protocol was introduced by this research which would prepare samples with high degree of quality and accuracy.

Materials and Methods: Morphological analysis of prepared polymeric nanocarriers (micelles, nanogels) by this protocol were done. Filtration, dilution and sonication as three crucial and effectiveness steps of sample preparation were assessed through DLS data and EM images.

Results: This research has tried to introduce a facile method with novelty of simplicity and rapidity. These triple steps could improve the quality of morphological data. The obtained results indicated that sample preparation methods have the most effective factors on sample size distribution and homogeneity of desired samples.

Conclusions: The suggested optimized preparation method will be helpful for all soft nanomaterial's samples.

Keywords: Dynamic light scattering (DLS), EM, Soft nanocarriers, Triple steps.

1. Background

The characterization and quantification of nanoparticles (NPs) are a difficult analytical challenge since measurements are highly depended on particle size and nature, sample concentration, solution's physicochemical properties, and principles of the analytical technique (1, 2). Furthermore, most samples containing NPs are dynamic, which makes NPs determination harder. Particle aggregation as a remarkable impediment can camouflage the signal of NPs and affects Dynamic Light Scattering (DLS) results (3). This is a proper method to study nanomaterial properties including size, intensity, number, surface charge and etc. As a privilege, DLS could provide series of data, while has its own simplicity and repeatability (4). Electron microscopes, on the other hand, are powerful tools in sample's morphological analysis, due to their ability for monitoring nanoparticles in detail form (5). these include scanning electron microscope (SEM) and transmission electron microscopy (TEM). Resolution of them depends on sample preparation and voltage intensity of devices. In order to obtain high-quality images, with excellent contrast between particles and their surroundings, scientists have suggested several protocols for sample preparation (6).

2. Objective

This research tried to set up a facile method for soft nano-scale carriers such as micelles and nanogels with aim of boosting up DLS and EM outcomes. In this regard, three main parameters such as dilution, filtration and sonication have been tested in various conditions. The final optimized protocol could be applied for all the soft nanomaterial samples and gathered precise information by DLS and EM assays.

3. Materials and Methods

3.1. Materials

Herbal drug extracts (curcumin, solanin, grape seed oil extract, apple extract, fersulfate iron), polyethylene glycol (PEG) and oleic acid all were purchased from sigma aldrich company. N-isopropylacrylamide (NIPAM), 2-(dimethylamino) ethyl methacrylate (DMAEMA), N, N'-methylenebisacrylamide (MBA) Deionized water, 5 µm pore size Millex Syringe Filter was purchased from Merck Company. Brominated lignin was prepared from by polimerization lab (Tarbiat Modares University). Carbon grids (used in TEM imaging sample preparation) were prepared from Sigma Aldrich Company.

3.2. Methods

3.2.1. Polymeric Nanocarriers Synthesis

The polymeric micelles (mPEG-Oleate (Oleic acid)) was synthesized via esterification of oleoyl chloride (0.01 mol) and monomethoxy PEG 2000 (0.01 mol) according to previously described method by our lab (7). The esterification reaction was performed in the presence of triethylamine (0.012 mol) at 25 ^{o}C for 2 h, where chloroform was used as the solvent. Organic and biological components investigated in this study were dissolved in the nano-micelle solution in appropriate proportion (1:25). Nanogel as the other systems which composed of Lignin-g-P(NIPAM-co-DMAEMA) was synthesized via atom transfer radical polymerization (ATRP) reaction.

3.2.2. Filtration

In order to remove large-sized particles from prepared samples for analyzing with DLS and EM devices, all samples (micelles and nanogels) were filtered with 5 µm pore size Millex Syringe Filter.

3.2.3. Dilution of Samples

In this regard, drug-loaded polymeric nanocarriers were prepeared in dilution of 1:10 and 1:100 ratios. To do this, concentration of main source have to be determined. Preparation of serial dilution needs instrumentation (accurate pipette) and proper pipetting technique (at least 10 to 15 repetition, slow and exact).

3.0.4. Sonication

To disperse particles and reduce their aggregation, different sonication times including 5, 10, 15 and 30 minutes at constant frequency (55 Hz as device power) were tested by ultrasonic bath (WUC-D10H from Witeg company, German). All sonicated samples were used for analysis via DLS and EM devices.

3.2.5. DLS Procedure

Particle size and stability of drug-loaded polymeric nanocarriers were studied through measuring of hydrodynamic radius and zeta potential. The average particle size and zeta potential of polymeric nanocarriers (micelles and nanogels) were measured by Zetasizer Nano ZS instrument (Malvern Instruments, UK) at 25 °C.

3.2.6. EM Visualization

For TEM visualization, carbonic grid was immersed in the solution of considered polymeric nanocarriers and samples were assessed by a Zeiss - EM10C - 80 KV TEM (Philips cm30, japan). Accelerating voltage of 200 kV was used for emitting electrons and imaging process was conducted for different magnifying from 200 nm to 20 nm. All images were captured from different parts of fixed carbonic grids therefore could demonstrate the accurate estimate of micelles' shape and size. The applied procedure in SEM imaging was the same as the TEM imaging. however, the only difference is about coating samples with thin layer of gold in SEM imaging process. In this regard, polymeric nanocarriers dripped on the aluminum foil and dried in the air and coated with layer of gold in vacuum condition.

4. Results

4.1. Improvement of DLS Data Quality Based on Triple Simple and Rapid Steps

Filtration of polymeric nanocarriers solution make more homogeneity of particles structures and DLS measurements was done with more accuracy. According to the results, filtered and sonicated samples have appropriate size distribution and homogeneity compared to non-filtered and non-sonicated samples. All the presented samples in **Table 1** showing the best condition of mentioned parameters (syringe filters with 5 µm pore size and 15 minutes for sonication time). in fact, filtration used to remove dust particels or lumps prior to analysis samples (8). Concentration rate as the other problem courses to particles agglomeration and exerts an inhibitory force on their distribution. Dilution approach was the surest way to eliminate this limitation (**Table 1**).

4.2. The Effects of Triple Steps on Quality of EM Images

Sample filtering has led to suitable size and uniform distribution of polymieric nanocarriers (**Fig. 1**). Operating the sonication is the second main parameter

Table 1. DLS results of filtered and sonicated various samples.

Product name	Dilution	Before filtration and sonication Size; nm (PDI)	After filtration and sonication Size; nm (PDI)
Nano-polymeric micelles loaded by curcumin	1/10	579.7 (0.527)	68.18 (0.572)
	1/100	397.7 (0.420)	20.17 (0.429)
Lignin-based nanogel compounds	1/10	525.1 (0.594)	98.38 (1.00)
	1/100	374.1 (0.538)	39.71 (0.418)
Nano- polymeric carrier loaded by fersulfate iron	1/10	971.1 (0. 629)	129.4 (0.516)
	1/100	651.9 (0.673)	203.9 (0.373)

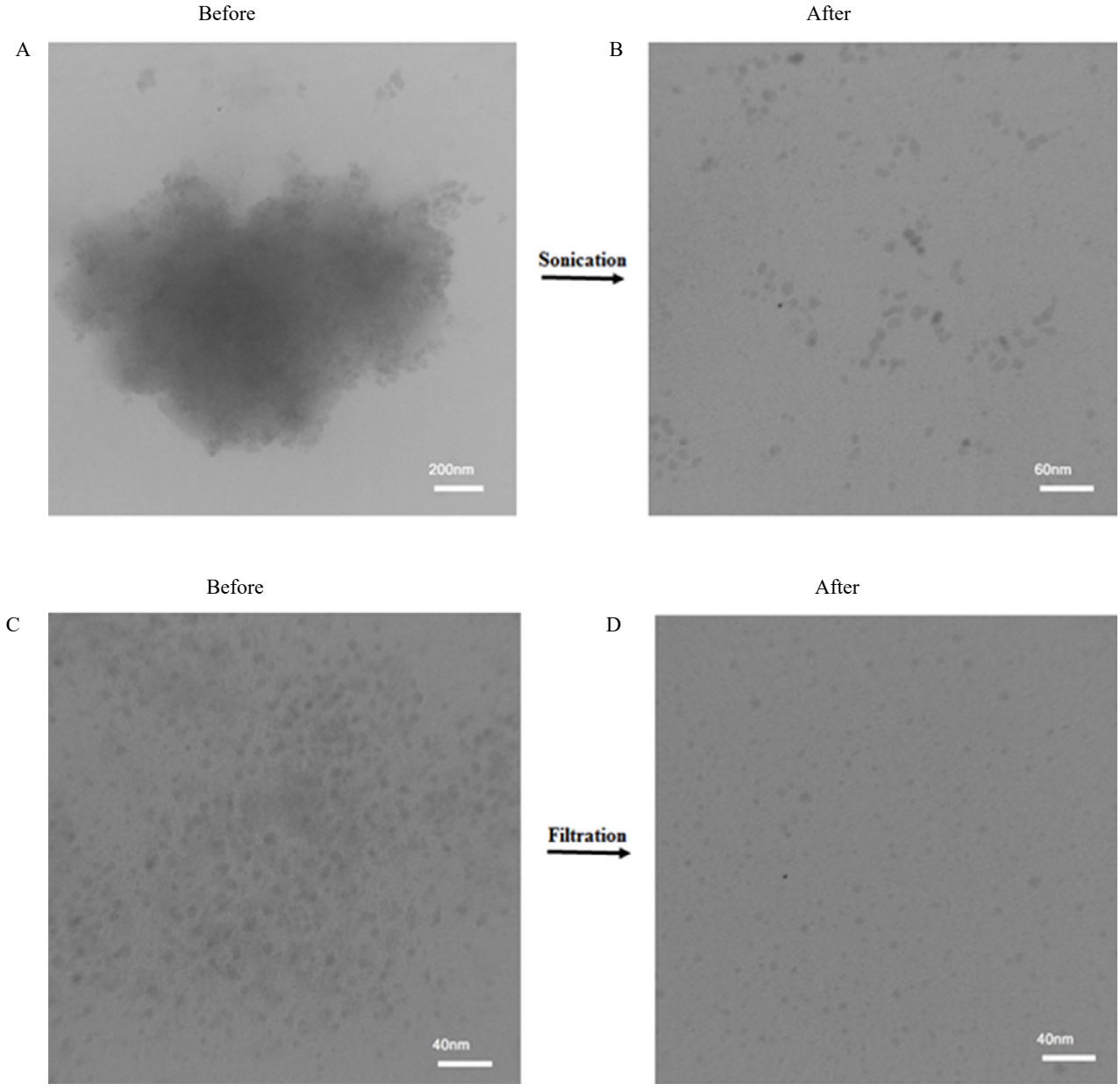

Figure 1. The effects of sonication and filtration on TEM results A) Particles agglomeration in nonsonicated sample. B) Particles with proper dispersion. C) Smeared particles by dust in non-filtered sample. D) Filtered once.

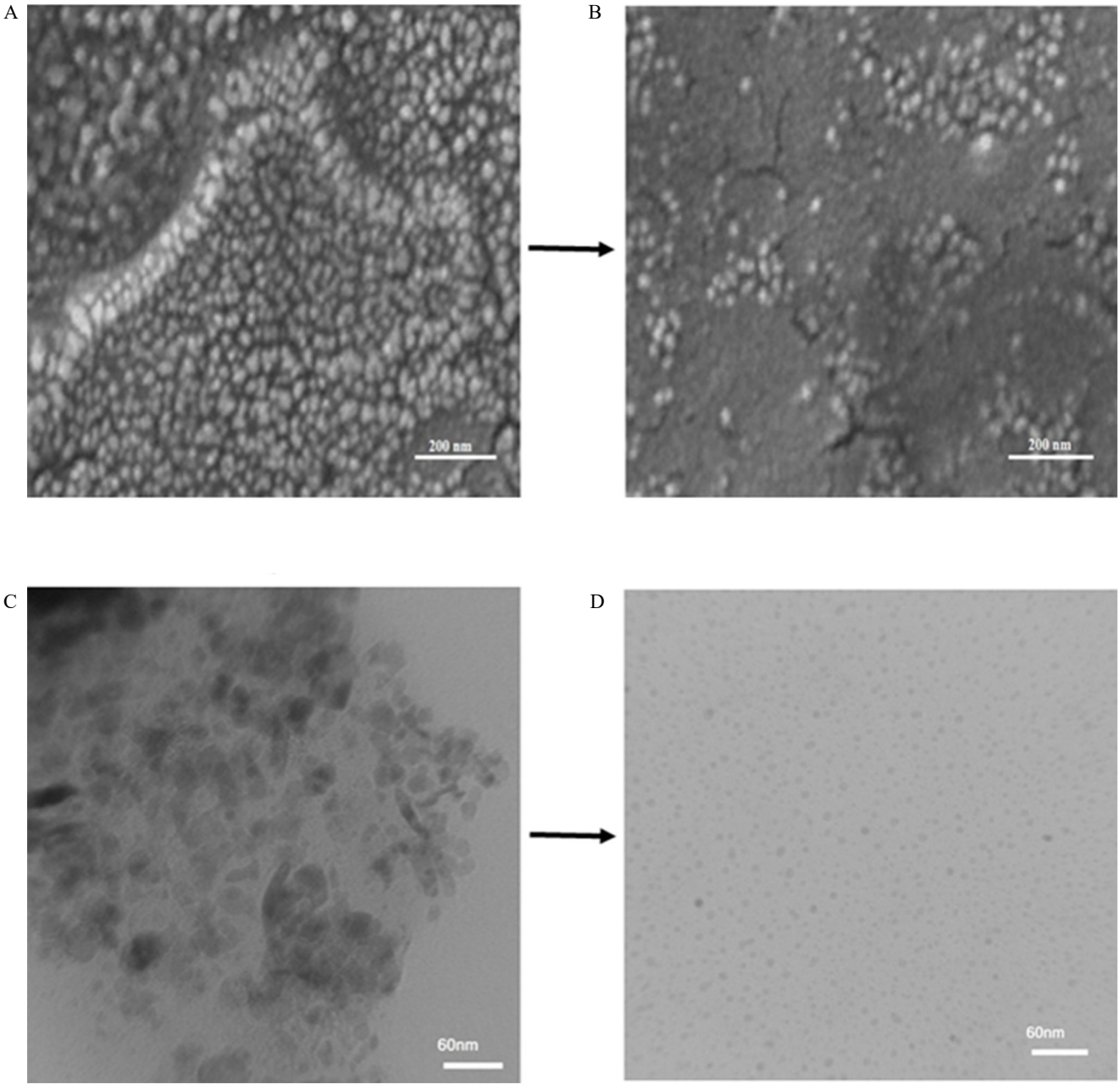

Figure 2. EM images of polymeric nanocarriers. A) SEM image of aggregated nanogels in high concentration (excess of 100 µg/ml^{-1}) samples. B) The same sample prepared in optimized method (filtration, dilution (50 to 100 µg.ml$^{-1)}$ and sonication (15 min)). C) TEM image of agglomerated particles (micelles) in high concentration (excess of 100 µg.ml^{-1}) samples. D) The same sample prepared in optimized method.

that has very influential in EM imaging. Observations of this research indicates, sonication of high-concentrated samples (excess of 100 µg.ml^{-1}) for more than 20 minutes, causes to interlocking of polymer chains, complexity of system and consequently, generates particles with micron size. Polymeric nanocarriers samples without sonication also showed large and interconnected structures. however, it should not be neglect from impact of dilution on EM imaging's quality. Samples with higher concentration shows aggregated and fusioned NPs with out of nanometer size range (**Fig. 2**). In addition, increasing the thickness and density of samples leads to electron beams diffraction with more intensity, which causes dark and unclear images. According to EM images, polymeric nanocarriers with treatments of filtration, dilution (1:100) and sonication (15 minutes) illustrated the best resolution and contrast. All suggested polymeric nanocarriers were tested for

morphological characterizations and similar results were obtained.

5. Discusion

There is copious communication and brief repoerts which emphasize on simplicity, facility, low cost and efficiency of methods (9). Determining NPs features, (especially the soft ones) such as particle size, morphology, and etc, has encouraged scientists to seek out an optimal method for enhancement of DLS data and EM imaging's quality (10). In this study, contrast of polymeric nanocarriers with their surroundings was improved by the optimized method. As mentioned, morphological characterization of drug-loaded polymeric particles was studied with DLS and EM devices. Different conditions of samples preparation was applied to investigation of three critical parameters (filtration, dilution and sonication) effects on research data's quality. Data accuracy of each sample was conformed in three experimental repetitions. It is worthy to mention that, dilution shows the important effects on inhibition of particles agglomeration and their distribution. however, particles with different size will be find in final products of dilution. The best dilution has range of 50 to 100 $\mu g.ml^{-1}$ typically (4). Actually, dilution can influence the particles number in final diluted product, but there is no effect on their size. However, filtration and sonication steps play the main role in size distribution. In addition, refractive index, light absorption and sample concentration have influenced quality and accuracy states of obtained results and their effects should be considered.

Effects of preparation steps on EM imaging's quality and resolution as another important part of present study, has been evaluated. Sonication procedure, which was optimized between 10 to 15 minutes (best frequency at 55 Hz) should be carefully considered. It is noteworthy that, sonication of polymeric NPs for long period of time (up to 24 h) can also leading to a solution of stable polymeric NPs with homogeneous dispersion (11). Our results showed that appropriate concentration of sample is also necessity to obtain perfect data. In NPs imaging system, type of NP has a great impact on images' resolution, contrast and quality. Typically, in SEM and TEM imaging techniques, inorganic nanoparticles repersent better contrast and their images have higher quality and resolution. However, capturing the images from soft materials such as polymers (synthetic and natural) and biopolymer such as proteins is much more difficult. Related to discrepancy of images quality, two reasons are imaginable. Firth, electron beams can hit organic tissues and destroy them, so that, the more power of electron beams the more organic material damages. Secondly, TEM images resolution will be reduced considerably in low voltage (12, 13). Previous reports noted that the best TEM images of polymeric nanopartials next to the gold nanorod (GNR) particles (as inorganic ones) can be obtained in 200 kV, while, in low voltage (80 kV and lower) these observation could not be obtained (14, 15). However, the mentioned triple steps of sample preparation could resulted to excellent images. For example, **Figure 2** shows the images of polymeric nanocarriers (micelles and nanogel) in the same quality as the images of inorganic ones. Browsing above mentioned images in two different states shows the efficacy of these three main steps clearly. Studying the morphology of natural or synthesized NPs as the first one step of investigations, has an impotent role to design rational and practical researches. Since DLS and EM imaging (SEM and TEM) are the most useable devices for characterizing particles, we tried to optimize a protocol for organic NPs preparation to obtain the best evaluation by DLS and EM imaging. Based on our achievements, filtration, dilution (50 to 100 $\mu g.ml^{-1}$) and sonication (15 minutes) in format of an appropriate method will illustrate the highest resolution and the most accurate results in soft nanocarriers. This protocol can be suggested for all the soft samples preparation in DLS and EM assays.

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Conflict of Interests

I certify that no actual or potential conflict of interest in relation to this article exists.

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