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Effects of gelatin coating glass coverslips on fungal attachment and their morphological demonstrations

Supattra Suwanmanee[1] and Natthanej Luplertlop[2]*

[1]Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.
[2]Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.

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Gelatin obtained from collagen, an extracellular matrix (ECM) protein, plays a crucial role in facilitating cell attachment. This study aimed to determine the effects of gelatin on fungal attachment and morphology. Samples of *Aspergillus niger* were grown on gelatin-coated coverslips and non-gelatin-coated coverslips, and prepared for examination under both light and scanning electron microscopes. The results show that the attachment and morphology of *A. niger* grown on gelatin-coated coverslips differ from that grown on non-gelatin-coated coverslips. *A. niger* grown on gelatin-coated coverslips exhibited higher levels of attachment, and less evidence of fungal detachment and dissemination during histological process than non-gelatin-coated coverslips. In addition, gelatin-coated coverslips presented more complete structures of fungi and better image quality than non-gelatin-coated coverslips. It is suggested that gelatin coating may be used in fungus slide preparation to prevent fungal damage during experiments, at the same time improving image quality.

Key words: Attachment, coverslip, fungus, gelatin, morphology.

INTRODUCTION

The extracellular matrix (ECM) is the material found on the outside of cells. It typically provides structural and biochemical support to surrounding cells. ECM is composed of a variety of proteins and polysaccharides. ECM proteins play a crucial role in supporting cell attachment and growth. One of the most prominent ECM proteins is collagen (Engler et al., 2006; Frantz et al., 2010).

Gelatin is a translucent, colorless, odorless, and tasteless solid substance, derived from collagen, which is one of the most well-known ECM proteins in animals. It is a denatured protein obtained either by partial acid hydrolysis of pig skin type I collagen (gelatin type A) or alkaline hydrolysis of bovine collagen (gelatin type B) (Kommareddy et al., 2005; Mohanty et al., 2005). It does not produce toxic byproducts. Therefore, it is a very suitable protein for use in many different applications. A previous report found that gelatin is used clinically as a plasma expander, as well as a stabilizer in vaccines (Kommareddy et al., 2005; Elzoghby et al., 2012). The results from an *in vivo* study in rats demonstrated that gelatin can be used in wound care as a wound healing agent (Kale et al., 2011).

The use of gelatin in biological applications is becoming increasingly widespread. Gelatin contains informational signals such as tripeptide sequence Arg-Gly-Asp (RGD), which modulates cell adhesion, migration, differentiation, and proliferation, thereby improving the final biological

*Corresponding author. E-mail: natthanej.lup@mahidol.ac.th. Tel: 66-02 306-9172. Fax: 66-02 643-5583.

behavior of cells (Sakai et al., 2009; Tan and Marra, 2010; Wang et al., 2012). Many species of fungi have structures that are fragile such as hyphae and conidia. During the microscopic method with conventional fungal slide culture, these fragile structures may extremely be prone to damage and slip off even if they are handled with care. Incomplete structures of fungi in microscopic slides are difficult to identify and can cause misinterpretation and diagnostic error. To overcome this problem, new techniques to improve fungal slide cultures will be explored. Previous data found that gelatin is the most frequently used medium for histological purposes. To preserve tissue sections on histological slides during the staining and washing steps, slides need to be coated with adhesive substances. Gelatin-coated slides can enhance tissue section attachment. Gelatin-coated surfaces can decrease section detachment in staining and washing steps (Frost et al., 2001). Therefore gelatin may promote adhesion of fungal on culture slide and prevent the destruction of fungi during the slide preparation.

There are many reports on the use of gelatin in biological applications. However, data on the benefits of using gelatin, in regard to fungal attachment and morphology, is still lacking. Therefore, the purpose of this research was to examine the effects of gelatin on fungal attachment and morphology via light and electron microscopy.

MATERIALS AND METHODS

Fungal strain and growth conditions

A. niger were used in this experiment because they have fragile structures that are extremely prone to damage, and can slip off even when handled with care. *A. niger* is also an opportunistic pathogen and is the most common fungi found in contaminated environment. In addition, they grow easily and fast on agar. The *A. niger* used in this research were obtained from the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Thailand. They were cultured in Sabouraud dextrose agar (SDA), SDA plus yeast extract (SDA+YE), and trypticase soy agar (TSA) (Difco Laboratories, Detroit, MI, USA) prior to the study.

Preparation of 10% gelatin-coated coverslips

The 10% gelatin solution was prepared by dissolving gelatin (Sigma-Aldrich Co., St. Louis, MO, USA) in autoclaved deionized H_2O. The sterilized 10 mm round coverslips were placed into the wells of a sterile 12-well plate (one coverslip per well). Then gelatin solution was added in each well to cover each coverslip. After the coverslips were incubated for 10 min at room temperature, the gelatin solution was removed. The coverslips were allowed to air dry in a laminar flow hood under UV light for at least 2 h. The coated coverslips can be stored at room temperature for further use.

Coverslip culture

Agar plugs of *A. niger* were inoculated onto agar in 6-well plates (Sample number (n) = 30 per type of agar, three replicates for each experiment). The 10% gelatin-coated coverslips and non-gelatin-coated coverslips were placed separately either over or under the inoculated fungi. Fungal cultures were incubated at 25-30°C for 3 days to allow the fungi to grow on coverslips before morphological examination.

Morphological studies

Preparation for light microscopy

The coverslip culture was wetted on the specimen side with a drop of 90% ethanol. One drop of lactophenol cotton blue was applied to the specimen, before the coverslip culture was gently lowered onto a drop of lactophenol cotton blue on a glass slide. The slide was left to dry and then sealed with Permount. For hematoxylin and eosin (H&E) staining, the coverslip culture was dipped in 100 and 95% ethanol, rinsed in distilled water, and stained with hematoxylin and eosin (Ajello et al., 1963). Fungal morphology was examined under a light microscope (Axio Lab.A1; Carl Zeiss, Oberkochen, Germany).

Preparation for scanning electron microscopy

The coverslip culture was mounted on an aluminum stub, coated with a thin layer of gold particles using a sputter coater (K550; Emitech, Kent, England), and observed under a scanning electron microscope (JSM-6610LV; JEOL, Tokyo, Japan) (Bozzola and Russell, 1992).

RESULTS

After the preparation of fungal coverslip culture (Figure 1a-c), the two groups of coverslips were observed, comparing fungal attachment and morphology. There was no significant difference in fungal density between gelatin-coated coverslips and non-gelatin-coated coverslips independently by the fungal agar media employed (data not shown). The results show that the attachment and morphology of *A. niger* grown on gelatin-coated coverslips differed from those grown on non-gelatin-coated coverslips, as described below.

A. niger stained by lactophenol cotton blue (Figure 2a, b) and H&E (Figure 2c, d) were observed under a light microscope. The fungal attachment on non-gelatin-coated coverslips (Figure 2a, c) was less efficient, with a higher rate of detachment during the staining and washing steps than with gelatin-coated coverslips (Figure 2b, d). In the case of the non-gelatin-coated coverslips, incomplete structures of *A. niger*, such as broken conidiophores, dispersed conidia, or lost conidia were observed. Some structures of *A. niger* on non-gelatin-coated coverslips, such as conidia or vesicles, were stained as pale color or colorless. Some areas of the image had multiple layers. Some areas were blurred and out of focus, resulting in the loss of image detail. When using gelatin-coated coverslips, there were fewer incomplete fungal structures.

There were no pale colored or colorless specimens in the samples. The fungi were brightly colored. In addition, images showed clear details of the structure of the fungi, such as conidia. These results were confirmed by scanning electron microscopy. The electron microscopic results were similar to the light microscope.

Gelatin-coated coverslips showed more complete

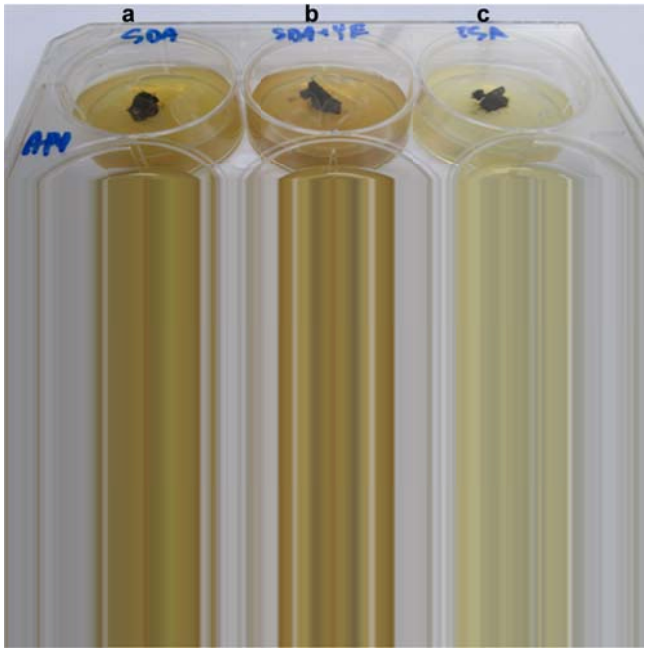

Figure 1. Sequence of preparation of fungal coverslip culture. (a) Samples of *A. niger* were inoculated on fungal media and then coverslips were placed on the inoculated fungi. (b) Fungi started to grow on media. (c) After 3 days of incubation, fungi grew out onto a part of the coverslips.

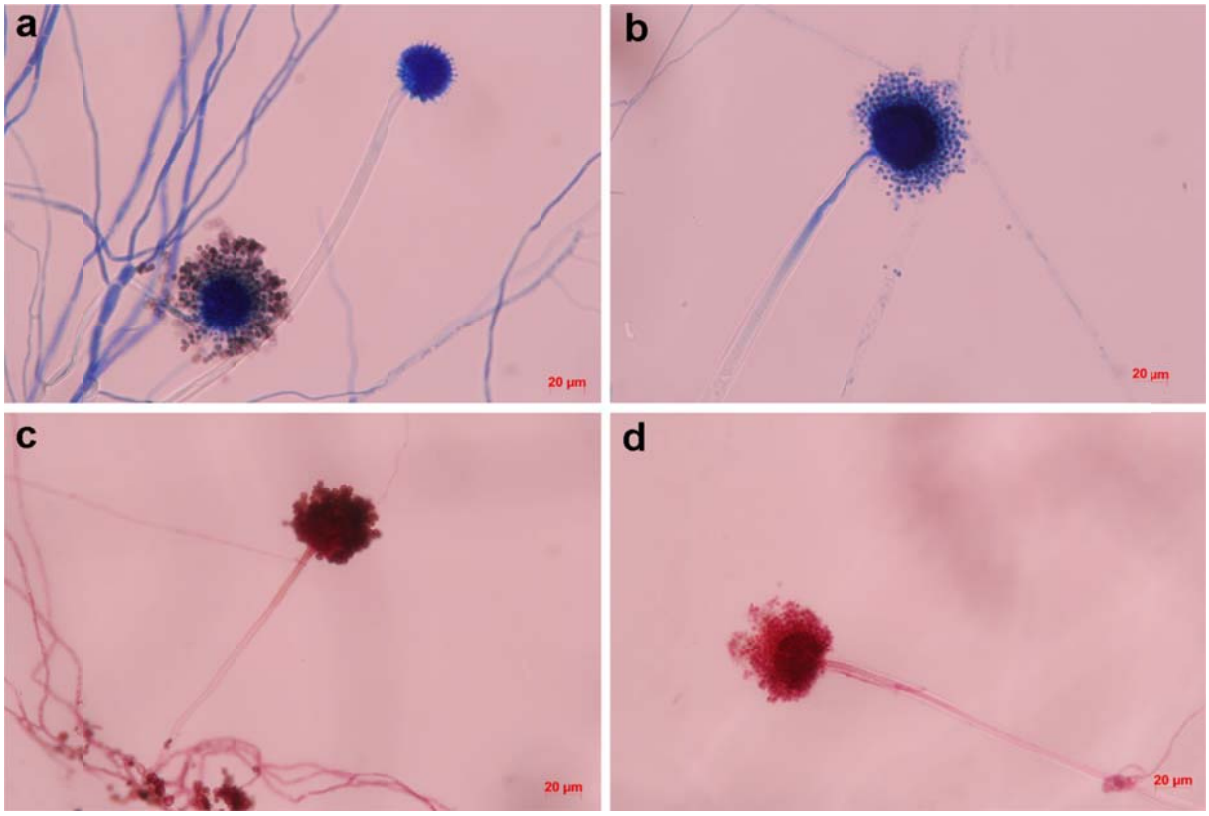

Figure 2. Light micrographs from lactophenol cotton blue (a, b) and H&E (c, d) staining of *A. niger* formed on different types of coverslips. (a, c) Non gelatin-coated coverslips, showing incomplete structure of *A. niger* (a) and blurred image (c). (b, d) Gelatin-coated coverslips, demonstrating complete structure of *A. niger*, with clear details of image (×40).

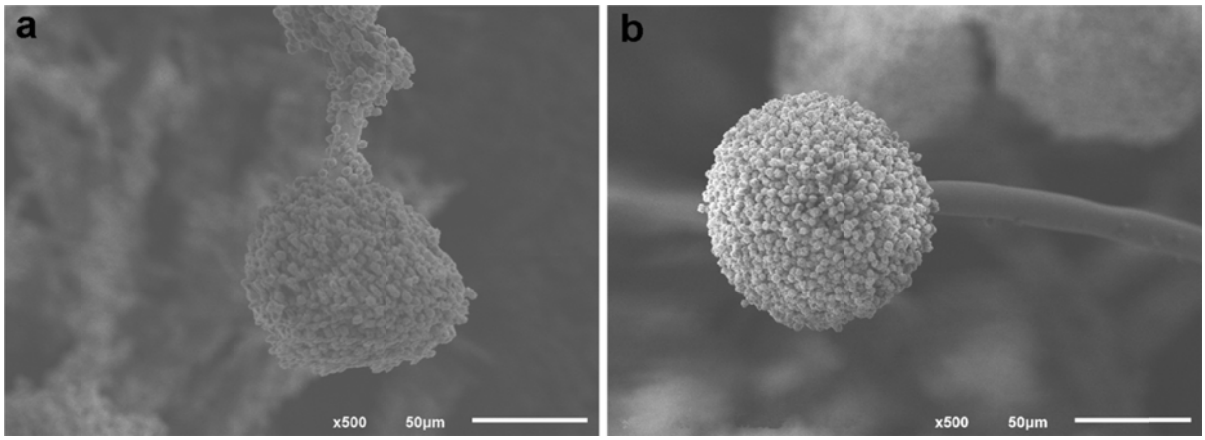

Figure 3. Scanning electron microscope images of *A. niger* grown on different types of coverslips. (a) Non gelatin-coated coverslip, demonstrating out of focus image. (b) Gelatin-coated coverslip, illustrating complete structure of fungus with clear details of image.

structures of *A. niger* and provided more detailed images (Figure 3b). In contrast, higher numbers of incomplete structures of fungi and blurred images were observed in non-gelatin-coated coverslips (Figure 3a).

All the experiments were conducted in triplicate. The quality of the results including attachment and morphology of *A. niger* achieved by growing on gelatin-coated coverslips was better than non-gelatin-coated coverslips. 73% of the total fungal slides of gelatin-coated coverslips could be interpreted, compared with 48% of the total fungal slides of non-gelatin-coated coverslips.

DISCUSSION

Many species of fungi form very delicate structures which are at least partially destroyed by even the most careful hands. It is very difficult to prepare slide cultures of fungi for microscopic examination. Many researchers have modified a variety of slide culture methods to solve this problem (Reiss, 1970; Mitchell and Britt, 1981; Ellis and Ajello, 1982; Harris, 1986; Fujita, 2013). In this study, a new culture method was applied, which prevented fungal damage during the experiment. This method permitted the growth of fungi on the coverslips. It is known that most cell types can be grown on soft materials, but in this study, *A. niger* started growing on agar (SDA, SDA+YE, TSA), and was then allowed to grow on a rigid substrate composed of coverslips coated with a thin film of gelatin.

The results of the study revealed that gelatin can promote the attachment of *A. niger* to coverslips and reduce the loss of samples of *A. niger* from coverslips during histolo-gical processes. Moreover, gelatin-coated coverslips showed more complete fungal structures and more detailed images than non-gelatin-coated coverslips.

Gelatin obtained from collagen, ECM protein, plays an important role in promoting cell attachment. The microscopic data of this experiment showed that gelatin can enhance the adhesion of fungi to coverslips. This finding was similar to previous reports of many cell types in culture. An *in vitro* study found that gelatin incorporated into poly(lactide-co-glycolide) (PLGA) nanofibers can promote the material surface properties for fibroblast cell attachment and proliferation (Hu et al., 2013). Gelatin combined with poly(3,4 ethylenedioxythiophene)-tosylate (PEDOT (TOS)) can support bovine brain capillary endothelial cell (BBCEC) adhesion and growth on well plates. BBCECs on gelatin/PEDOT(TOS)-coated well plates were elongated, which was a sign of initial adhesion, whereas BBCECs on other well plates remained round and non-elongated (Bongo et al., 2013). Cell adhesion is the binding of a cell to a surface or substrate, such as an ECM or other cell. Many factors are known to mediate adhesion between the cell and another surface or substrate. It is known that the substrate's surface morphology has an effect on adhesion, proliferation, and function of cells (Zhu et al., 2003). The data from a previous study found that the roughness values of gelatin and glass slide were 1.7±0.9 nm and 0.6±0.5 nm, respectively (Bongo et al., 2013). This increased roughness provides opportunities for greater fungal adhesion, as shown in this study. Therefore, gelatin can help prevent fungal damage during experiments, by increasing fungal attachment on coverslips and reducing the loss of fungi from coverslips during histological procedures.

A previous report found that filamentous fungi can form biofilms, complex 3D structures with cells usually enclosed within an extracellular matrix consisting of polymeric substances (EPS). A biofilm is characterized by cells that are securely attached to a surface and/or other cells within EPS (Stoodley et al., 2002; Harding et al., 2009; Siqueira and Lima, 2013). In this study, gelatin may stimulate EPS production or act as biofilm that result in the increase of the attachment of *A. niger* to coverslips.

In this study, lactophenol cotton blue, the most conventional stain for microscopic examination of fungi and H&E, the most conventional stain for light microscopy, were able to stain fungal cell wall. The results show that *A. niger* grown on gelatin-coated coverslips had more attach-ment and better morphology than that grown on non-gelatin-coated coverslips. The results were also confirmed by scanning electron microscopy. Our study demonstrates that lacto-phenol cotton blue and H&E staining were not significantly different, but no data exists concerning other staining methods, fungal attachment and morphology.

The data from this study is very beneficial for preparing fungal slides for histology. Gelatin is an alternative substrate which creates a surface which can hold fungi on coverslips more securely, therefore preventing the separation of fungi from coverslips. Gelatin makes coverslips rough for the fungi to adhere more strongly. This new technique helps to protect against misinterpretation and diagnostic error from incomplete structures of fungi in microscopic slides. Gelatin-coated coverslips can prevent the destruction of fungi during the experiment and improve image quality, making it easier to observe fungal cells. In contrast, blurred images resulted from using non gelatin-coated coverslips. Gelatin is also very easy to prepare, convenient, and relatively inexpensive. Prepared gelatin solutions can be kept for up to 1 month. Gelatin-coated coverslips can be stored in closed boxes at room temperature for several weeks. In addition, fungal slides using gelatin-coated coverslips can be stored for a longer time than non-gelatin-coated coverslips. This new technique is more easily performed and saves time.

Conflict of interests

The authors have not declared any conflict of interest.

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