J Nanostruct 15(4): 1569-1576, Autumn 2025

RESEARCH PAPER

Silver Nanoparticles Enhanced the Cytocompatibility of two Dental Adhesive Agents (*In vitro* study)

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ARTICLE INFO

Article History:
Received 27 May 2025
Accepted 15 August 2025
Published 01 October 2025

Keywords:
Cytocompatibility
Dental adhesive agents
Silver nanoparticles

ABSTRACT

The purpose of this study was to look at the direct and indirect cytotoxic effects of two universal dental adhesive agents combined with silver colloidal dispersion on a human gingival fibroblast cell. Peak universal (Ultradent, USA) and Optibond Universal (Kerr, America) commercial dental adhesive agent systems were infused with 8% by mass of colloidal dispersion containing silver nanoparticles (AgNps). Adult rabbits were used to create a cell line of human gingival fibroblast cells. The cytotoxic activity of four adhesive agent groups on fibroblast-like cells was investigated using two cytotoxic assays: GA: Peak Universal (control), GB: Peak Universal (8% AgNps incorporated), GC: Optibond universal (control), and GD: Optibond (8% AgNps incorporated). From the adhesive agent groups, forty adhesive agent samples (5 x 1 mm discs) were manufactured and utilized for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test, and 32 discs were used for the High-content screening (HCS) assay. The cytotoxic assay results revealed that all adhesive agents examined had a high degree of cytocompatibility. However, the 8% incorporated adhesive agent Groups (GB and GD) had much lower cytotoxicity than their controls. Furthermore, groups GC and GD had significantly better cytocompatibility than groups GA and GB. In comparison to their control groups, the incorporation of 8% by mass colloidal dispersion of silver nanoparticles greatly improved the biocompatibility of the evaluated universal adhesive agents.

How to cite this article
Al-Abbas M., Al-Badr R., Shamash M. Silver Nanoparticles Enhanced the Cytocompatibility of two Dental Adhesive Agents (In vitro study). J Nanostruct, 2025; 15(4):1569-1576. DOI: 10.22052/JNS.2025.04.007

INTRODUCTION

Cytocompatibility tests are screening assays used to evaluate a living cell's interaction with a material in a cell culture, including measuring cell survival and growth. They are in vitro tests for examining a material's biocompatibility because they are performed outside of a living creature. These tests necessitate the use of the examined materials in conjunction with an isolated biological system (i.e., cells, enzymes, or something else) [1].

Cytotoxicity testing are either direct or indirect. The direct assays are used to assess cell damage caused by the presence of the tested materials in direct contact with the cells without a barrier [2]. The multiparametric High Content Screening Toxicology (HCS) assay, for example, provides a high degree of accuracy. In contrast, indirect cytotoxic tests are used to evaluate the toxicologic

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effect of a substance released from a material that has penetrated past a barrier and may cause cell damage. As with the MTT cytotoxic assay (a colorimetric assay). It is a sensitive and dependable indication of the metabolic activity of the cell [3].

In response to variations in professional judgment regarding the number of steps or the adhesive agent strategy used, manufacturers have introduced more versatile adhesive agent systems that can be used in either the etch-and-rinse (i.e., two steps) or self-etch mode (i.e., one or two steps) [4]. This type of adhesive agent is known as a "Multi-mode Universal" adhesive agent system. However, achieving a tight stable seal between the adhesive agent and the dentin substrate is still difficult due to hydrolytic degradation and the action of lytic dentinal matrix enzymes, which results in destabilizing the adhesive agent bond interface and reducing bond stability [5].

With the advancement of nanotechnology, there has been a great increase in the use of nanoparticles in several industries (for example, antimicrobial materials, electronics, drug delivery, cosmetics, and others) [6]. The physicochemical properties of silver nanoparticles vary. It is insoluble in water, very stable, and non-toxic.

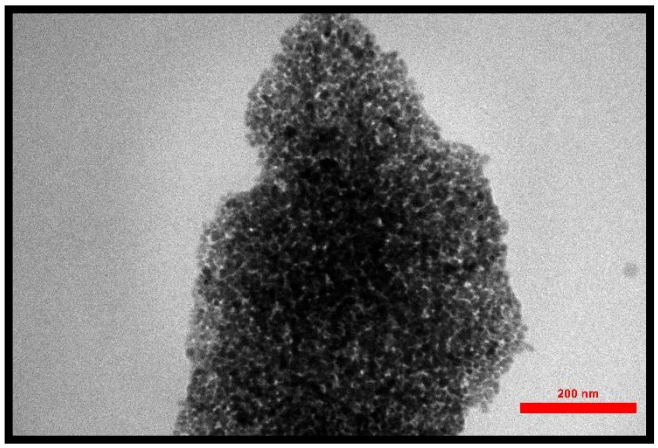

Fig. 1. Transmission electron microscope image for the prepared colloidal dispersion of AgNps nanoparticles.

Table 1. lists the chemical makeup of the all-purpose adhesive agents employed in this study.

Adhesive agent	Composition
Peak Universal	Urethane dimethacrylate, Hydroxyethyl methacrylate, methacrylate hydrophilic monomers, methacrylate acid monomers, ethanol, water, camphorquinone, ethyl 4-dimethylamino-benzoate, surfactant, sodium fluoride
Optibond universal	Methacryloyloxydecyl dihydrogyne phosphate,methacryloxyethyl, methacryloyloxyalkyl thiophosphate methacrylate methacrylate monomer, acetone, water, initiator, silica

AgNps nanoparticles have recently been used in dentistry due to their high antibacterial activity. They have better antibacterial activity than chlorhexidine and are biocompatible [7].

Thus, the current in-vitro investigation will look into the cytocompatibility of two universal adhesive agents combined with a silver nanoparticle on a human gingival fibroblast cell line using two cytotoxic assays (MTT and HCS). This is the first study to use AgNps colloidal dispersion to improve the chemical and biological characteristics of universal adhesives.

MATERIALS AND METHODS

Preparation and incorporation of the silver nanoparticles colloidal dispersion into the adhesive agents

The protocols specified by Cave and Mundell (2015) [8] were followed to make a colloidal dispersion of AgNps nanoparticles (AgNps, US nano, high purity, 99.9+%, 50 nm) (Fig. 1). The generated colloidal dispersion was added at 8% by mass (0.20gm/5gm) to two universal dental adhesive agents, Peak universal (FGM, Brazil) and Optibond Universal (GC, America), according to the mass fraction formula (Eq. 1):

Mass Fraction= [mass of colloidal dispersion / Total mass of adhesive agent] x 100

Preparation of the cell line

Human Gingival Fibroblast Cell Vials were purchased from Innoprot (Parque Tecnológico de Bizkaia, Spain) and were isolated from healthy human gingiva (Fig. 2a) fluorescence microscopy image, (b) phase-contrast microscopy image. At passage one, these cells were cryopreserved and given frozen. Every vial contains almost 500.000 live cells [9]. Flow cytometry was used to count 25 104 gingival fibroblast like cells in 5 mL tubes for this study. They were then incubated at 37 °C with 5% CO2 in Dulbecco's Modified Eagle's Medium, a medium developed for chondrogenic growth.

Preparation of the adhesive discs

72 Adhesive agent discs were manufactured for this study using ready-made silicone rubber moulds of 5x1 mm dimensions and 55L capacity in 2009 according to ISO regulation 4049-10 for cytotoxic screening for both MTT and HCS tests [10]. This was accomplished by first filling the adhesive agent disc gaps halfway with solvent, which was then gently evaporated with a warm air tooth dryer for 20 seconds. The remaining halves of the disc voids were then filled, and the solvents were evaporated once more before being coated with celluloid strip and light cured for 40 seconds using an LED light curing apparatus. Finally, the adhesive agent discs were removed from the mould and their thickness and diameter were measured. Following that, all discs were polished using #1500-grit paper.

MTT screening assay

Samples distribution

Forty adhesive agent discs were made from the control and 8% AgNps included adhesive agents as

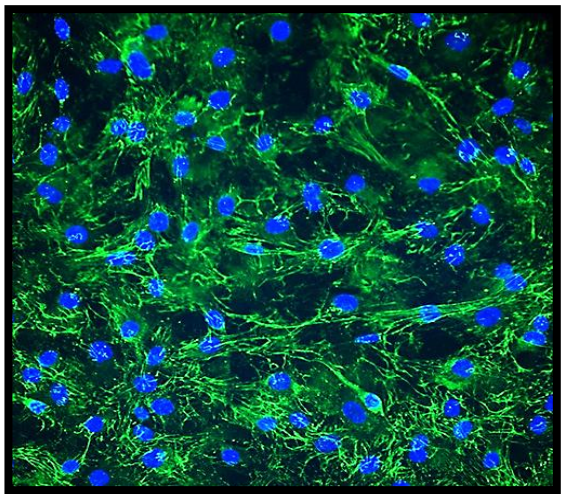

Fig. 2. Gingival fibroblast like cells: fluorescent microscopy image.

stated above and distributed as follows:

Group A: 10 adhesive agent discs (Peak Universal) were immersed in 6 ml DMEM in an incubator at 37Co for 72 hours (Control).

Group B: For 72 hours in an incubator at 37°C, 10 adhesive agent discs (8% AgNps Peak Universal) were immersed in 6 ml DMEM.

Group C: 10 adhesive agent discs (Optibond Universal) were immersed in 6 ml DMEM in an incubator at 37 °C for 72 hours (Control).

Group D: 10 adhesive agent discs (8% integrated AgNps Optibond Universal) were immersed in 6 ml DMEM for 72 hours at 37Co in an incubator.

The culture media containing the adhesive agent extracts to be used for the MTT screening assay was left in place after the adhesive agent discs were carefully removed from the DMEM media after the 72-hour immersion period.

Cultivation of the Test Cells

In an incubator with 5% CO2 at 37 °C, human gingival fibroblast cells were grown and activated in RPMI 1640 culture media (Sigma, Irvine, CA, UK) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin. Following that, aliquots of the separated cells were subcultured after the cells were detached using 0.25% trypsin [11].

MTT assay protocol

Human gingival fibroblast cells were cultured at 1.8x104 cells per microliter and 0.44x104 cells per well on 96-well Costar® plates (Corning, USA). After one, two, and three days, the grown cells are separated using 0.05% trypsin, and their proliferation index is calculated by counting them in a Neubauer chamber. Following a gentle stir and a 24-hour incubation period at 37 °C and 5% CO_2, the plates were covered with sterile parafilm and 200 ul of the culture fluid containing the adhesive agent eluents (extracts). Next, each well received the application of 12 L of MTT solution (8 mg/mL in PBS) and 80 L of DMEM medium, respectively. Cells were incubated at 37 °C, 5% CO2, and 95% air humidity for three hours. After three hours, the MTT solution was replaced with the 110 L dimethyl sulfoxide solvent solution. The plate was then incubated for a further fifteen minutes at room temperature. The viability of the cells was next ascertained by measuring the optical density of the wells at a wavelength of 590 nm using a spectrophotometer. The experiment was repeated twice in the exact same settings to ensure accuracy.

High-Content Screening Assay (HCS)
Sample grouping

Following preparation, thirty-two adhesive agent discs were split up into four groups:

Group A: Eight Peak Universal adhesive agent discs were grown in close proximity to the cells (Control).

Group B: Eight adhesive agent discs (eight percent AgNps Peak Universal) were grown in close proximity to the cells.

Group C: Eight Optibond Universal adhesive agent discs were grown in close proximity to the cells (Control).

Group D: Eight adhesive agent discs with 8% AgNps Optibond Universal integrated were grown in close proximity to the cells.

High-Content Screening assay procedure

Each well (32 wells total, 8 wells per group) contained 1.5x104 human gingival fibroblast cells/mL of seeding. After that, the adhesive agent discs were grown in direct contact with the cells (one disc in each well), and they were incubated for a day at 37 °C and 5% CO2. On the other hand, untreated cells were sown in the culture media as the negative control. 50 microliters of HCS cell viability after a day. For each well, staining solution was applied. After that, the cells were cultured for 30 minutes at 37°C. The plate was then incubated for 20 minutes at room temperature. Afterwards, each well received 100 microliters of 1x wash buffer once the fixing solution had been gently aspirated. 50 L of the primary antibody solution was added to each well after the blocking buffer was added, and the combination was then incubated at room temperature for 60 minutes in a light-protected environment. The plate was cleaned three times using 100 l of one wash buffer per well after the primary antibody solution was aspirated [12].

Following the removal of the wash buffer, each well received 100 microliters of 1X permeabilization buffer, and the wells were then incubated for 10 minutes at room temperature in the dark. After that, the plate was washed twice and the permeabilization buffer was aspirated after 100 microliters of 1X wash buffer were added to each well. There was a seal on the plate. The plates were sealed and kept for a day at 4°C in the dark. Subsequently, the plates are placed into the HCS automated fluorescence microscopy apparatus to

get simultaneous readings of multiple parameters, such as the number of viable cells, total nuclear intensity, and membrane permeability of the cells [12].

RESULTS AND DISCUSSION

Results of MTT test

Descriptive Statistics

Table 2 and Fig. 3 exhibit the findings of the

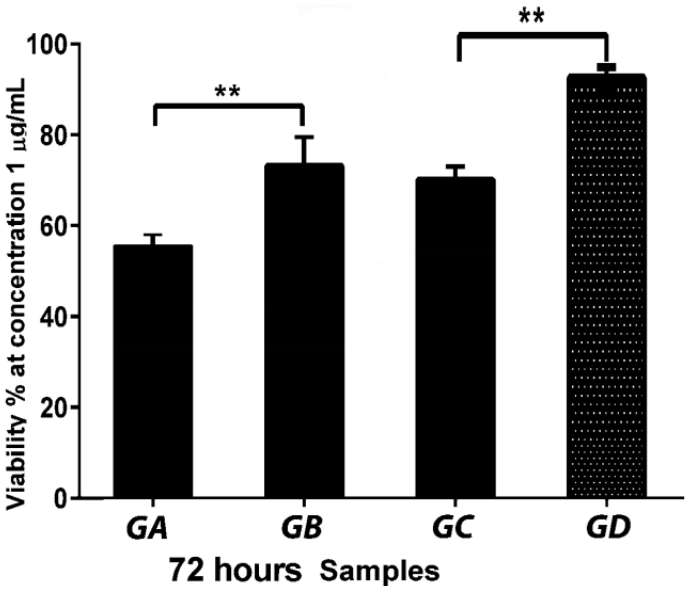

Fig. 3. Graph showing the means of cells viability (%) in response to the adhesive agent extracts at three 72 hours immerse.

Table 2. MTT cell viability results (%) at 72 h immersion time.

Groups	Immersion period	Mean	Std. Deviation	Std. Error
GA	72hours	56.90	0.455	1.311
GB	72hours	74.61	1.355	1.145
GC	72hours	75.51	0.415	0.654
GD	72hours	82.65	0.725	0.564

Table 3. To determine the significance of differences in the mean viability of the cells between the control groups and the 8% AgNps contained adhesive agents, an independent samples t-test was employed.

Groups	P value (t-test)	Significance
GA vs GB	0.015	HS
GC vs GD	0.001	HS
GA vs GC	0.003	HS
GB vs GD	0.002	HS

descriptive statistics for the viability of human gingival fibroblast cells in response to the adhesive agent extracts of a 72-hour immersion time. The means, standard deviations, and standard errors were used to express the data.

The 8% AgNps integrated adhesive agents had significantly higher cell viability mean values than their control groups, as Table 2 and Fig. 3 demonstrated. Optibond with 8% AgNps incorporation demonstrated superior cell survivability compared to Peak Universal adhesive agents with 8% AgNps incorporation. With the lowest cell viability, the Peak Universal (control) exhibited the most cytotoxic effect.on time.

Inferential statistics (MTT assay)

The significance of the variations in the cell viability mean values ($p \leq 0.05$) between the adhesive agent groups was assessed using an unpaired t-test. The test revealed remarkably substantial group differences (Table 3).

Results HCS assay

HCS assay Descriptive statistics.

Table 4 and Fig. 4 display the findings of the descriptive statistics of viable cell count for the tested adhesive agent groups and the untreated control. In comparison to the control adhesive agent groups, the 8% AgNps integrated adhesive

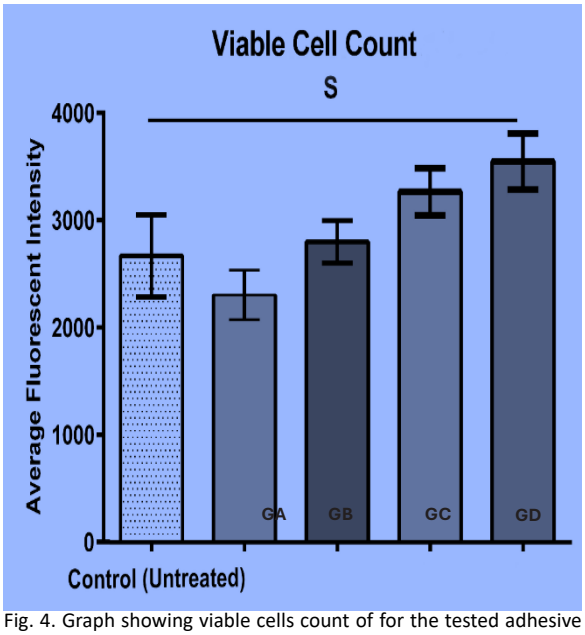

Fig. 4. Graph showing viable cells count of for the tested adhesive agent groups in comparison to the control untreated cells.

Table 4. The Viable Cell Count of cells in response for the tested groups and the negative control of the untreated cells.

	Negative Control (Untreated)	GA	GB	GC	GD
Mean	2832	2775	2784	3248	3449
Std. Deviation	165.4	277.1	110.8	148.6	121.28
Std. Error of Mean	155.2	260.8	112.7	174.5	181.44

agent groups had a much greater viable cell count, according to the data. Optibond outperformed Peak universal adhesive agent groupings in terms of viable cell count. The control, amber universal, had the lowest number of viable cells.

Inferential statistics

Table 5 displays the findings of the inferential statistic used to determine if the viable cell count differences between the groups of tested adhesive agents and the untreated control were statistically significant. The groups' differences were found to be statistically greater using the independent t-test.

Dental adhesive agents contain polymers that can be exposed to the cells of the oral tissue, particularly the gingival tissue. When the polymer products are released from the adhesive agents and move to the adjacent periodontal tissues or towards the pulpal tissue through the dentinal fluids, this exposure can be either direct or indirect during the adhesive agent application procedure [13]. Regarding the biocompatibility of various materials, in vivo investigations can yield the most accurate results, and for moral reasons, the majority of cytotoxicity tests are carried out on cell line cultures [14].

The literature presented a number of in vitro experiments to examine the cytotoxic properties of dental adhesive agent systems. In general, the MTT test is the most widely used due to its speed, affordability, ease of use, repeatability, and dependability. However, by evaluating many cell viability characteristics, the HCS assay can offer more accurate information on the vitality of the cells [15].

In this study, two cytotoxic assays (MTT, HCS) were carried out to examine the cytotoxic effects of the commercial dental adhesive agents on human gingival fibroblast cells, taking into account the likelihood that the composition of these adhesive agents will change after the AgNps nanoparticles are colloidally dispersed.

The results of this investigation demonstrated that gingival fibroblast cells were subjected to metabolic effects by all investigated adhesive agent groups. Furthermore, the cell viability of the 8% AgNps incorporated adhesive agents was significantly higher than that of the non-incorporated control adhesive agents.

It is likely that the action of the integrated AgNps nanoparticles is related to the study outcomes. The remarkable biocompatibility of silver and its alloys makes them popular biomaterials for the synthesis of dental and clinical implants [16]. Furthermore, research revealed a connection between silver nanoparticles' biocompatibility and antibacterial capabilities [17]. This is due to AgNps's low electrical conductivity, which is linked to its low rate of electrochemical oxidation, which reduces oxidative stress and releases reactive oxygen species, which are the most harmful, resulting in the formation of a thin oxide layer [18].

Furthermore, the investigation demonstrated

Table 5. presents a comparison of the significance of differences ($p \leq 0.05$) in viable cell counts between the four adhesive agent groups and the negative control (untreated) with the 8% AgNps incorporated and control (non-incorporated adhesive agents).

Groups	independent t-test	Significance
Control (Untreated) vs. GA	0.004	HS
Control (Untreated) vs. GB	0.001	HS
Control (Untreated) vs. GC	0.001	HS
Control (Untreated) vs. GD	0.004	HS
GA vs. GB	0.005	HS
GC vs. GD	0.004	HS
GA vs. GC	0.005	HS
GB vs. GD	0.001	HS

that the Peak universal adhesive agent exhibited notably greater cytotoxicity in comparison to Optibond universal, a finding likely related to the distinctions in their molecular makeup. While Optibond Universal is not, Peak Universal's composition includes UDMA and HEMA monomers in its chemical structure. Because of its hydroxyl group, which might impact cell viability by raising reactive oxygen species levels, HEMA has a low molecular weight and dissolves readily in aqueous conditions [19].

The HSC assay results, which showed a high degree of viable cell count and are corroborated by the fluorescent images of the cells in close contact with the tested adhesive agents, corroborate the results of the MTT investigation. The assay showed that the integrated adhesive agent groups had a substantially greater viable cell count than the non-incorporated adhesive agent groups.

CONCLUSION

The 8% mass incorporation of silver nanoparticles greatly increased the cytocompatibility of the universal adhesive agents when compared to the control non-incorporated adhesives, within the confines of this in vitro study. Furthermore, there was a noticeable increase in cytotoxicity with the Peak bond universal adhesive agent in comparison to the Optibond universal adhesive agent.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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