Effect of nano-sized bioactive glass particles on the angiogenic properties of collagen based composites

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Abstract Angiogenesis is essential for tissue regeneration and repair. A growing body of evidence shows that the use of bioactive glasses (BG) in biomaterial-based tissue engineering (TE) strategies may improve angiogenesis and induce increased vascularization in TE constructs. This work investigated the effect of adding nano-sized BG particles (n-BG) on the angiogenic properties of bovine type I collagen/n-BG composites. Nano-sized (20–30 nm) BG particles of nominally 45S5 Bioglass® composition were used to prepare composite films, which were characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The in vivo angiogenic response was evaluated using the quail chorioallantoic membrane (CAM) as an model of angiogenesis. At 24 h post-implantation, 10 wt% n-BG containing collagen films stimulated angiogenesis by increasing by 41 % the number of blood vessels branch points. In contrast, composite films containing 20 wt% n-BG were found to inhibit angiogenesis. This experimental study provides the first evidence that addition of a limited concentration of n-BG (10 wt%) to collagen films induces an early angiogenic response making selected collagen/n-BG composites attractive matrices for tissue engineering and regenerative medicine.

1 Introduction

Biodegradable composite systems incorporating nano-sized bioactive glasses (n-BG) have emerged recently as a new family of nano-structured biomaterials for biomedical applications [1]. The combination of bioactive glass nanoparticles or nanofibers with biocompatible polymers, including polyesters such as poly(lactic acid), poly (hydroxybutyrate) and poly(caprolactone), and natural-based polymers such as proteins (collagen, silk fibroin) or polysaccharides (chitin, chitosan, starch) enables the production of nanocomposites with potential to be used in tissue engineering and regenerative medicine [1–3].

The physico-chemical, mechanical and biological advantages of incorporating n-BG in biodegradable nanocomposites in comparison to conventional (micron-sized) bioactive glasses (µ-BG) have been reviewed [1]. The use of n-BG is expected to improve both the mechanical and biological properties of polymeric matrices. The higher specific surface area of n-BG particles allows not only for a higher protein adsorption but also a faster release of ionic dissolution products, especially critical concentrations of biologically active, soluble silica and calcium. In addition, n-BG will induce nanostructured features on the composite surfaces, which are likely to improve cell behavior. These effects have been previously shown in n-BG/poly (3-hydroxybutyrate) composites [4, 5] and in other n-BG containing biopolymers [6, 7].

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Enhancement of the angiogenic potential of implantable tissue scaffolds is the focus of considerable research efforts in tissue engineering (TE) strategies [8–10]. Increasing evidence in the literature shows that the use of bioactive silicate glasses may stimulate angiogenesis and improve neovascularization [11–15]. For example, the pro-angiogenic properties of μ-BG and n-BG filled poly (D, L lactide) (PDLLA) composites both in vitro and in vivo have been recently demonstrated [15].

The use of collagen-based biomaterials in the field of tissue engineering and regenerative medicine applications has been intensively growing over the past decades [16–21]. Type I collagen is the major constituent of the extracellular matrices to which proliferating endothelial cells are exposed in an injured tissue [22–24]. In an attempt to increase the angiogenic potential of collagen-based biomaterials it is conventional to administer proangiogenic growth factors like vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF) [25–27]. As compared to conventional approaches, bioactive glasses can serve as inorganic angiogenic agent to induce increased vascularization when incorporated in TE constructs without the need of expensive and potentially risky growth factors [11–15].

The aim of the present study was to evaluate, for the first time, the angiogenic potential of collagen composites containing silicate bioactive glass nanoparticles using the quail chorioallantoic membrane (CAM) as an alternative to the traditional mammalian models of angiogenesis. The research is relevant considering also the increasing interest in developing bioactive glass/collagen matrices for tissue engineering and regenerative medicine [28–30].

2 Materials and methods

2.1 Composite preparation and characterization

Composite films were fabricated using uncross-linked bovine type I collagen (1 % w/v in 0.5 M acetic acid neutralized with 0.1 M sodium hydroxide, Laboratorio Celina, Buenos Aires, Argentina) and bioactive glass nanoparticles (n-BG, 20–30 nm) of nominal composition close to 45S5 Bioglass® (46 wt% SiO_2, 23 wt% Na_2O, 27 wt% CaO, 4 wt% P_2O_5) [31] (kindly supplied by W. Stark, ETH Zurich, Switzerland). To prepare the composites, n-BG (in as-received condition) was blended with collagen (10 and 20 wt% n-BG) in a batch mixer and composites were compression molded into films with thickness of 40 μm. Collagen films without n-BG were also prepared to be used as a control. The films samples were sterilized with gamma radiation (25 kGy) for bioassays. The micro- and nanostructure of the films was examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

2.1.1 Scanning electron microscopy (SEM)

After mounting on stubs and gold sputtering, the collagen and composite films were examined with a scanning electron microscope (JEOL JSM 6480 LV, Japan).

2.1.2 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was performed with a Phillips CM30 T/STEM, operated at 300 kV acceleration voltage using a LaB_6 filament. TEM images were acquired with a charged coupled device (CCD) camera (FastScan-F114, from TVIPS), having an image size of 1024×1024 pixels. In order to avoid damage caused by the electron beam all TEM investigations have been carried out with a Gatan model 636-DH double-tilt liquid nitrogen cooled specimen holder, which enables TEM investigations at −170 °C specimen temperature.

2.1.3 Cryo ultramicrotomy

For the preparation of thin TEM lamellae of the collagen/n-BG composites cryo ultramicrotomy was employed which represents a typical preparation method for this kind of material [32]. Starting from round shaped specimens with a diameter of about 5 mm and a thickness of approximately 40 μm triangular shaped pieces were cut with a dissection scissors and mounted on an atomic force microscopy sample holder of the ultramicrotome (Leica EM UC/FC 6). Careful parameter variations delivered the following settings to be the best for the sample preparation: −25 °C for the specimen and the knife, as well as −40 °C for the glass transition temperature. Before final sectioning the areas, being possibly destroyed by the use of the dissection scissors, were removed with a 45° diamond cryo trimming tool (cryotrim 45, Diatome).

Since dry sectioning resulted in serious problems concerning electric charging, high compression effects and folding of the sections cryo wet sectioning was employed. A mixture of 60 % DMSO (dimethyl sulfoxide) and 40 % purified water was used as floating liquid. For sectioning of the collagen with 10 wt% n-BG a cryo 35° diamond knife (Diatome) at a clearance angle of 6° and a sectioning speed of 3 mm/s was applied for achieving a section thickness of 300 nm. Finally, the sections were picked up with a perfect loop tool (Diatome) and prepared on commonly used TEM copper grids coated with a continuous carbon film for TEM investigations. No staining has been applied in order to best preserve the structure of the composites.

2.2 Bioassay

Quail egg culture and experimental methods were approved by the institutional ethics committee of the School of Dentistry, University of Buenos Aires, Argentina. Humane care of the animals were carried out according to the Guide for the Care and Use of Laboratory Animals, National Research Council (USA), 2010.

2.2.1 Embryonic quail chorioallantoic membrane (CAM) assay

Experiments were performed on Japanese quail (*Coturnix coturnix japonica*) embryos grown by the shell-free culture method [33]. In brief, fertilized eggs of quails were incubated *in ovo* at 37 °C under ambient atmosphere, cracked at embryonic day 3, into 6-well tissue culture polystyrene plates, and cultured further *ex ovo* at 37 °C. Neither tissue culture medium nor antibiotics were added to the cultures.

Discs (5 mm diameter) of sterilized films were gently placed onto the chorioallantoic membrane (CAM) at 7 days of total incubation (Fig. 1). Two films were placed on each CAM with at least one control (collagen) and one composite film (10 wt% n-BG or 20 wt% n-BG containing collagen) per embryo. In negative controls no material was implanted. Each experiment included ten embryos per group and was repeated twice. The embryos were incubated further at 37 °C for 24 and 72 h. At the end of these periods, the CAMs were fixed in situ with

Fig. 1 A shell-less quail at embryonic day 7, the stage when collagen and composites films were placed on the chorioallantoic membrane (CAM)

4 % paraformaldehyde/2 % glutaraldehyde in PBS pH 7.4. The specimens were fixed for at least 3 days at room temperature and the discs together with the underlying CAM were dissected and examined grossly with a stereomicroscope maintained at one focal plane to quantify angiogenesis and then processed for embedding in paraffin. Histological sections (10 μm thickness) were stained with hematoxylin-eosin for histological evaluation by light microscopy.

2.2.2 Quantification of angiogenesis

Quantification of angiogenesis was accomplished by counting the number of blood vessel branch points within the confined regions of the discs. Ten samples per implant material and per time point from two independent experiments were used to count and calculate the number of blood vessel branch points. Bifurcations were also quantified for native CAM areas of the same size in the untreated controls. The number of blood vessel branch points is relative to the number of newly sprouting angiogenic vessels [34].

2.3 Statistical analysis

An ANOVA was used for statistical analysis of data taking $\alpha = 0.05$ and $\beta = 0.01$. Data are presented as mean ± SD.

3 Results

3.1 Composite characterization

Typical microstructure images of collagen and collagen/n-BG composites are shown in Figs. 2a–c and 3. Figure 2a is a SEM image of the collagen film without n-BG. The collagen fibrillar network and micron-sized clusters of n-BG particles are clearly visible in SEM images of the surface of the composite films (Fig. 2b, c). Since SEM has limited resolution and can only reveal the distribution of n-BG at the very surface of the films, TEM investigations were carried out on thin sections cut from the bulk of the films by cryo-ultramicrotomy. In order to investigate the influence of the n-BG particles on the sectioning behaviour of the samples, collagen specimens with 0, 10 and 20 wt% n-BG particles content were sectioned. Different characteristics depending on the bioglass content were observed. With increasing n-BG content the material seemed to be less ductile and compression effects in sectioning direction were reduced. Furthermore, a different influence of the floating liquid on the sections was observed. The pure collagen specimen suffered a compression by sectioning of
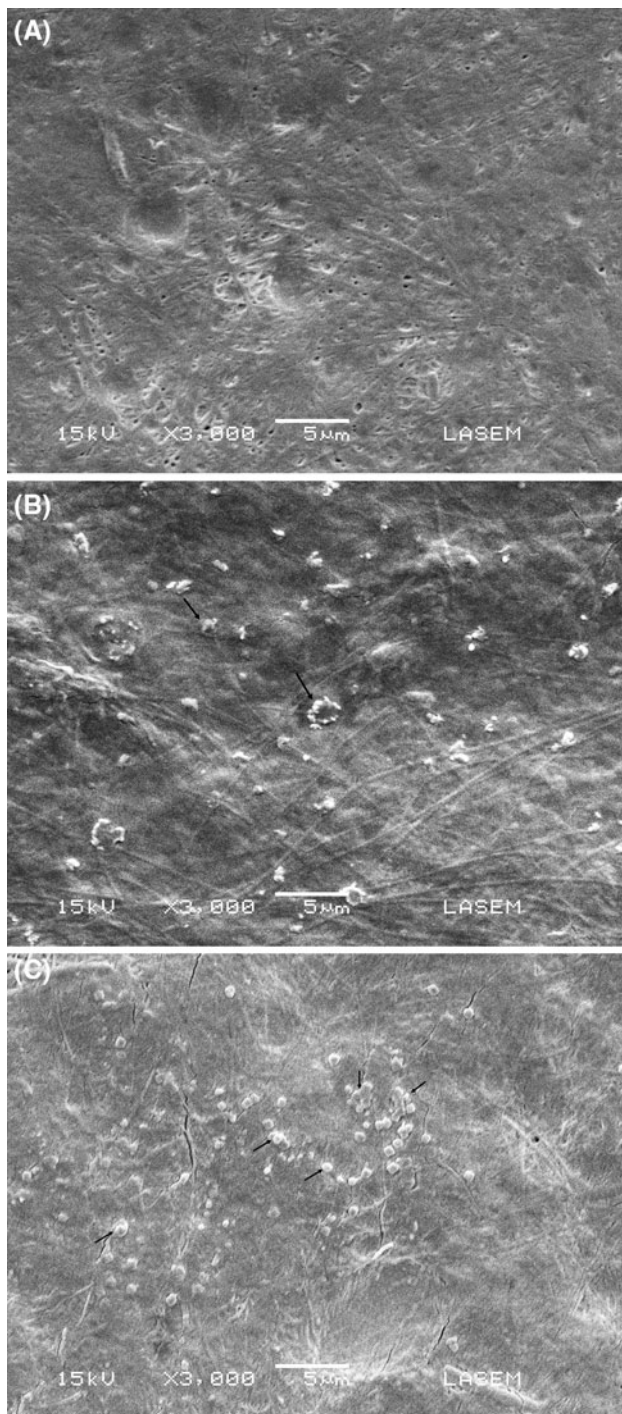
Fig. 2 Typical microstructure of as made films: **a** SEM image of the surface of a collagen film without n-BG. **b, c** SEM images of the surface of a collagen/n-BG composites. **b** 10 wt% n-BG containing collagen film **c** 20 wt% n-BG containing collagen film. Notice the micron-sized clusters of n-BG particles (*arrows*)

about 31 % at a sectioning thickness of 100 nm. But the effect of compression was superimposed by an extension of about 37 %, leading to a compression in sectioning direction of 6 % and an extension of 37 % perpendicular to the

sectioning direction but also to a minimization of crumpling. The sections seemed to soak up the floating liquid. The influence of the floating liquid on the sections decreases with increasing n-BG content and sectioning thickness. These effects are considered regarding the interpretation of the TEM images. Figure 3 shows bright-field TEM images of the collagen film without n-BG and the collagen/n-BG composites. The approximate cutting direction (after cryo ultramicrotomy) is indicated by white dotted arrows. As observed by SEM, collagen fibrils are also visible in TEM (Fig. 3a–c), showing an axial periodicity of 67 nm, being characteristic for this type of collagen. The reason why the collagen fibrils are not clearly visible in Fig. 3a might due to the higher sample thickness in this case, which limits the resolution in TEM and thus resulting in a blurred image contrast. In the collagen/10 wt% n-BG composite (Fig. 3b) collagen fibrils and BG particles are clearly visible. The BG particles show a clear image contrast difference, compared to the collagen matrix. The diameter of the BG particles is between 90 and 130 nm, which deviates from the expected size of 20–30 nm in diameter. This can be related to agglomeration of n-BG particles into larger clusters. The TEM image (Fig. 3c) of the collagen/20 wt% n-BG composite shows exemplarily that the BG particles form agglomerates, with a size of several hundreds of nanometers, or several microns as observed by SEM. However, TEM emphasizes that the BG agglomerates are intercalated between collagen fibrils constituting a composite with intimate contact between organic and inorganic components. Moreover, the BG agglomerates were found to be widely spread in both collagen/n-BG composites (data not shown) investigated in the present work.

3.2 Angiogenesis examination

The angiogenic response was evaluated using the CAM as an in vivo model of angiogenesis and results are summarized in Figs. 4, 5 and 6. In initial experiments we found that pure collagen films had no effect on CAM angiogenesis at 24 and 72 h after implantation. They had similar levels of blood vessel densities as compared to native CAM (data not shown). However, at 24 h post-implantation, the number of blood vessels branch points was increased to 41 % on CAMs implanted with 10 wt% n-BG containing collagen films in comparison to those treated with collagen alone (Figs. 4, 5). In contrast, at 24 h after implantation, films containing 20 wt% n-BG resulted in an anti-angiogenic response, depicted by an statistically significant decrease (49 %, $P < 0.05$) in the number of blood vessels branch points compared with the pure collagen films (Fig. 4, 5c).
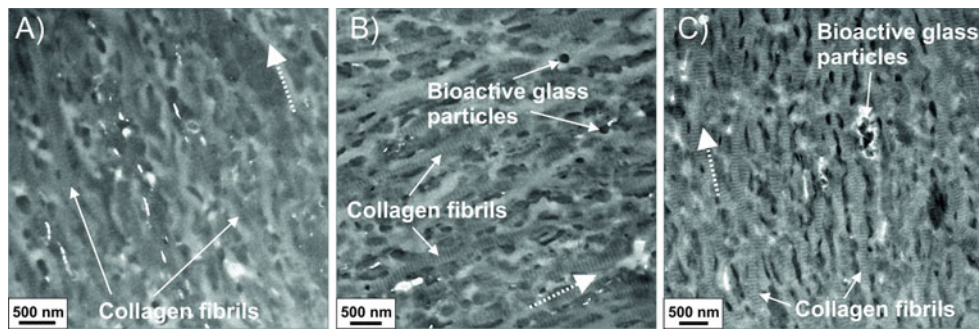
Fig. 3 Bright-field TEM images of **a** collagen without n-BG, **b** collagen/10 wt% n-BG composite and **c** collagen/20 wt% n-BG composite. The n-BG agglomerates are deposited and intercalated between collagen fibrils

Fig. 4 Quantification of angiogenesis. Values are mean ± SD; *$P < 0.05$ compared with control (collagen) 24 h values ($n = 10$). Due to toxicity, the data of 72 h, collagen/20 wt% n-BG group were not collected
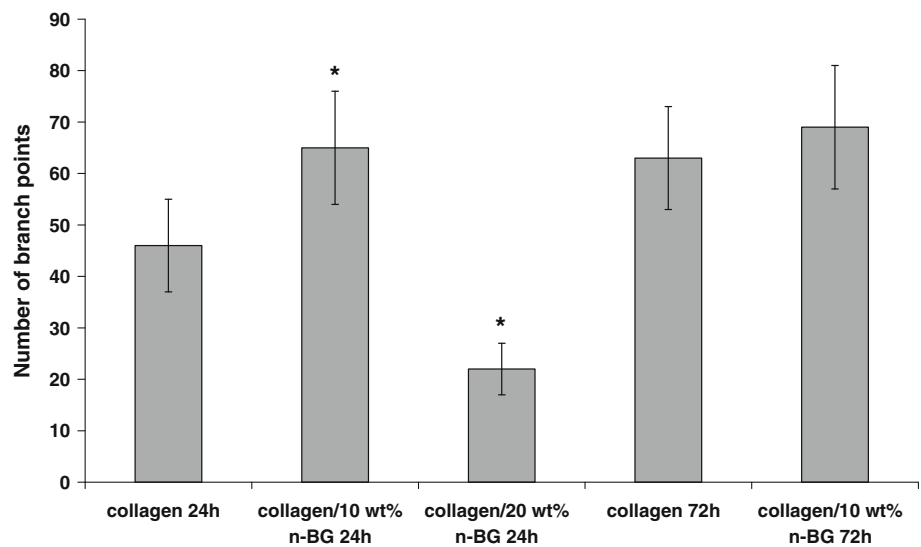
Interestingly, the average number of branch points in the treated CAMs for 24 h with composite films containing 10 wt% n-BG was comparable to the vessel density detectable at 72 h in CAMs implanted with pure collagen or with 10 wt% n-BG containing collagen films (Fig. 4). Although increases in the number of blood vessel branch points in CAMs exposed during 72 h to composite films containing 10 wt% n-BG were generally somewhat greater than with the pure collagen films, these differences were not significant (Fig. 4).

The histological examination revealed that at 24 h after implantation, films were adherent to the chorion without invading the mesenchyme (Fig. 6a, b). At some points, epithelial cells from the chorion invaded the composite films (Fig. 6b). Moreover, no significant increase of the mononuclear cell infiltrate was observed in the CAMs treated with composite films containing 10 wt% n-BG with respect to collagen-treated samples (Fig. 6b), ruling out the possibility that the early angiogenic activity exerted by the 10 wt% n-BG containing composite films was the consequence of the triggering of an inflammatory response. No migration of vascular cells towards the implant was observed in the CAMs treated with composite films containing 10 wt% n-BG 72 h after implantation (Fig. 6c).

On other hand, in contrast to the CAM tissue response to pure collagen films (Fig. 6d), a strong perivascular inflammatory infiltrate was found in the CAMs treated for 24 h with composite films containing 20 wt% n-BG (Fig. 6e). Concomitantly, this inflammatory response do not sustain further new blood vessel formation (Fig. 4), affecting furthermore the embryo survival (near 0 at 72 h post-implantation).

4 Discussion

The CAM assay represents a useful preliminary screening procedure to investigate the angiogenic activity of several agents (i.e. cytokines, growth factors, drug delivery systems and biomaterials) providing a reliable alternative method for further examination in mammalian models [35, 36]. In previous studies using the chick embryo CAM assay, it was found that a significant angiogenic response occurred when growth factors (i.e. FGF, VEGF) were
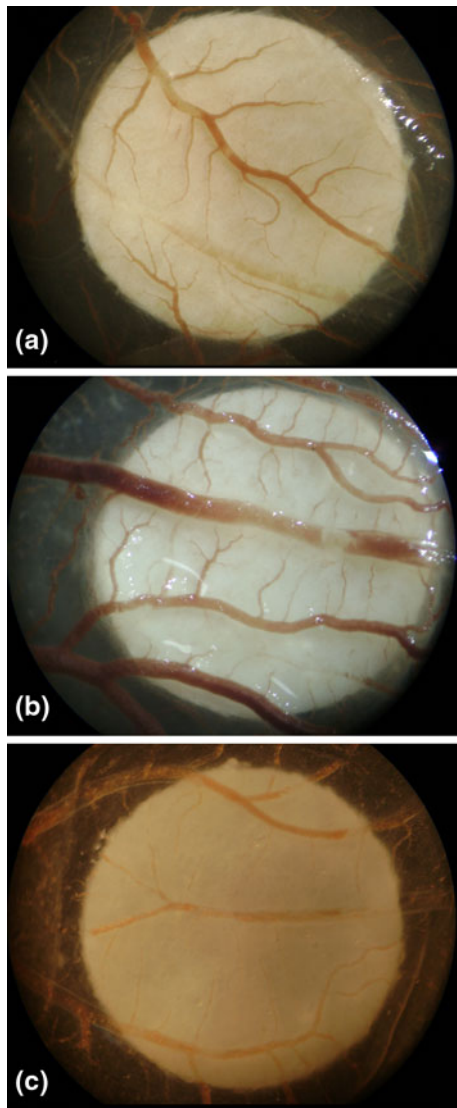
Fig. 5 Stereomicroscopic views of CAM tissue response at 24 h post-implatation. **a** collagen film **b** 10 wt% n-BG containing film **c** 20 wt% n-BG containing collagen film. Orig. Mag. × 35

added to the bovine fibrillar type I collagen implants [25, 37, 38]. Similar effects were observed by exposing chick embryo CAM to microporous collagen spheres [39] and cross-linked and/or heparinised three-dimensional collagen scaffolds [40, 41], as well as, acellular collagen-based TE matrices containing endogenous angiogenic factors were able to induce a strong angiogenic activity on the chick embryo CAM [42, 43]. The quail embryo CAM has been used extensively as a convenient model for in vivo study both angiogenesis and anti-angiogenesis in response to different factors [33, 44–46]. The present study evaluated, for the first time, the response of the quail embryo CAM to collagen composite thin films containing n-BG particles. Thin films have been successfully used in a variety of tissue engineering/regenerative medicine

applications both in preclinical studies and in clinical applications [47, 48]. Biocompatible polymer thin films and ultra-thin films have many useful and advantageous biological and physical properties for their application as a wound dressing, such as sufficient flexibility, transparency, and adhesiveness [47, 48].

The uncross-linked collagen films when used alone did not stimulate inflammatory angiogenesis in this CAM assay. Therefore, the capability of 10 wt% n-BG containing collagen films to induce a rapid, robust angiogenic response may be attributable to the direct angiogenic activity exerted by the presence of n-BG particles in the composite film, because inflammation, which itself could induce angiogenesis, was not detectable by the histological evaluation at 24 h post-implantation in the CAM mesenchyme beneath the implant. It has been previously shown [15] that human fibroblasts grown on n-BG containing PDLLA composite films secreted significantly increased amounts of VEGF into the cell culture medium compared with fibroblasts grown on pure PDLLA films. We hypothesize that the early angiogenic activity exerted by 10 wt% n-BG containing collagen films was as consequence that these films may have stimulated CAM's fibroblasts to secrete VEGF as indicated by the presence of small tortuous, corkscrew-like blood vessels sprouting from larger pre-existing vessels, a hallmark of VEGF signaling [49] (Fig. 5b). It is also possible that the increased angiogenic capacity of films containing 10 wt% n-BG may be the result of a more rigid structure of the composite film. Actually, it has been reported that cross-linked collagen matrices were found to exhibit increased angiogenic activity compared with uncross-linked collagen when assessed in the chick embryo CAM model [39, 40]. Further studies involving examination on mechanical properties in these composites will be needed to draw final conclusions on the angiogenic potential of our system. Additionally, because the applied approaches do not allow statements on the vascular quality, it would be worth investigating if the newly formed blood vessels shows mature characteristics, i.e. the capillaries consisted of endothelial cells layered on a basal membrane surrounded by pericytes and smooth muscle cells.

On the other hand, it has been demonstrated that a weak inflammatory response co-incident with angiogenesis would be a useful adjunct to a well vascularized tissue engineered construct [50]. Surprisingly, in our study, the inflammatory infiltrate observed at 24 h after implantation failed to enhance angiogenesis in the CAMs treated with composite films containing 20 wt% n-BG. We think that these results were probably due to an excessive inflammatory response. Since bioglass microparticles are more exposed in the samples with a higher concentration, this might lead to a quicker solution and higher reaction
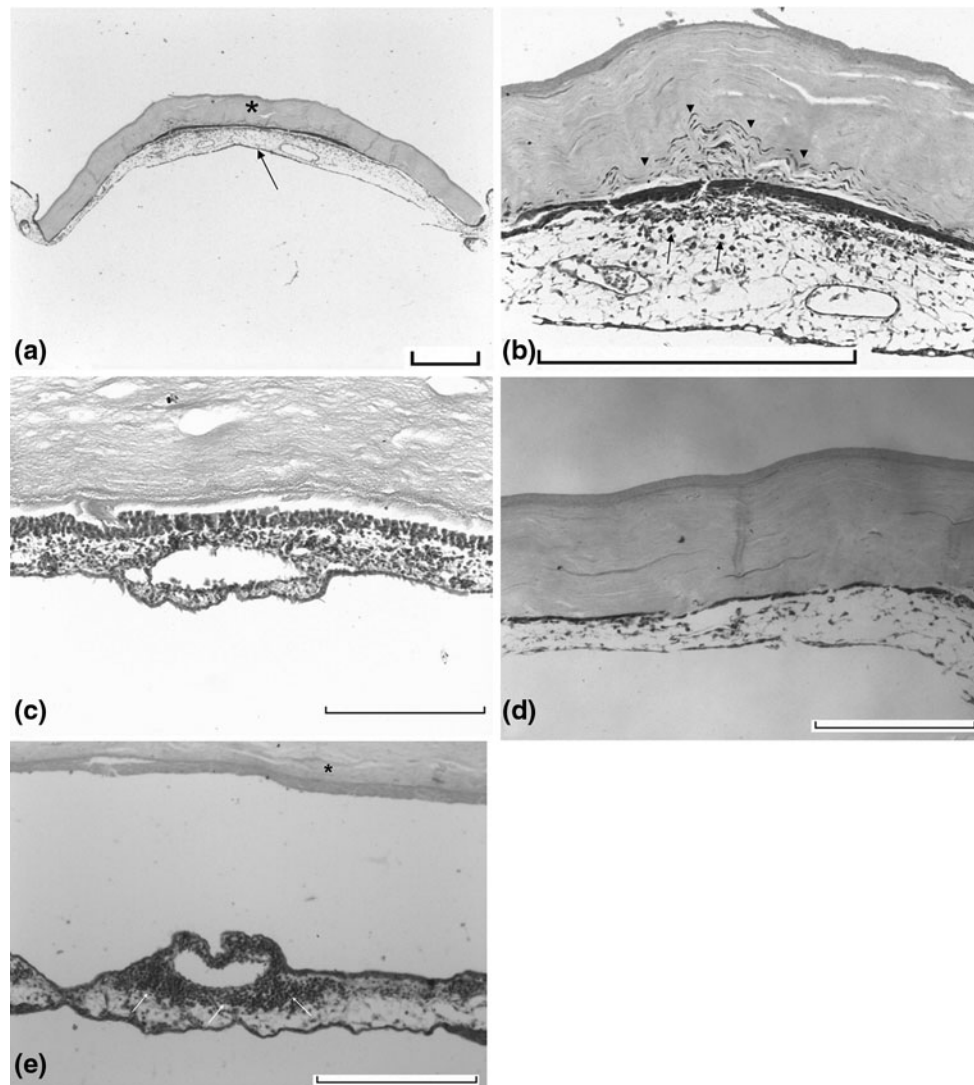
Fig. 6 Photomicrographs showing the CAM tissue response to composite films. At 24 h after implantation, 10 wt% n-BG containing collagen films (*asterisks*) were adherent to the chorion without invading the mesenchyme (**a, b**). *Arrow* in Fig. 6A indicate the CAM. Scarce mononuclear cells (*arrows*) was observed in the CAMs treated with 10 wt% n-BG containing collagen films. Note the epithelial cells from the chorion invading the composite film (*arrowheads*) (**b**). No migration of vascular cells towards the implant was observed in the

CAMs treated with composite films containing 10 wt% n-BG 72 h after implantation (**c**). In contrast to the CAM tissue response to pure collagen films (**d**), a strong perivascular inflammatory infiltrate (*white arrows*) was observed in the CAMs treated for 24 h with composite films containing 20 wt% n-BG (**E**). These composite films were frequently displaced from underlying CAM during tissue processing. *Scale bar* 100 μm

kinetics between the glass and the medium. As a result of this, higher ionic concentrations and dramatic pH changes will undergo on the surface of the bioglass particles, perhaps leading to this enhanced inflammatory response. Further studies, therefore, will be necessary to understand the cellular and molecular mechanisms for which the 20 wt% n-BG containing collagen films inhibited angiogenesis in quail CAM.

As presented above, the BG particles appear as agglomerates (up to several micrometers in size), instead of being present as single nanoparticles with the expected size

between 20 and 30 nm in diameter. It was previously shown for BG/polymer composites [4, 5] that the size/morphology of BG particles clearly affects the biological response and cell behavior of such materials. These studies support that BG particles when being in the nm-range improve the cell behavior and allow for instance a faster release of ionic dissolution products. Similar effects are expected for the collagen/n-BG composites investigated in the present work, thus influencing the angiogenic potential of the collagen/n-BG composites. As observed by TEM the size of the BG particles in the collagen/10 wt% n-BG

composite was significantly lower, than the one in the collagen/20 wt% n-BG composite. While in the case of a collagen/10 wt% n-BG composite the angiogenesis was stimulated, the same was found to be inhibited in the case of a collagen/20 wt% n-BG composite. The amount of adding n-BG to collagen seems to end up in a different n-BG morphology (smaller agglomerates at smaller BG concentrations), which might cause a different angiogenesis response, associated by the specific surface area of the BG particles and thus a specific biological material response.

The development of an extensive CAM capillary network is of great importance for the avian embryo as it serves as its respiratory organ until the time of hatching [35]. In the present study, we have found that composite films containing 20 wt% n-BG resulted in an anti-angiogenic response and increased the mortality of quail embryos. It has been recently reported that n-BG particles induced cytotoxic effect on human fibroblasts seeded on 20 wt% n-BG containing PDLLA composite films [15]. Impaired in vitro cell viability with 20 wt% n-BG filler content has also been found in P3HB/n-BG composite films seeded with osteoblast-like cells [5]. It is noteworthy that possible toxic effects of the composite films containing 10 wt% n-BG on quail embryos might not be discovered due to inhibited solubility of the n-BG since n-BG within the composite is most likely fully coated with collagen, due to strong protein adsorption on the bioactive glass surface. Since CAM model is suited for short-time assays, further long-term studies involving wound healing or tissue engineering animal models are required to further elucidate this matter.

Overall, improved understanding of the angiogenic effect of biodegradable/bioactive glass nanocomposites will increase the attractiveness of these materials for applications in tissue engineering and regenerative medicine. We anticipate further application of the quail embryo CAM model to investigate angiogenic potential of a variety of bioactive glass compositions, in which different doped ions are being considered to stimulate angiogenesis [12].

5 Conclusion

This experimental study provides the first evidence that addition of a limited concentration (10 wt%) of silicate bioactive glass nanoparticles (n-BG) to collagen films induces an early angiogenic response making selected collagen/n-BG composites attractive matrices for tissue engineering and regenerative medicine.

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