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Surface functionalization of bioactive glasses with natural molecules of biological significance, part II: Grafting of polyphenols extracted from grape skin

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ABSTRACT

Polyphenols, as one of the most important family of phytochemicals protective substances from grape fruit, possess various biological activities and health-promoting benefits, for example: inhibition of some degenerative diseases, cardiovascular diseases and certain types of cancers, reduction of plasma oxidative stress and slowing aging. The combination of polyphenols and biomaterials may have good potential to reach good bioavailability and controlled release, as well as to give biological signaling properties to the biomaterial surfaces. In this research, conventional solvent extraction was developed for obtaining polyphenols from dry grape skins. The Folin&Ciocalteu method was used to determine the amount of total polyphenols in the extracts. Surface functionalization of two bioactive glasses (SCNA and CEL2) was performed by grafting the extracted polyphenols on their surfaces. The effectiveness of the functionalization was tested by UV spectroscopy, which analyzes the amount of polyphenols in the uptake solution (before and after functionalization) and on solid samples, and XPS, which analyzes the presence of phenols on the material surface.

1. Introduction

Grapes as table food and winemaking sources had a long and abundant history, dated to the Ancient Greek and Roman civilizations. Due to the various nutrient elements in grape, such as vitamins, minerals, carbohydrates, edible fibers and phytochemicals, nowadays substantial scientific attention has been focused on the potential biomedical effect of grapes and grape products. Various biomedical research studies have been centered on the investigation of the role of nutrients from grape in several areas including cardiovascular health, cancer development and progression, Alzheimer's disease and other neurodegenerative disorders, aging and alterations in cognitive and motor function, antiviral activity, oral health, immune function, and diabetes [1,2]. Among the phytochemicals protective substances in grape, polyphenols play an important role according to their notable biomedical properties and health-promoting benefits [3]. The phenolic compounds mainly include simple phenols, phenolic acids (both benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins. In plants, phenolic compounds may act as phytoalexins, antifeedants, and attractants for pollinators, contributors to the plant pigmentation, antioxidants, and protective agents against UV light, among others [4]. In grape skin, the phenolic compounds usually contain proanthocyanidins, ellagic acid, mycicetin, quercetin, kaempferol, trans-resveratrol, etc. [5]. Pastrana-Bonilla's [6] research, have quantified the antioxidant activity of muscadine grape skin extracts as 12.8 μmol TE/g expressed according to the Trolox Equivalent Antioxidant Capacity (TEAC) assay. Falchi et al. [7] have found that the ischemic reperfusion injury was significantly inhibited in the rats isolated of heart after 30 days' consumption of the extracts from flesh and grapes skin which exhibited equal effect of cardioprotection. Hudson et al. [8] have reported that extracts from grape skin could induce prostate tumor cell lines apoptosis with high efficiency.

In order to obtain these useful components, different extraction methods of polyphenols have been developed. Liquid–liquid extraction is the main method used for grapes [9]. The extraction conditions of phenolic compounds are influenced by solvent, temperature, extraction time and the ratio of sample-to-solvent. The common solvents used for extraction includes ethanol, methanol, acetone or formic acid and water in different ratio. Besides, some improved methods have been developed Hong et al. [10] used microwave-assisted extraction technique to optimize the isolation

Abbreviations: GA, gallic acid; PH, phenols extracted from grape skins; CA, citric acid.

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of phenolic compounds from grape seeds. Ultrasound-assisted extraction has been employed to extract resveratrol from grapes [11]. In addition, supercritical fluid extraction [12] and aqueous two-phase system extraction [13] have made advances in grape phenol extraction. Total phenolic content is typically determined by way of the Folin and Ciocalteu method using gallic acid as standard [14]. In order to improve the molecular stability and bioavailability of the molecules of biological interest, an interesting approach their grafting on a carrier. Polymeric matrices have been widely used as carrier for osteogenic growth factors [15,16]. It is also possible to graft biomolecules on the surface of biomaterials for implants with different grafting strategies such as simple adsorption, covalent bonding or release from a degradable carrier [17,18]. In this way the functionalized biomaterial surface acts as localized carrier for the biological principle and this represents a challenging strategy to send specific signals to cells and tissues via *in situ* delivery. Many research papers deal with the surface grafting of proteins, enzymes and drugs [15,16,19–21] on different kinds of biomaterials, although few are related to polyphenols and natural biomolecules [22,23].

Among the different classes of biomaterials, bioactive glasses have been widely studied for orthopedic and dental applications, due to excellent biocompatibility and osteoinduction ability [24,25]. A number of surface functionalization techniques made it possible to graft, on bioactive glass surfaces, various kind of biomolecules such as proteins, growth factors and enzymes [26,27], however, few research have been focused on the combination of bioactive glass ceramics and natural bioactive phytochemicals. Some researches indicated phenol functionalization of carbon nanotubes to improve their mechanical and dynamic properties [22]. It has been observed that the incorporation of resveratrol (stilbenes from grapes, peanuts and berries) with porous poly-ε-caprolactone (PCL) surface can improve the alkaline phosphatase (ALP) activity of rat bone marrow stromal cells and can enhance mineralization of the cell–scaffold composites *in vitro* [23].

The aim of this research is to graft polyphenols, extracted from grape skin, to bioactive glasses in order to combine the bioactive properties of glass with the biological activities and health-promoting benefits of polyphenols. Compared with the commercial molecules, the utilization of nutraceuticals from natural sources offers the opportunity to exploit the waste products of food industry for obtaining high added value products for healthcare applications. In this paper, attention is mainly paid to the extraction of polyphenols, the grafting conditions and the analysis after surface functionalization.

2. Materials and methods

2.1. Extraction of phenol compounds from grape skin

Fresh red grapes (Barbera) were provided by a small-scale producer in the north of Italy (vineyard situated in Vaglio Serra, Asti, Piedmont, Italy). The fresh grapes were divided into four parts: flesh, skin, seed and stem (in this case only grape skin was employed). Fresh red grape skin was dried in oven at 60 °C and then grinded into small pieces.

Conventional solvent extraction was performed in a water–ethanol mixture (20:80, volume ratio) with the solid–liquid ratio 1:20 [28]. The extraction temperature was of 60 °C and the extraction time of 60 min in a thermostatic bath. The extraction solution was separated from the grape skin through filtering and put into an oven, at 60 °C, until the ethanol was completely evaporated. The extract was finally freeze dried and weighted. Phenols extracted from grape skins will be indicated as PH from now on.

2.2. Determination of total phenol content

Total phenol content of the extracts was determined by the Folin&Ciocalteu method [14]. In brief, 2 ml of the test solution were introduced in the test bottle and add 6 ml double distilled water, followed by adding 0.5 ml Folin&Ciocalteu reagent (Folin&Ciocalteau phenol reagent 2 M with respect to acid, 47,641, Sigma Aldrich). After 3–5 min mixed, 1.5 ml Na_2CO_3 (20%) was added. The final solution was tested after 2 h reaction by UV absorption at 760 nm (CARY 500 VARIAN). Three different samples were prepared and tested for each type of functionalization.

Gallic acid (GA 97.5–102.5% titration, G7384, Sigma Aldrich) was used as standard for phenols quantification. Six gallic acid solutions with increasing concentration (0.0025, 0.005, 0.01, 0.02, 0.03 and 0.04 mg/ml) were prepared and tested with the Folin&Ciocalteau method in order to obtain the calibration standard curve. The curve was used to calculate the total phenol content of the extracts.

2.3. Materials preparation

Two kinds of bioactive glasses were considered in this research as substrates for surface functionalization: SCNA (57% SiO_2, 34% CaO, 6% Na_2O, 3% Al_2O_3 mol%), which is characterized by a simple composition and a relative low bioactivity, and CEL2 (45% SiO_2, 3% P_2O_5, 26% CaO, 7% MgO, 15% Na_2O, 4% K_2O mol%) which presents a more complex composition and an higher bioactivity.

These two glasses were previously characterized in terms of characteristic temperatures, degree of bioactivity and surface reactivity after different washing treatments for surface functionalization with biomolecules [26,27]. Both glasses were prepared by traditional melt and quenching techniques as described in [27,29] and considered both in the bulk and powder forms. Reagents were melted in a platinum crucible and poured on a brass plate to obtain bars, or in water to obtain a frit. Glass bars were annealed in a furnace and then cut and polished in order to obtain homogeneous slices. Glass frits were milled and sieved up to obtain powders with a grain size lower than 20 μm.

2.4. Hydroxyl exposure

The first step of functionalization consisted in a cleaning and surface activation treatment. A procedure optimized in previous works on the surface modification of these glasses was employed [26,27]. In brief, samples were washed in an ultrasonic bath once in acetone for 5 min and three times in water for 5 min each time. After these processes, samples were dried in air at room temperature.

2.5. Polyphenol grafting

An amount of 100 mg phenol extracts were dissolved in 20 ml double distilled water and mixed for about 2 h under magnetic stirring. Samples were soaked in phenol (PH) solution for 24 h at 37 °C. For each sample 5 ml of 5.0 mg/ml phenol solution was employed.

The addition of citric acid (CA) in the PH uptake solution was performed (for bulk samples functionalization) in order to evaluate the effect of pH on the glass and molecular behavior. In fact, the ion release of bioactive glasses in the functionalization medium causes an increase of pH to basic values, as discussed in the results and discussion section and in previous papers [29,30]. A solution of 0.5 M citric acid (CA, Citric Acid Monohydrate, ACS reagent 99.0–102.0%, Sigma Aldrich) was added drop wise to the PH uptake solution up to pH 3.0. A part of samples was functionalized with CA-modified PH solutions.

Solution pH was measured before and after samples soaking on three different samples and indicated as mean ± standard deviation.