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Title: Fiber-reinforced nano-pigmented PMMA as an improved denture base

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Abstract: Objectives: The aim of this study was to incorporate three different fibers to a synthesized PMMA powder for denture bases, to evaluate their physical properties, Candida albicans adherence and toxic effect.

Methods: Pink poly(methyl methacrylate) powder was synthesized using TiO₂ and Fe₂O₃ nanoparticles as pigments, and 1% of glass fibers, polyethylene fibers and flock fibers were added. PMMA and fibers were examined by Scanning Electron Microscopy. PMMA with each type of fibers was mixed with methyl methacrylate and peroxide benzoyl for thermopolymerization in a water bath. Samples were prepared to test elastic modulus, flexural strength, porosity, Candida albicans adherence and cytotoxicity. Lucitone 199 was compared with the experimental groups. One-Way ANOVA ($p < 0.05$) and Tukey Test were applied.

Results: PMMA were synthesized into spherical shapes. Polyethylene and Lucitone 199 fibers are a bundle of thin fibers, whereas glass fibers and flock fibers are single fibers. There were no differences in elastic modulus ($p > 0.05$) but a difference was found in flexural strength ($p < 0.05$), having the PMMA with glass fibers the highest value. Lucitone 199 showed the higher porosity and the acrylic resin with more than the 50 % of more Candida albicans adherence compared with the PMMA with all the reinforced fibers. All the tested groups resulted non-toxic material after being in contact with mouse fibroblast culture during 24 h.

Significance: The PMMA containing nanopigments and reinforced with glass fibers, polyethylene fibers and flock fibers are suitable means for producing physical/mechanically adequate and non-toxic reinforced materials with antimicrobial properties for dentistry applications.

Dear Editors of the Dental Materials:

The **Fiber-reinforced nano-pigmented PMMA as an improved denture base** by Moreno-Maldonado V, Barceló-Santana FH, Vanegas-Lancón D, Plata-Rodríguez ME, Castaño VM, Acosta-Torres LS was prepared with our results on the synthesis, physical-chemical characterization, *Candida albicans* adherence and cytotoxicity evaluations of reinforced acrylic resins for denture bases colored with nanopigments.

Looking forward to here the comments of the reviewers.

Sincerely yours.

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Fiber-reinforced nano-pigmented PMMA as an improved denture base

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1. Introduction

Fractures in poly(methyl methacrylate) (PMMA) dentures occur mainly after 3 years in service [1]. Several factors in the clinical environment can induce the denture bases failure, such as occlusal disharmonies, overload, repeated chewing stress, handling and mechanical impacts caused by accidents [2].

In spite of the current developments in polymer technology, PMMA is used in the 95 % of thermopolymerized acrylic resins for denture bases, because of its color, durability, solubility and biocompatibility properties [3,4]. Therefore, it is important to enhance the strength and fatigue of denture base resins [5,6]. One approach to resolve this problem is to incorporate reinforcing fibers into the denture base polymer during the processing [7].

Previous in vitro studies claim that the reinforcing fibers used in denture bases allow an increase in their mechanical properties, as well as avoiding crack propagation [8,9].

Physical and mechanical properties of polymers are crucial for achieving clinical success and durability of complete dentures. The denture base must be able to withstand high impact forces in addition to normal chewing forces [10].

The goal then is to formulate a material that can be used in exactly the same way as conventional denture base PMMA [1].

Some commercially acrylic resins for denture bases have used reinforcing fibers in their formulation naming the products as “high impact” or “impact resistance” like Lucitone 199 (Dentsply), a commercial product containing plastic fibers [11].

The glass fibers were firstly used as reinforcement in the mid-late 40's, they are good thermal non-conductors and have been popular in many industrial applications. Until 1970, glass fibers were used in dentistry to reinforce denture bases [12]. It is necessary to carry out a silanization process of glass fibers to modify its surface layer, thus producing a rough surface that increase the contact area and also, showing chemical changes in the fiber creating covalent bonds with the PMMA [13,14]. This modification of the fiber's surface has another important advantage, because water sorption decreases significantly, as compared to glass fibers without silanizing [15].

Polyethylene is chemically inert, it has good and high impact resistance [12]. Because of its global production (~60 million tons annually) is a very cost-effective product, which has gained popularity and makes a perfect choice for reinforcement with fibers.

The flock fibers or rayon fibers are obtained from cellulose base manufactured disulfide. Currently, the flock fibers are used in the Maxillofacial Prosthetic Clinic in the Dentistry Faculty at the Universidad Nacional Autónoma de México, as a material of characterization of PMMA in soft tissue implants and in ocular prostheses due to its different colors availability [16].

Denture stomatitis is a common form of oral Candidiasis and it develops among complete and partial denture wearers, and is defined as an inflammation of the palatal mucosa in contact with the denture [17]. *Candida albicans* is one of the opportunistic pathogens in human oral cavity and introduction of predisposing factors such as systemic disease, immunosuppressive drugs, xerostoma, or dentures result in fungal infections [10].

It is difficult to avoid pathogenic microorganism's adhesion to the surface of the dental materials though some efforts have been made [18]. The one crucial characteristic virulence of *Candida albicans* is its ability to form biofilms and adhere to the surface of biotic and abiotic materials [10].

The condition begins with *Candida* biofilm growth onto the denture-mucosa interface. Biofilm growth progresses over the denture surface, leading to inflammation of the denture-exposed palatal mucosa [19].

The objectives of the present study were to synthesize PMMA particles and to incorporate different types of reinforced fibers, evaluating physicochemical behavior, cytotoxic effect and *Candida albicans* adherence. The fundamental hypothesis was that using reinforces fibers increase the physical-mechanical properties of PMMA denture base polymer, besides present lower *Candida albicans* adherence and being a non-toxic polymer.

2. Materials and Method

Materials used are summarized in Table I.

2.1 PMMA synthesis

PMMA was synthesized by the suspension polymerization technique using methyl methacrylate as monomer (200 g), benzoyl peroxide as initiator (1 %) and gelatin (2.5 %) as suspending agent. The reaction was carried out in a five neck flask using reflux and nitrogen atmosphere during 2 h. The last 30 min of the reaction, 0.01 % of Fe₂O₃ (70 - 299 nm), and 0.01 % of TiO₂ (50 - 225 nm) nanoparticles [20] were dissolved in water and incorporated into the reaction system to obtain a pink polymer. The resulting particles were carefully washed and dried at 60 °C during 24 h.

2.2 Control group

The commercial acrylic resin Lucitone 199 was used as control group. The fibers of Lucitone 199 were separated from the powder using a mesh No. 400 to perform the physicochemical characterization of the fibers.

2.3 Reinforcing fibers

Three types of fibers were incorporated in the synthesized PMMA formulation: glass fibers, polyethylene fibers (red fibers, 3 mm in length) and flock fibers (red fibers; 500 µm in length).

2.4 Glass fibers silanization

The glass fibers were cut at 3 mm in length and wetted in 1% methacryloxypropyltrimethoxysilane during 24 h. After that period of time, the glass fibers were dried at 60 °C for 24 h.

2.5 Spectroscopy

To confirm the silanization procedure, the glass fibers were analyzed by, Fourier Transformed Infra-Red (FT-IR) spectroscopy, conducted in a Bruker Vector 33 Instrument by the transmittance technique. The FT-IR spectra were obtained at room temperature, in the region 400 and 4000 cm⁻¹.

2.6 Scanning Electron Microscopy

SEM observations of polymer particles (PMMA and Lucitone 199) and fibers (glass, polyethylene and flock fibers) were carried out with a JSM-6060LV Scanning Electron

Microscope (JEOL, Peabody, MA). The samples were coated with gold by vacuum evaporation and examined with X500 magnifications.

2.7 Samples preparation

Samples were prepared by mixing PMMA powder with 1 % of each kind of fibers, then the mixture was incorporated to MMA (powder:liquid ratio was 3:1), with 1 % initiator (benzoyl peroxide) and packed into metallic flasks at the dough stage. The thermopolymerization was conducted by immersing the flasks in 70 ± 1 °C water for 90 min followed by another immersion in boiling water for 30 min. Lucitone 199 samples were prepared according to the manufacturer's directions. Samples were trimmed with wet abrasive paper of grit 100 and 300 (Fandelli, Mexico), in order to obtain 65 X 10 X 2.5 mm samples for flexural behavior analyses, 30 X 10 X 2.5 mm for porosity test and 10 X 2 mm discs for cytotoxicity assay and *Candida albicans* adherence.

2.8 Flexural behavior

Ten samples of each acrylic resin were prepared (n=40) and after being immersed in a 37 °C water bath for 48 h, the flexural behavior was analyzed in a tension-compression cell (Mecmesin, Horsham, England), using a cross head of 0.5 kg/min. Samples were loaded to failure in a three-point bending machine. The elastic modulus (E) and flexural strength (S) were calculated through the formulas $S = 3PL / 2bh^2$ and $E = FL^3 / 4\delta bh^3$, where P is the load at break, b and h are the width and the thickness of the specimen, respectively, L is the length between supports (10 mm), δ is the maximum deflection of the center of the beam, and F is the slope of the tangent to the initial straight-line portion of the load-deflection curve.

2.9 Fracture Surface

After the flexural test, the fractured segments were axially observed by SEM to evaluate the fracture surface to assess surface quality and porosity.

2.10 Porosity

Ten samples per group were initially weighted and placed into silica gel desiccators. Every 24 h sample's weight was recorded until constant mass (± 0.0005 g). Internal porosity (V_{ip}) of each sample was calculated with the equation $Wa = (dr-da)(V_{sp}-V_{ip})$, where Wa is the sample's weight (g), dr is the acrylic resin density (1.198 g/cm^3), da (0.00123 g/cm^3) is the local air

density at 21°C and 585 mmHg, V_{sp} is volume of samples and V_{ip} the volume of internal porosity (cm³).

2.11. *Candida albicans* adherence

Candida albicans ATCC 90026 (American Type Culture Collection, Manassas, VA) was cultured overnight in yeast broth (Sigma-Aldrich). Cells were harvested by centrifugation at 3,000 rpm for 5 min and pellet was adjusted to obtain a suspension with 0.15 optical densities at 540 nm. Three sterilized resin specimens in each acrylic group (n=12) were placed into 24-well sterile culture plates (Nunc) and 500 µL yeast suspension was added. After a 24 h incubation period at 37 °C, non-adherent cells were removed from specimens by washing for 10 min under sonication, followed by 3 washings with distilled water for 1 min under shaking. Adherent fungi were extracted by incubation with 1.0 mL of benzalconium chloride for 15 min. Finally, a microbial cell viability assay based on luminescent ATP measurement (Bac Titer-Glo, Promega, Fitchburg, WI) was performed in order to determine the number of viable cells adhered to composite resins. Briefly, extract aliquots (20 µL each) were mixed with 30 µL BacTiter-Glo reagent in 1.5 mL-ependorf clear tubes and luminescence was recorded after 5 min in a 20/20 luminometer (Turner Biosystems, Promega) at wavelength of 590 nm emission. Relative luminescence intensity, in 10 sec-integration periods, was measured in triplicate (n=36).

2.12 Cytotoxic Assay

Three acrylic samples in each group (n=12), were sterilized by exposure of both planes to ultraviolet irradiation during 5 min. 3T3-L1 mouse embryonic fibroblast-like cells were exposed to samples and proliferation was assessed by measuring reductase enzymatic activity by transformation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a colored reduced form [21]. The cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% bovine fetal serum (Gibco) and 100 U/mL penicillin-streptomycin in 95 % humid, 5 % CO₂ atmosphere at 37 °C. For cytotoxicity experiments, culture medium was prepared following the ISO10993-5 specifications [22], then 1x10⁴ cells were sown in 24-well sterile plates (Nunc-Thermo Fisher Scientific, Roskilde, Denmark) and incubated in these extraction media for 24 h. Three acrylic samples of each group were placed in contact with the cells and incubated during 24 h at 37 °C. After that,

the acrylic resins were removed and the cell viability was measured by the MTT assay following the manufacturer instructions (Sigma, St. Louis, MO). The absorbance was measured in a microplate reader (Bio-Rad 680) at a wavelength of 655 nm. Cell cultures with no resins were used as controls. Each experiment was ran in triplicate (n=36).

Statistical Analysis. One-Way ANOVA ($p < 0.05$) and Tukey Test were applied to the elastic modulus, flexural strength, porosity, *Candida albicans* adherence and cytotoxicity results.

3. Results

3.1 Spectroscopy

FT-IR glass fibers and silanized glass fiber spectra showed the characteristic stretched bands corresponding to the Si - OH and Si - O - Si bonding in 900 and 1100 cm^{-1} . The silanized glass fibers presented the ester carbonyl C = O stretching vibrations at 1200 cm^{-1} , proving the silanization interaction in the fibers (Figure 1).

3.2 Morphology of fibers and polymers particles

Figure 2a and 2b shows that glass fibers and flock fibers are similar in width and shape; they are single fibers around 12 and 15 μm in diameter. The polyethylene and Lucitone 199 fibers (Figure 2c and 1d) are formed by many joined thin fibers (3 – 5 μm) forming a bundle; final fibers are 25 – 30 μm in diameter. Figure 2e and 2f presents spherical particles for the synthesized PMMA and Lucitone 199. Both materials have spherical shapes and particles size between 5-120 μm and 12-150 μm respectively.

3.3 Flexural behavior and porosity

In the elastic modulus values there was no significant statistically difference ($p > 0.05$) between all groups, but in the flexural strength there PMMA with glass fibers and Lucitone 199 showed the higher values, compared to the PMMA, with polyethylene and flock fibers presenting a statistically difference ($p < 0.05$). The porosity values indicated that incorporating flock fibers reduced the porosity percentage (Table 2), but all the synthesized polymers with the different fibers showed lower porosity than Lucitone 199, which is the material with the higher porosity value ($p < 0.05$).

The images in Figure 3 correspond to the fracture surfaces of the polymers after the flexural test. The PMMA with glass fibers (3a) and Lucitone 199 (3d) showed similar structure without voids and with similar surface. PMMA with flock fibers and PMMA with polyethylene fibers showed that fibers pulled-out from crack surface and voids corresponding to the pulled-out fibers of the other half of the samples.

3.4 Candida albicans adherence and cellular compatibility

Figure 4 shows a statistically significant difference ($p < 0.05$) between the PMMA experimental groups with different kinds of fibers and Lucitone 199, the commercial resin. Lucitone 199

showed more than the 50 % of the adherence of *Candida albicans* compared with the PMMA with polyethylene, glass and flock fibers.

The higher LRU's value indicates the higher *Candida albicans* adherence in the acrylic resin surface. The PMMA groups with polyethylene fibers, flock fibers and glass fibers showed no significant difference.

The activity of fibroblast-like cells cultured in the presence of materials with different kind of fibers was explored. An enzyme metabolic assay, reflecting viability of cultured cells, showed no toxic effect on the exposed cell population, so all the reinforced PMMA have a biocompatibility behavior similar to that of the commercial acrylic resin (Figure 5).

4. Discussion

It has been reported the synthesis of PMMA with metal-oxide nanopigments to get a pink like-gum acrylic resin with lower porosity but with no better flexural behavior [20]. Due to the above, in this study pink PMMA was synthesized and three kinds of reinforcing fibers were added to the PMMA powder and process in water bath in order to improve the mechanical properties, considering the need for prosthesis fractures are a common time consuming and costly problem for patients [1].

The synthesized PMMA particles, using gelatin as suspension agent, showed spherical shape and similar particle size compared to the commercial acrylic resin. Previous studies reported that using gelatin during the PMMA synthesis as suspension agent produce spherical particles and it is easy to remove during washing [23].

Glass fibers present good aesthetic characteristics and can chemically bond to denture base resins through a silane treatment [5], thus improving the flexural properties and wear resistance [24].

In the present study, the results of FTIR spectroscopy confirmed the chemical silane integration to the structure of the glass fibers, so it was assured the correct usage of the fibers into de PMMA composition hopping the best integration.

According to other researches, the best reinforcing agents are considered to be nonwoven bundle filaments of 10 to 20 μm diameter of high-density polyethylene or polypropylene fibers combined with custom-made composite resin pontics [25]. In this study the SEM observations showed that polyethylene and Lucitone 199 fibers are a bundle of fibers with a final diameter of 25 – 30 μm , unlike the flock and glass fibers which consist of a single filament around 12 – 15 μm . However, it was not found an influence of the shape of the fibers on the physical properties of the fibers incorporated to the PMMA and with Lucitone 199.

It is very difficult to place accurately the reinforcement fibers in the desired position in the denture base resin [26]. Continuous unidirectional fiber-reinforced composites are anisotropic and provide superior reinforcement; despite they are difficult to place in the correct position in the denture [24,27]. It has been reported the use of chopped fibers with distribution in all directions (isotropic) providing comparable properties than the polymers with anisotropic fiber

distribution [24], so, in the present work fibers were mixed with the PMMA powder to obtain a random-oriented fibers distribution in the matrix of the processed polymer.

The properties of the final products are related to the kind of fibers, the length of the fibers and the concentration of fibers. There was found that reinforce a commercial acrylic resin adding glass fibers (1 %; 4 mm) improved the elastic modulus and the transverse strength [24]. In the present research the PMMA powder was reinforced with glass fibers (3 mm), polyethylene (3 mm) and flock fibers (0.5 mm) using 1 % of fibers in all groups. The comparison was carried out with Lucitone 199, a commercial high impact acrylic resin containing organic pink fibers. The elastic modulus was similar in all groups, but the flexural strength was higher when the glass fibers were used, it can be justified because glass fibers had a previous silanization process providing a chemical interaction with the PMMA molecule resulting in higher resistance [27]. Therefore, denture base resins reinforced with polyethylene fibers lack adequate strength [5] and there were no publications about denture base reinforcements with flock fibers.

The Lucitone 199 results showed in this study are according to previous publications in flexural strength value (78.2 ± 2 MPa) and in the appearance of the fracture surface of showing a layered pattern [28].

The porosity was lower when experimental fibers were used in the PMMA formulation compared with Lucitone 199 resulting in good a good quality for the synthesized PMMA, because significant porosity can severely weaken the acrylic resin prosthesis and a spongy denture tissue surface, full of nutritive substances, is an ideal incubator for species such as *Candida albicans* [29].

The first interactions leading to plaque formation is the microbial adherence to surface prosthetic materials. Herein in this works *Candida albicans* was cultured under aerobic conditions to obtain a cell suspension and incubated with the acrylic samples to assess the attachment of *Candida albicans*, the most common oral-associated pathogen, to the fiber reinforced PMMA. The inability of current antifungal therapy to cure denture stomatitis emphasizes the importance of treatment methods directed towards reducing initial fungal attachment, to oral surfaces, including mucosa and denture surfaces [30]. Some researchers have tried to incorporate antibacterial components into dental materials, hoping to reduce the

microorganism adherence, but it could not produce an everlasting effect and may affect some physical and mechanical performance of the dental materials [18]. The present results showed decreased *Candida albicans* adherence in the three experimental tested groups compared with Lucitone 199 which showed the higher *Candida albicans* adherence. This behavior can be associated not to the reinforced fibers but to the nanopigments used during the PMMA synthesis. Recent research was performed using a synthesized PMMA with and without nanopigments and the pink PMMA showed less *Candida albicans* adherence. The TiO₂ and Fe₂O₃ nanopigments are metallic oxide particles with photocatalyst activity and with reported antimicrobial effect because of their crystal structure of rutile and anatase (TiO₂) and hematite (Fe₂O₃) [20].

One of the criterions for biocompatibility is that the material is not toxic to cells. In vitro cytotoxicity tests are required steps in the screening of new materials for use in vivo. The MTT test method was selected for use in the present study. It is based on an evaluation of the mitochondrial function after exposure to potential toxic substances. Biological properties of dental materials are important in relation their clinical use, because in some clinical situations the fibers may be covered only by a thin layer of polymer or come directly in contact with oral tissue [31].

As shown in Figure 5, cells incubated for 24 h with PMMA reinforced fibers indicated that the new formulations and Lucitone 199 was devoid of toxicity.

According to the hypothesis of the study, these results demonstrated that nanostructured metal coloring additives and glass fibers, polyethylene fibers and flock fibers are suitable means for producing physical-mechanically adequate and nontoxic reinforced materials with antimicrobial properties for dentistry applications.

Further research on the reinforced denture bases is therefore encouraged for future prosthodontics development.

5. Conclusions

This work points out a potential of fiber reinforcement for the improvement of resin-based dental materials.

The flexural strength was notably improving using glass fibers; herein the porosity was decreased with the three kinds of reinforced fibers.

Candida albicans adherence showed a marked decrease when the synthesized PMMA with nanopigments and fibers were used.

Non-toxic effect was demonstrated in all the acrylic resins tested by the cytotoxicity assay.

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Captions.

Table 1. List of materials used in this study, manufacturer and batch number.

Table 2. Mean values and standard deviation for the flexural behavior and porosity test.

Figure 1. FT-IR spectra of a) silanized and b) not silanized glass fibers.

Figure 2. Scanning Electron Micrographs of a) glass fibers, b) flock fibers, c) polyethylene fibers and d) Lucitone fibers. Particle polymer images for e) synthesized PMMA and the commercial acrylic resin f) Lucitone 199.

Figure 3. Fracture surface observations after the flexural behavior tests. a) PMMA with glass fibers, b) PMMA with flock fibers, c) PMMA with polyethylene fibers and d) Lucitone 199.

Figure 4. Luminescence assay results of *Candida albicans* adherence onto PMMA, PMMA reinforced with glass, flock and polyethylene fibers, and Lucitone 199.

Figure 5. Biocompatibility of PMMA fiber reinforced assessed through a metabolic assay in 3T3 fibroblast-like cell line.

Table 1. Nanopigments, reagents, fiber reinforcing materials and a commercial acrylic resin used.

Product	Manufacturer	Batch number
Titanium oxide (TiO ₂)	González Cano y Compañía S.A. de C.V. (México)	R-F9400
Iron oxide (Fe ₂ O ₃)	González Cano y Compañía S.A. de C.V. (México)	R-4511
Benzofenone-2	Sigma (St. Louis, MO)	34156
Methyl methacrylate	Sigma (St. Louis, MO)	MKBC5616
Benzoyl peroxide	Sigma (St. Louis, MO)	01720DH
Trimethoxysilil prophylmethacrylate	Sigma (St. Louis, MO)	115K0058
Gelatine	Knox, Unilever (México)	0114ABAP
Acetic acid	J.T. Baker (USA).	
Glass fibers	VVG, Fibras y Resinas (México)	
Polyethylene fibers	Plásticos Sonora (México)	
Flock fibers	Navi Empaques, S.A. (México).	
Lucitone 199	Dentsply (York, PA)	20122

Table 2. Mean values of the flexural behavior and porosity test.

GROUP	ELASTIC MODULUS (GPa)	FLEXURAL STRENGTH (MPa)	POROSITY (% w)
PMMA + GF	2.6 (0.3)	79.2 (0.4)	4.2 (0.7)
PMMA + FF	2.5 (0.3)	76.4 (0.3)	3.9 (0.5)
PMMA + PF	2.4 (0.2)	76.4 (0.3)	4.6 (0.8)
LUCITONE 199	2.5 (0.2)	78.2 (0.2)	6.8 (1.0)

Standard deviation in parenthesis.

Figure 1
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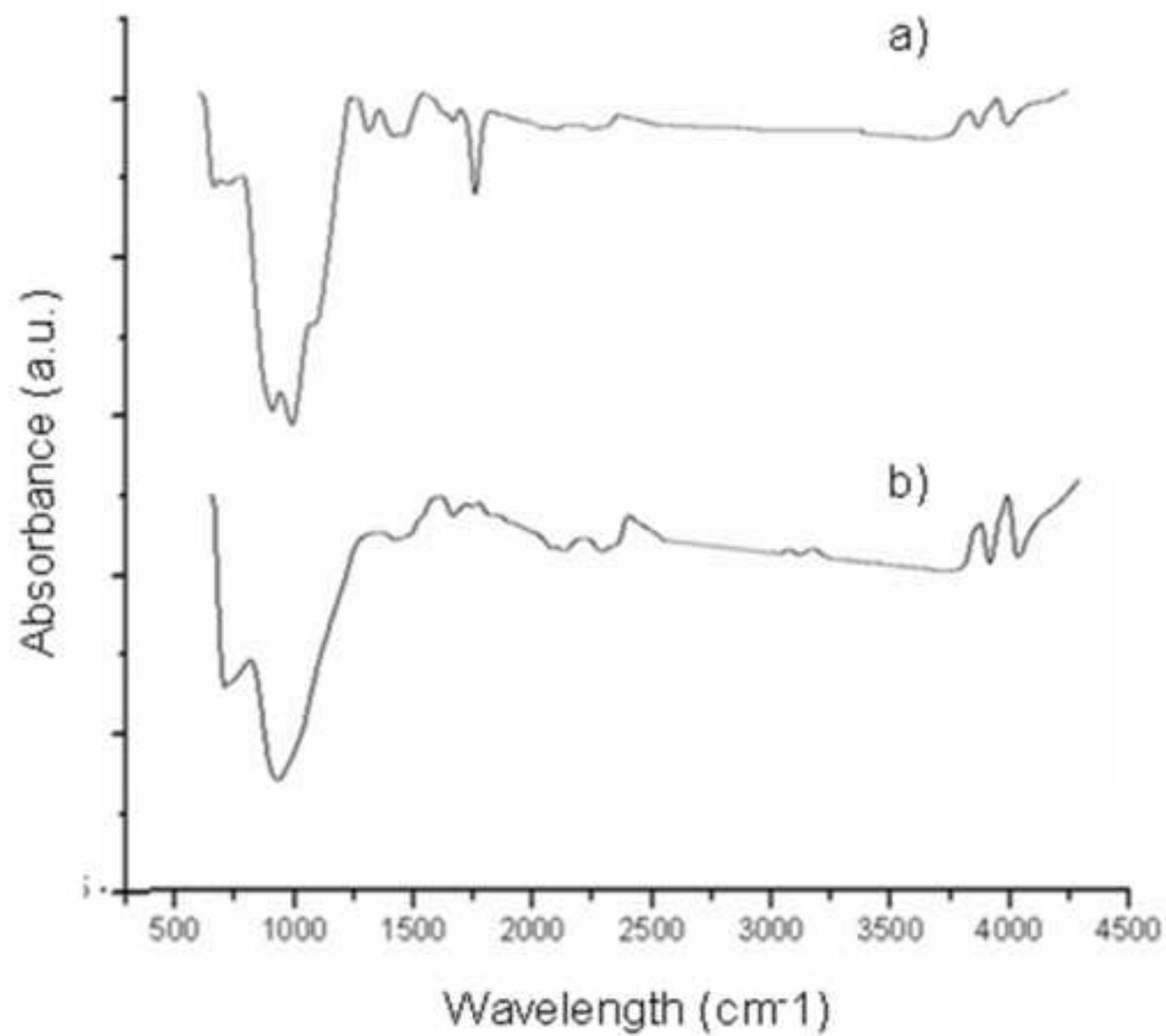


Figure 2
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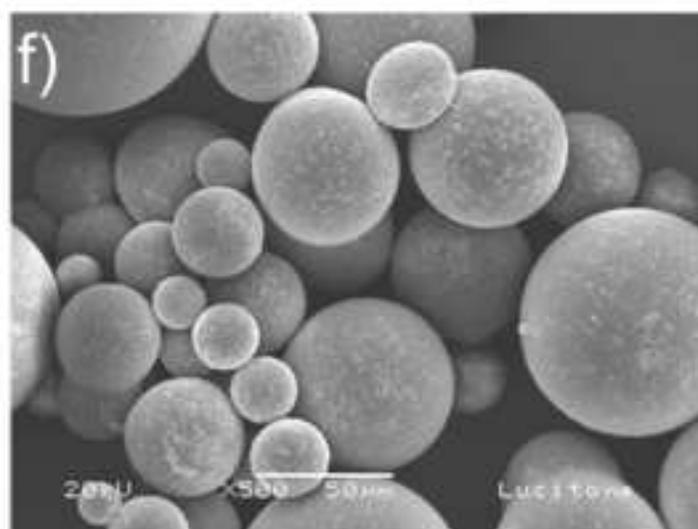
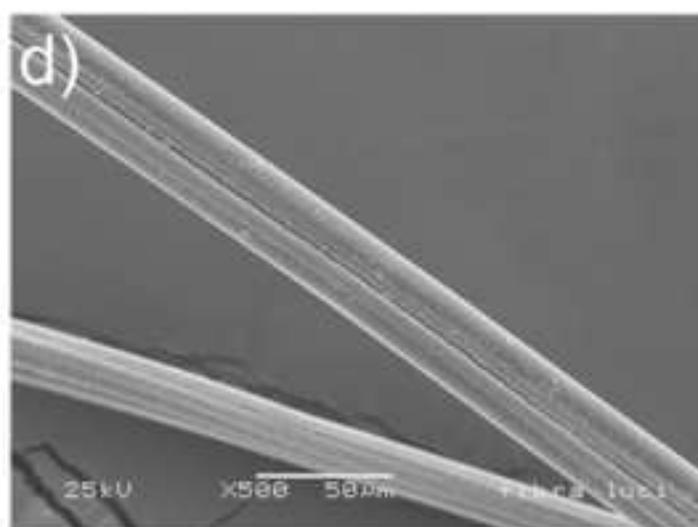
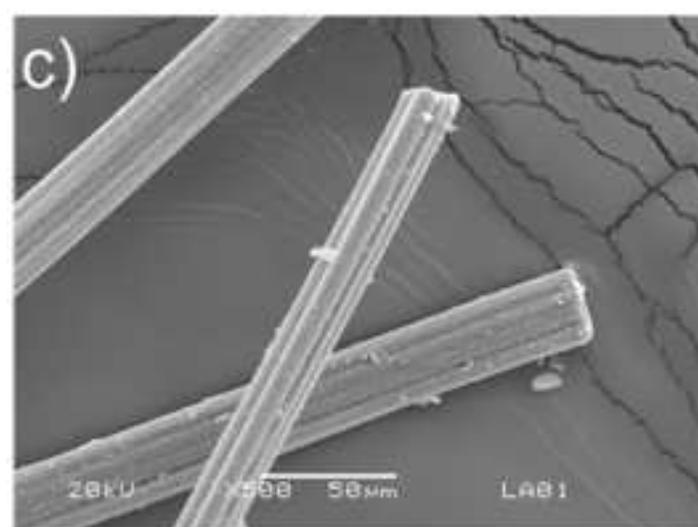
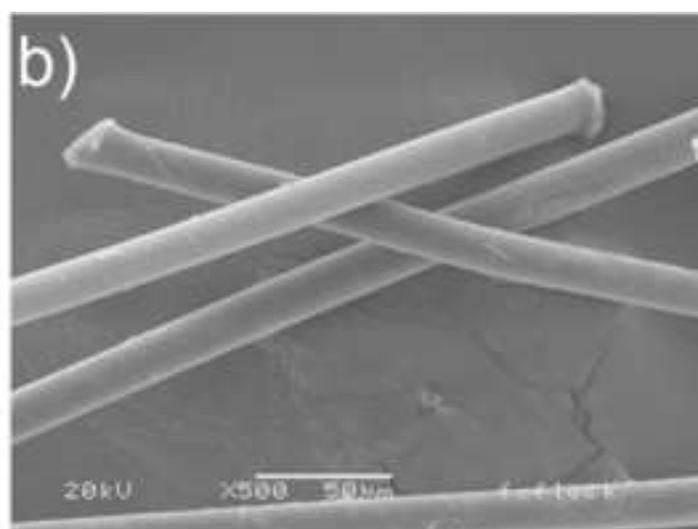
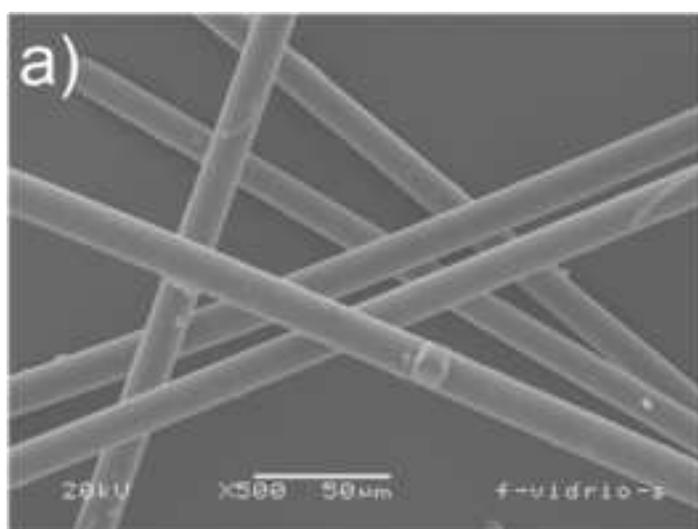


Figure 3
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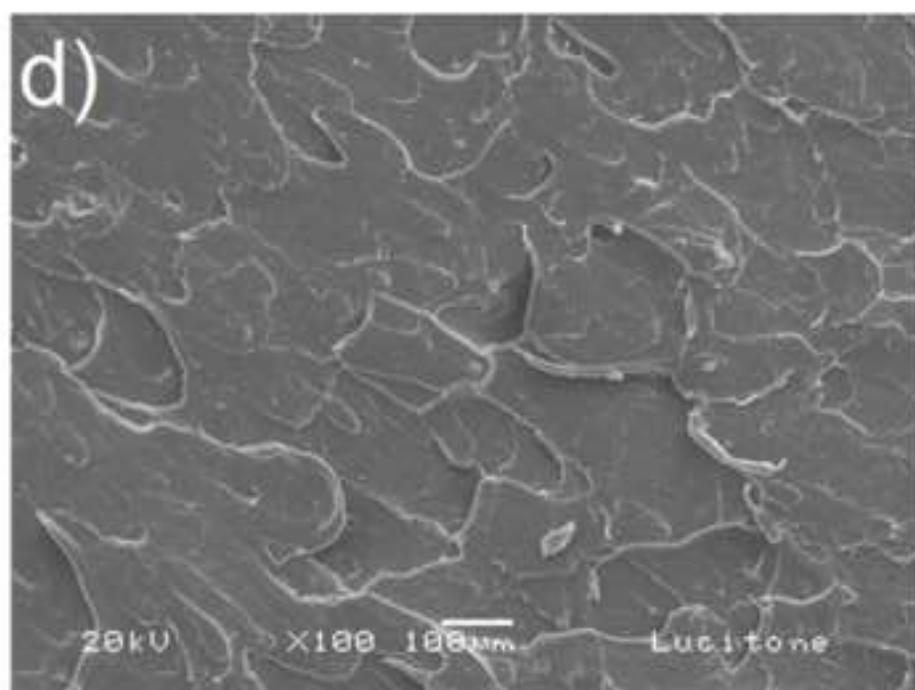
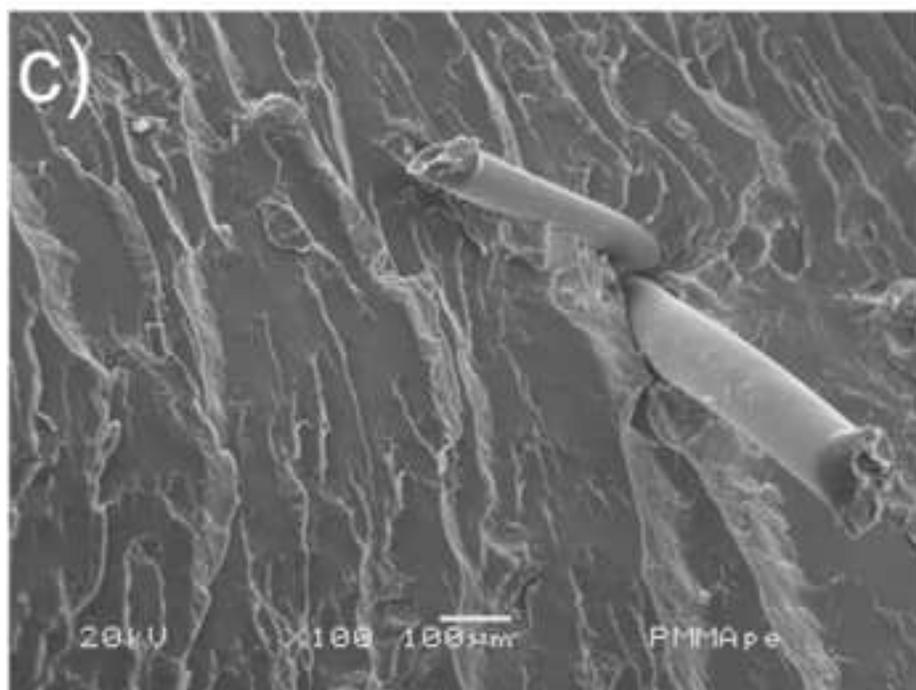
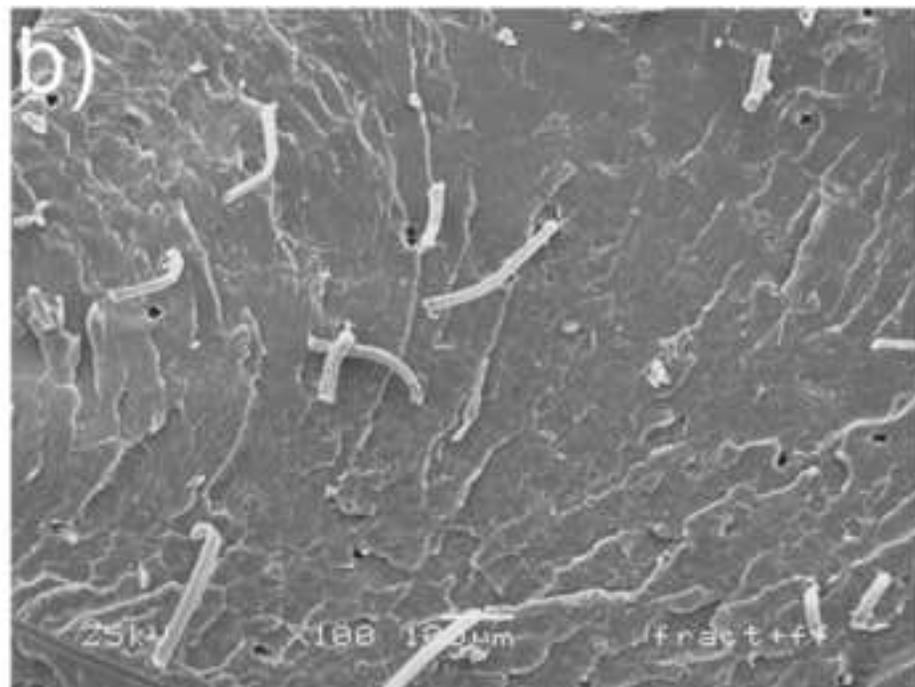
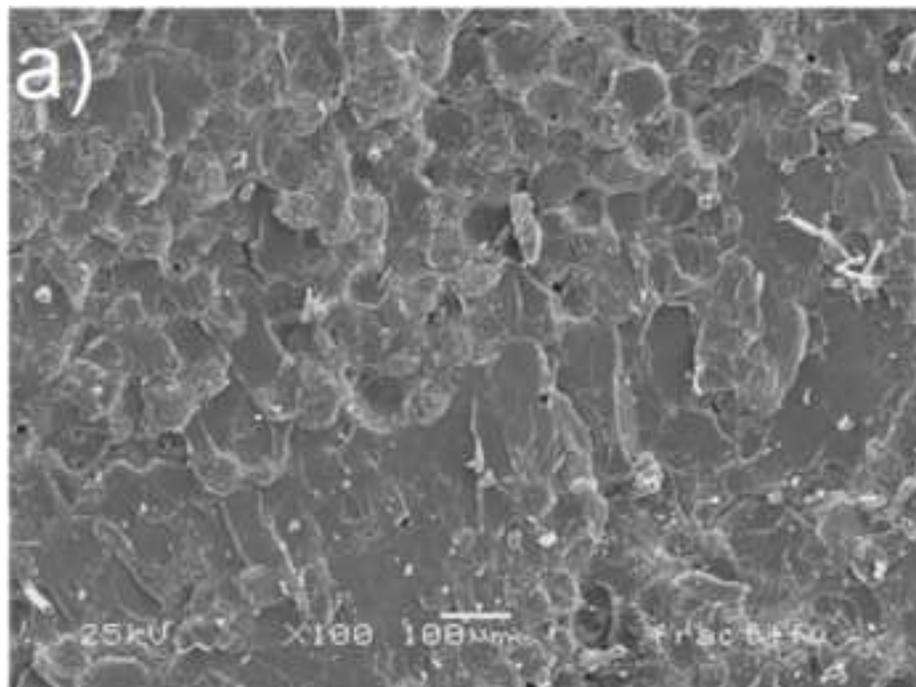


Figure 4
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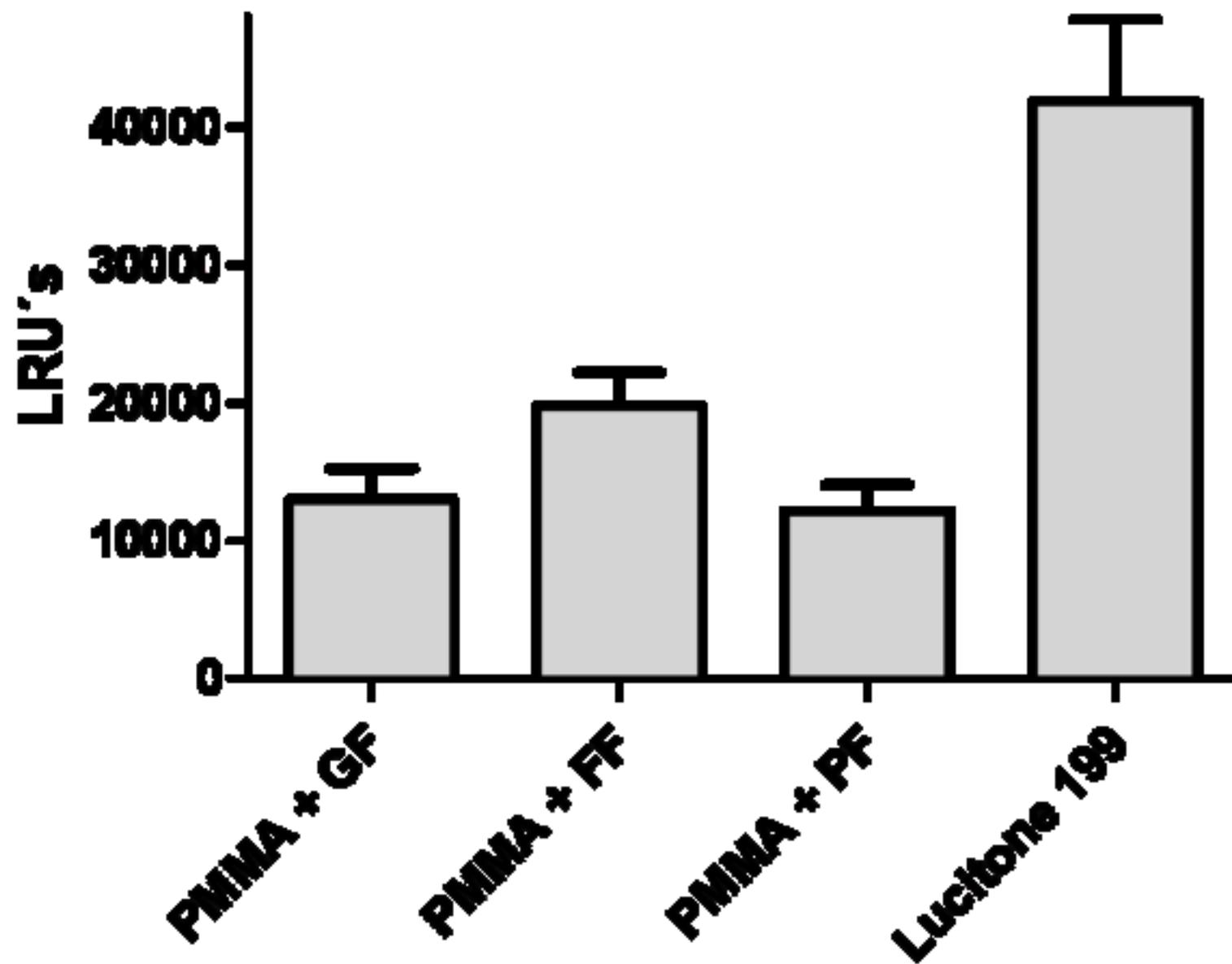


Figure 5
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