Preparation and evaluation of doxorubicin containing an intratumorally injectable, click-cross-linked hyaluronic acid hydrogel

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Statement of purpose: The primary clinical treatment for solid tumors is surgery for tumor removal. In addition, radiation therapy and drug therapy are additionally used to remove cancer tissue that has not been completely removed after tumor incision and the therapies are used to prevent cancer metastasis by remaining cancer tissue and cancer cells. Among many anti-cancer drugs to treat cancer patients in clinicals, Doxorubicin (Dox) suppresses tumor growth by inhibiting RNA transcription. Doxorubicin is widely used in the treatment of breast cancer, bladder cancer, sarcoma, and lymphoma, and it is known for its brilliant anti-cancer effect as it was registered as an essential drug by the WHO in 1974 However, It comes with many side effects, such as heart disease, hair loss and etc. For those reasons, the drug concentration in plasma should be precisely controlled and it is used to inject the drug directly into tumors to diminish the side effects. In this study, a drug called doxorubicin was injected into the tumors with hyaluronic acid (HA) together, which is easily obtained from nature and used in various fields due to its excellent biocompatibility, biodegradability, and low toxicity, as a hydrogel. However, when Doxorubicin loaded HA hydrogels are injected into the cancer, the biodegradation periods of HA within the cancer tissue occurs very quickly, and the release of doxorubicin occurs also very quickly following by the rapid biodegradation periods of HA hydrogel. When the HA hydrogel is degraded in short time, the drug concentration in plasma dramatically increased and it may occur numerous side effects. To increase the drug efficacy and to make the drug concentration sustainably maintained in therapeutic level, we made Doxorubicin loaded in-situ forming HA hydrogels. We prepared trans-cyclooctene (TCO)-conjugated HA (TCO-HA) and tetrazine (Tet)-conjugated HA(Tet-HA). Since the biorthogonal reagents, trans-cyclooctene and tetrazine are immediately reacted in bodies and known for low toxicity, we prepared trans-cyclooctene (TCO)-conjugated HA (TCO-HA) and tetrazine (Tet)-conjugated HA(Tet-HA). As Dox loaded TCO-HA and Dox loaded Tet-HA are injected into tumors, the solutions are immediately formed as a hydrogel then, the click-crosslinked HA hydrogels are degraded in long periods and make Dox release sustainably.

Methods: To prepare the animal tumor model, B16F10 cell were subcutaneously injected. To prepare click cross linkable TCO-HA and Tet-HA, trans-cyclooctene-amine hydrochloride salt (Click Chemistry Tools, AZ, USA) and Methyltetrazine-PEG4-amine hydrochloride salt (Click Chemistry Tools, AZ, USA), were introduced to hyaluronic acid respectively. In situ cell death detection kit (Sigma, St. Louis, MO, USA) was used to confirm tumor cell apoptosis To determine the anti cancer activity *in vitro* and *in vivo,* MTT assay and TUNEL staining were perfomed.

Results: The rheological properties measured with MCR 102. To confirm which cross-linking density is suitable for Dox releasing system, we prepared Cx-HA-250(Cx-HA with crosslinking density 250), Cx-HA-500(Cx-HA with crosslinking density 500) and Cx-HA-750(Cx-HA with crosslinking density 750). The complex viscosities of cross-linked hydrogels increased as the cross-linking densities become larger. The results indicated Cx-HA-250, Cx-HA-500 and Cx-HA-750 were successfully prepared and we couldpredict the larger cross-linking density of the HA hydrogels, the more sustained Dox release. *In vitro* result indicated that the optical densities of Dox containing cross-linked HA (Cx-HA-Dox) and Dox solution which was repeatedly treated reacted to zero at 4 day. On the other hand, Dox solution which were treated once and Dox containing HA hydrogel (HA-Dox) make the B16F10 cells died at early experiment time but the cells were proliferated at 4 day. The *in vitro* experiment indicated the Cx-HA hydrogel degraded slowly than uncross-linked HA and the Dox amount from Cx-HA were sustainably maintained. Dox solution, Dox solution which was treated repeatedly (Dox repeat), HA-Dox and Cx-HA-Dox solutions were treated when the tumor sizes reached 150 mm^3 to 200 mm^3. Dox repeat and Cx-HA-Dox solution suppressed tumor growth and the tumor size increased less than 3 times at 18 day than that of at 0 day. Otherwise, tumor size in HA-Dox group and Dox solution group increased more than 10 times than initial times. To find the reason for tumor growth size experiments we measured the remaining Dox amounts into tumors by HPLC. The remaining Dox amounts in Cx-HA-Dox group decreased gradually and maintained 20% at 18 day. In addition, the remaining Dox amounts in Dox solution group and HA-Dox group decreased rapidly and no Dox amount were measured from 12 day. As other *in vivo* experiments, TUNEL positive cell percentage in Cx-HA-Dox recorded the largest number from 6 day to 18 day comparing to other experiment groups.

Conclusions: In this study, we have successfully manufactured Cx-HA-Dox with longer biodegradation period. Cx-HA-Dox exhibited sustained Dox release from injected site in tumors for prolonged periods, as well as demonstrated enhanced therapeutic effect of Dox over defined injected sites. Thus, we successfully developed a feasible Click cross-linked HA for a sustained Dox delivery system.