Ph.D. Thesis Booklet

Preparation and characterization of biopolymeric microparticles for surface-enhanced Raman spectroscopy and fluorescent microscopy imaging

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1. Introduction

Nowadays, polymeric materials are a vital part of everyday life. Hundreds of thousands of polymer building blocks – monomers – have been discovered, together with a large number of preparation and modification methods, allowing to fabricate polymers with broad variety of sizes, shapes, chemical and physical characteristics. Hydrophilic or hydrophobic, soft or hard, bulk or porous, insulating or conducting, light emitting or light absorbing, nano-, micro- or kilometer sized – polymers allow to fabricate materials with wide spectrum of properties – in many cases even from the same monomer.

Polymeric micro- and nanoparticle systems are utilized in many different areas such as in drug delivery systems, vaccines, biomedical diagnostics, aerosol research and tracking, as fillers, reference particles in simulation experiments, process troubleshooting, etc.

Some applications require highly monodisperse microparticles that are easiest to fabricate from polymeric materials. In addition to those inherited from the monomer, the properties of a polymeric structure can strongly be influenced and controlled using additives. The incorporation of organic dyes into polymer matrixes enables the creation of novel and desirable structures for a variety of applications in the technology, including life sciences as biomarkers for biological imaging, flow devices and biomedical areas. While the conventional organic dyes and fluorescent proteins exhibit high photobleaching rate during in vivo or in vitro imaging, making long-term imaging difficult

due to sample photodamage, the incorporation of the fluorescent molecule into a host polymer microparticle can be a good strategy for reducing photobleaching and photodamage.

Another promising application of polymeric microparticles is their use as supports for surface-enhanced Raman spectroscopy (SERS). Raman spectroscopy is based on the inelastic scattering (Raman scattering) of photons by the characteristic vibrations of molecules, crystal lattice, and other structural components. This method can be used to investigate a wide variety of organic and inorganic, solid, liquid, and gaseous, ordered and disordered materials. With this technique, no sample preparation is required, and the sample does not deteriorate under proper measurement conditions. The Raman signal is inherently weak, but a strong signal enhancement can be observed if the analyte molecule is adsorbed on a surface consisting of active metal nanostructures like gold and silver. This effect known as surface-enhanced Raman scattering. SERS is working on metallic nanoparticles or nanostructures in colloidal or solid substrate form. Being a promising technique with proven single-molecule detection development of efficient SERS agents is targeted by many research projects. In SERS substrate development gold coating of nano- or micro-sized morphologies fabricated by lithography, chemical modification of gold and silver, attachment of metallic nanoparticles to different substrates are the main development areas. In spite of the huge efforts and successful developments, there is still a lack of easy, reproducible, effective and cost-efficient preparation methods for SERS substrates.

While the group of metals suitable for SERS is limited, there are much more possibilities in case of the supports, and polymers could be promising materials for that. According to the Web of Science search engine, SERS agents on polymeric supports were fabricated earlier by others. Polymeric microparticles have several advantageous properties as SERS supports. They can be used to fabricate large area SERS substrates from dense microparticle monolayers or flow-through SERS cells by filling channels with the spheres; as single microparticles, they can easily be delivered in fluid or gas streams for remote sensing. The exposed parts of the microparticle not covered with the metal can also be functionalized to facilitate the attachment to specific sites.

2. Objectives

The aim of my work was to develop polymeric microparticles for fluorescent microscopic imaging and surface-enhanced Raman spectroscopic applications. For this, diethylene glycol dimethacrylate (DEGDMA) has been selected as monomer. DEGDMA based polymers are widely used in dentistry, and this biocompatible material was found to be a highly flexible subject for polymer synthesis by gamma radiation initiated polymerization while having only a binary monomer mixture, the use of appropriate solvent and concentrations allows to fabricate bulk, micro- and microporous, and even particulate DEGDMA polymers. In general, precipitation polymerization can be used to obtain monodisperse DEGDMA microspheres.

My work included the following tasks:

1. Determine the conversion of DEGDMA polymers by Raman spectroscopy with different solvents. The monomer mixtures of different composition were irradiated by various doses of gamma radiation, and this research allowed also to study the reaction kinetics of the polymerization and to determine its parameters by fitting with the Avrami equation.

2. Optimize the preparation conditions for DEGEMA microparticles obtained by gamma radiation-initiated polymerization technique and characterization of the obtained microspheres with different methods.

3. Develop a method to synthesize fluorescent monodisperse microparticles through incorporation of FITC dye into the DEGDMA structure during gamma radiation-induced polymerization technique. Characterize the fluorescent microparticles with different methods and test their performance. This task included the building of a fluorescent light-sheet microscope.

4. Develop a technique to fabricate SERS substrates consisting of DEGDMA microparticles decorated with gold nanoparticles. Examine the performance and capabilities of the obtained SERS substrates by the detection of biomolecules.

3. Materials and methods

3.1. Preparation of polymeric microparticles

Several methods are known to produce polymeric microparticles, including suspension, emulsion, solvent polymerization. The main

disadvantage of these preparation methods is their dependence on surfactants and stabilizers as basic additives in the polymerization in order to maintain the polymer phase in the synthesized solution, reduce the accumulation of product particles, prevent adhering to the solution. In contrast, gamma radiation-initiated precipitation polymerization is a facile method to fabricate polymeric microparticles. The activation of the monomers by high-energy radiation requires only the monomer and the solvent for the chemical preparation, so the particles can be created without any initiator or stabilizer. This easy and relatively simple technique allows to control the shape, size, and size uniformity of produced particles through the composition of the monomer mixture, irradiation dose, dose rate, and even the reaction time. The wide pool of monomers allows to fabricate microparticles of specific surface properties.

3.2. Morphology

Scanning electron microscopy (SEM) is an versatile method was performed to evaluate the morphology of prepared DEGDMA microparticles, fluorescent DEGDMA microparticles, poly(DEGDMA)/AuNPs composite and to characterize the particle size and size distribution. The SEM measurements were performed at the Institute for Solid State Physics and Optics of Wigner Research Centre for Physics (Budapest, Hungary) by using a Tescan MIRA3 scanning electron microscope. Imaging was performed with 1-5 kV electron energies to avoid charging and damaging the samples. The SEM images were analyzed with the ImageJ software

3.3. Photoluminescence spectroscopy

Photoluminescence (PL) is an optical phenomenon related to light emission from any kind of matter after absorption of a photon of wavelength λ1 (in a process called photoexcitation). The absorbed photon excites an electron, which decays to a lower energy excited electronic state, and then emits light of wavelength λ2, as it radiatively decays to its ground electronic state. The wavelength of emission, λ2, is usually longer than the excitation wavelength (i.e., the emitted photon has lower energy).

Since the presence of photoluminescence characteristics in a particular material can be interpreted as evidence of luminescent characteristics of the material at certain excitation conditions. In my work, I measured PL spectra to characterize the intrinsic luminescent properties of the fabricated optical material of FITC/DEGDMA microparticles.

PL spectra of the prepared luminescent polymeric microparticles structures were investigated using a Horiba Jobin Yvon 3-22 Fluorolog fluorimeter with a 450 W Xenon lamp excitation source.

3.4. Light-sheet fluorescent microscopy

Light Sheet Fluorescence Microscopy (LSFM), technique for imaging of FITC/DEGDMA microparticles, is based on the Selective Plane Illumination Microscopy (SPIM) system, is an optical technique that has been used to image specimens by monitoring the scattered light after the specimen is illuminated with an appropriate wavelength. Here, it is

worth mentioning that the cylindrical lens is a basic unit for forming the light sheet of the laser beam. The cylindrical lens is a kind of lens that concentrates light into a line rather than a point. In other words, the main feature of the cylindrical lens is to compress light in the direction perpendicular to this line, but it remains unchanged in the direction parallel to it based on the lens design.

3.5. Raman spectroscopy technique

Raman spectroscopy is an effective light scattering technique for the detection of characteristic vibrations of molecules, solids, liquids and gases. The technique can be used to characterize the molecular structure and bonding configuration of materials by observing the frequency shift between the excitation photon and the photon scattered by the medium. Raman spectroscopy is based on the inelastic light scattering effect induced by the interaction between the incident photons and the building blocks of the medium. Nowadays, Raman spectroscopy is utilized in vast range of applications such as in environmental protection, quality monitoring, temperature measurements, medical diagnostics, biology and nanomedicine.

In this research, I used two instruments for Raman and SERS measurements on my samples: a Renishaw 1000 Raman spectrometer integrated with a Leica DM/LM microscope and a Renishaw inVia Raman system integrated with a Leica DM2700 microscope. The two devices are similar, with the latter being essentially an improved version of the former, with motorized mirror control and higher sensitivity and

higher resolution detectors. Several excitation light sources are available for these devices, the Raman measurements were performed at 785 nm excitation. While 785, 633, 532 nm excitation are used for SERS studies of proposed poly(DEGDMA)/AuNPS composite. The spectra were shown using Renishaw WIRE software on PCs linked to the spectrometers, where exposure duration, laser power, integration, and measurement range were configured.

3.6. SERS measurements

Surface-enhanced Raman spectroscopy or surface-enhanced Raman scattering (SERS) is a spectroscopy tool used to enhance the intensity of Raman scattering light by adsorbing the analyte molecules on rough metal (such as gold, silver) surfaces or nanoparticles as SERS active substrates. During SERS the Raman excitation and/or the Raman scattered light is amplified through its interaction with localized plasmons – collective oscillations of the free electrons in the metal.

Recently, the SERS phenomenon was accepted two forms of enhancement mechanisms: the electromagnetic and the chemical enhancement mechanisms.

SERS enhancement factor (EF) is a fundamental characteristic of the SERS effect. The EF of the proposed SERS substrate was determined using as low as 20 micromol/L solutions of R6G in ethanol. For SERS spectroscopic measurements, the solution was dripped to the poly(DEGDMA)/AuNPs SERS surface and, after drying, the spectra were recorded. while the normal Raman as a reference, I have used the

spectrum measured on a solution dropped onto a poly(DEGDMA) substrate without hot spots (AuNPs) with 5mmol/L R6G dye concentration.

In other words, in the case of using a solution as a test sample, the number of molecules absorbed on the surface is orders of magnitude greater than the number of molecules involved in normal Raman scattering. Therefore, the analytical enhancement factor (AEF) can be given as follows:

$$\text{AEF} = \left(\frac{C_{RS}}{C_{SERS}}\right) \times \left(\frac{I_{SERS}}{I_{RS}}\right) \tag{1}$$

where ISERS and IRS represent the Raman intensities of the analyte molecule at the same wavenumber in SERS and normal Raman spectrum, respectively, and CSERS and CRS are the concentrations of the analyte molecule under SERS and normal Raman conditions, respectively.

4. New scientific results

In my doctoral thesis, I was focusing on the preparation and laser spectroscopic study of polymeric microparticles for different applications. Firstly, I prepared microparticles and estimate their typical experimental conditions, then developed them to fabricate fluorescent microparticles to be used in imaging. Secondly, I proposed a new composite of polymer microparticles covered with gold nanoparticles to be used in surface-enhanced Raman spectroscopic applications to detect certain biomolecules.

The first step of my doctoral research work was to study the reaction kinetics of DEGDMA monomer in different solvents upon gamma irradiation with different doses and to determine the dose dependence of the degree of conversion with Raman spectroscopy and mass difference measurements. Both methods showed good agreement in the DC calculated by Raman spectroscopy and mass difference-based techniques, but, the Raman method allows determining the conversion rate of the polymer by a non-contact, non-destructive and, in terms of realization, simple manner, having the potential for real-time remote monitoring in the irradiation chamber. The results were fitted using the Avrami equation, and the observed variations in reaction rate were attributed to the fact that the DEGDMA polymerization process behaves differently in alcohols and other solvents, as determined by their solubility values.

In the second phase, I synthesized polymeric microparticles of DEGDMA monomer with ethyl propionate solvent by gamma radiation-initiated precipitation polymerization firstly, then FITC dye has incorporated into the polymer matrix to form luminescent FITC/DEGDMA microparticles. The morphology, size, bonding configuration, and emission properties of the microparticles were investigated by using SEM, Raman, PL spectroscopy, and LSFM, respectively. It was found that the structure of the FITC was preserved after the irradiation and the obtained microparticles have good emission properties.

In the last part of my study, I have developed the method to synthesize SERS enhanced substrate by attaching gold nanoparticles to polymeric microparticles prepared by gamma radiation-initiated polymerization. Firstly, I fabricated poly(DEGDMA)/AuNPs composite and investigated their enhancement efficiency as a SERS substrate using R6G the common fluorescent probe dye. Secondly, the practical applications of proposed SERS substrates were tested by DNA strand-specific to the parasite Giardia lamblia (the β-giardin gene).

<u>The new scientific results achieved during my doctoral research work are summarized in the following points:</u>

1- I studied the polymerization kinetics of diethylene glycol dimethacrylate (DEGDMA) upon gamma radiation in different solvents with Raman spectroscopy and mass difference measurements [I].

   (i) I found that the rate of polymerization strongly depends on the type of the solvent, and the process is faster in alcohols than in acetone, ethyl acetate, and ethyl propionate. This behavior was explained by the difference in the solubility factors of the solvents.

   (ii) By the detailed analysis of the Raman spectrum of DEGDMA with irradiation dose, I showed that there are intact C=C bonds in the polymer structure even well after reaching the plateau in the conversion curve. This indicates the presence of partially polymerized or entrapped

monomer molecules in the formed structure. The C=C Raman peak intensity was found to correlate with the type of the solvent and the reaction rate of polymerization, being smaller for alcohols than for acetone, ethyl acetate, and ethyl propionate.

2- I developed a method to synthesize fluorescent microparticles by incorporating fluorescein isothiocyanate isomer-I (FITC) dye into the diethylene glycol dimethacrylate (DEGDMA) polymer network by gamma radiation-initiated polymerization [II-IV].

   (i) I found that the addition of the dye to the DEGDMA monomer mixture increases the size and alters the spherical form of the resulting particles.

   (ii) I demonstrated that the emission properties of the obtained micro-objects are in good agreement with those of the FITC indicating, but with an extended excitation wavelength range, that can be attributed to the interconnection between the FITC molecules and the polymer structure.

3- I developed a method to synthesize surface-enhanced Raman spectroscopic (SERS) substrate by attaching gold nanoparticles (AuNP) to the polymeric diethylene glycol dimethacrylate (DEGDMA) microparticles prepared by gamma radiation-initiated polymerization [V, VI].

(i) I showed that the DEGDMA/AuNP composite has two plasmonic peaks in the absorption spectrum. The 550 nm band can be attributed to single gold nanoparticles, and the 700 nm one to their aggregates.

(ii) By studying the surface enhancement properties of the substrate with Rhodamine 6G (R6G) molecule I found that for 785 nm excitation wavelength its enhancement factor is $4.4*10^3$ and the detection limit with 20 micromol/L, and it shows good long-time stability performance over 2 months in SERS experiments. I proved the SERS substrate's biosensing capability through the detection of DNA sequences.

5. Publications related to the Ph.D. thesis

[I] **Malik H. Mahmood**, László Himics, Tamás Váczi, István Rigó, Roman Holomb, Barbara Beiler, Miklós Veres; "**Raman spectroscopic study of gamma radiation-initiated polymerization of diethylene glycol dimethacrylate in different solvents**", (Journal of Raman Spectroscopy, 52 (**2021**) 1735–1743),

doi.org/10.1002/JRS.6207 **Q2** **IF: 3.133**

[II] **Malik H. Mahmood**, László Himics, Péter Baranyai, László Péter, Miklós Veres; "**Preparation of luminescent monodisperse microparticles prepared by gamma radiation**

initiated polymerization", proceedings of the Annual Conference of the Hungarian Microscopic Society at Siófok, Balaton, May 23-25, **2019.**

[III] **Malik H. Mahmood**, László Himics, László Péter, Péter Baranyai, Miklós Veres; "**Luminescent monodisperse microparticles prepared by gamma radiation-initiated polymerization**", proceedings of the 14th Hungarian Aerosol Conference at Visegrád, October 2-4, **2019**.

[IV] **Malik H. Mahmood**, László Himics, László Péter, Péter Baranyai, Miklós Veres; "**Characterization of luminescent monodisperse microparticles prepared by gamma radiation-initiated polymerization**", (Optical Materials, 108 (**2020**) 110209),

doi.org/10.1016/j.optmat.2020.110209   **Q1**   **IF: 3.08**

[V] **Malik H. Mahmood**, Ali Jaafar, László Himics, László Péter,Ágnes Nagyné Szokol,István Rigó, Shereen Zangana, Attila Bonyár, Miklós Veres; "**Surface-enhanced Raman scattering substrates for DNA detection based on nanogold-capped poly(DEGDMA) microparticles**", proceedings of the 25th Saratov Fall Meeting Conference, Laser Physics and Biophotonics, at Saratov, Russia, September 27- October1, **2021**.

[IV] **Malik H. Mahmood**, Ali Jaafar, László Himics, László Péter, István Rigó, Shereen Zangana, Attila Bonyár, Miklós Veres; **"Nanogold-capped poly(DEGDMA) microparticles as surface-enhanced Raman scattering substrates for DNA detection"**, (Submitted for publication to J. Phys. D: Appl. Phys.),

6. Further scientific publication

[III] Ali Jaafar, **Malik H. Mahmood**, Roman Holomb, László Himics, Tamás Váczi, Anton Y. Sdobnov, Valery V. Tuchin, and Miklós Veres; **"Ex-vivo confocal Raman microspectroscopy of porcine skin with 633/785-NM laser excitation and optical clearing with glycerol/water/DMSO solution"**, (Journal of Innovative Optical Health Sciences, (**2021**) 2142003),

doi.org/10.1142/S1793545821420037   **Q1**   **IF: 1.77**